DEVELOPMENT AND OPTIMIZATION OF ELECTROCHEMICAL SENSORS TO DETECT BACTERIAL PATHOGENS FOR POINT-OF-CARE APPLICATIONS

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ABSTRACT

This dissertation focuses on the development and optimization of electrochemical sensors to detect bacterial pathogens for point-of-care applications. Recent spikes in hospital-acquired infections have resurfaced fears of antibiotic-resistant bacteria and their adverse effects on human health. Presenting new challenges in pathogenesis and resistance, infections caused by these microorganisms are becoming increasingly difficult to treat, with prompt administration of targeted therapies providing the best chances for patient recovery. To address this emerging threat, this dissertation aims to develop and improve biosensors that will allow for early and rapid detection of these bacterial pathogens.

*Pseudomonas aeruginosa* is a commonly isolated nosocomial pathogen frequently associated with infections in patients with cystic fibrosis and chronic wounds. *P. aeruginosa* secretes pyocyanin, a unique quorum sensing molecule that can be monitored electrochemically due to its redox-active nature. Detection of pyocyanin has been demonstrated to be a relatively simple, inexpensive way to screen for *P. aeruginosa* infections, but there remain significant challenges in the development of this sensing strategy before it can be used as a viable diagnostic technique.

Several enhancements were investigated for sensor improvement. One focus of this work was to use amino acids to up-regulate pyocyanin production to expedite identification methods at the onset of infection. The second focus of this work was to electrochemically screen additional pathogens for unique redox-active molecules. As no other redox-active molecules could be directly measured or identified, a secondary electrochemical detection method was developed using aptamers as highly selective
recognition elements. The third focus of this work was to microfabricate an electrochemical sensor to detect pH, a general marker of infection, and pyocyanin to detect the presence of *P. aeruginosa* for applications toward a smart bandage. Finally, the *P. aeruginosa* sensor was clinically validated with human and animal patient samples.

Future work and recommendations include continued clinical data collection and further optimization of the aptamer biosensor to detect other clinical pathogens. These sensors will lead to faster detection methods for *P. aeruginosa* and other clinically-relevant bacteria, allowing clinicians to promptly switch from broad-spectrum antibiotics to targeted therapies, lowering hospital expenditures, minimizing drug resistance, and improving patient care outcomes.
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1.0 Introduction

For more than a century, the primary clinical identification method for bacterial infections has been plate cultures where bacteria are isolated and purified overnight using nutrient-based agar medium. Rapid, automated instrumentation has been widely regarded as the next step towards advancing bacterial identification. These instruments, however, still require a pure bacterial colony obtained from a plate culture and thus a lead time of at least 18-24 hours before an identification can be made [1, 2]. Even novel molecular diagnostics such as the polymerase chain reaction require significant manpower and hours of processing, making them unfeasible as point-of-care tools in the clinic [3]. Therefore, there is an unmet need to develop rapid screening tools for identification of clinically-relevant bacterial species in the clinical setting.

The detection of bacterial infections in the hospital setting has gained significant interest as recent spikes in hospital-acquired infections have increased awareness of antibiotic-resistant bacteria and their adverse effects on human health. Both the Centers for Disease Control and Prevention and the Infectious Diseases Society of America have called for greater attention towards diagnosing and monitoring drug-resistant bacterial pathogens in hopes of minimizing the widespread problem of antibiotic resistance [4].

One of the leading causes of bacterial infections in the hospital setting is *Pseudomonas aeruginosa*, a common Gram-negative bacterium [5, 6]. This opportunistic pathogen is frequently responsible for infections in patients with cystic fibrosis [7-9], chronic wounds [10], severe burn victims [11], and immunocompromised hosts such as patients with AIDS [12]. Chronic infection in patients with cystic fibrosis is common and the prevention and treatment of these infections are difficult due to the lack of sensitive
detection methods for this bacterium [13]. Although it is seldom responsible for infections in healthy individuals, several factors contribute to the success of *P. aeruginosa* in the compromised, including its ability to thrive in the hospital setting and its increasing resistance to antibiotics [5]. *P. aeruginosa* infections pose serious concerns for this patient population, making it an important bacterium to study in the scientific and medical community.

Unique to *P. aeruginosa* is its production of pyocyanin, a redox-active quorum sensing molecule linked to biofilm formation and therefore a significant contributor in the bacteria’s pathogenesis [14-16]. Because it is redox-active, pyocyanin can be detected electrochemically [17-26]. With this information, the concentration of *P. aeruginosa* in a sample can be calculated and the infection correctly identified. However, electrochemical sensors are limited in their sensitivity and the concentration of pyocyanin present in solution at early stages of infection is very low. Given these challenges, there is a need to develop methods that increase the production of pyocyanin by *P. aeruginosa*.

Current diagnostic techniques rely on culture-based approaches that are time-consuming and require subjective interpretation [27, 28]. To address this, one aim of this dissertation is to develop a more rapid, sensitive identification technique for *P. aeruginosa* using molecules to up-regulate pyocyanin production. Recently published literature has shown that the addition of amino acids up-regulates the biofilm formation of *P. aeruginosa* [29, 30]. However, these studies do not address the link between pyocyanin production and *P. aeruginosa* growth. Research carried out in the 1950’s to determine how amino acids influence pyocyanin production has not been revisited or applied to sensing strategies [31, 32]. Other up-regulatory molecules for biofilm growth
have also been identified in past years including iron [33-35] and simple sugars [36]. The electrochemical detection of the up-regulation of pyocyanin production will lead to a better understanding of how this bacterium grows at the onset of infection as well as improve upon detection sensitivity for applications in the healthcare setting.

While this dissertation initially focuses on the detection of *P. aeruginosa*, the concept of detecting rapidly produced small molecules by cells, as a means of increasing sensitivity, can be extended to other pathogens. The second aim of this dissertation will be to electrochemically screen additional pathogens for production of redox-active small molecules, specifically in other prominent hospital-acquired infections to develop faster, alternative means for detection. If no redox-active molecules can be directly measured or identified, a secondary method of electrochemical detection will be to develop an aptamer-based electrochemical detection scheme. Aptamers are short DNA or RNA strands that act as highly selective recognition elements and have been incorporated in biosensor platforms for detecting molecules with high sensitivity and selectivity [37, 38].

The final aim of this dissertation is to apply these sensor technologies and detection strategies in the hospital setting, specifically in the form of a smart bandage containing a miniaturized electrochemical sensor that will be able to rapidly detect the onset of bacterial infections [39, 40]. The smart bandage will output useful information such as pH levels, an important variable in infection detection [41]. Finally, validation of the sensor with human and animal samples is necessary to translate this technology into the clinical setting. The development and optimization of these sensors will lead to an inexpensive, accurate, and rapid detection method for *P. aeruginosa* and other bacterial pathogens for point-of-care applications.
2.0 Critical Literature Review

2.1 ESKAPE Pathogens

The widespread use of antibiotics to treat bacterial infections has led to a sweeping increase in bacterial resistance as unnecessary, incorrect, or indiscriminate antibiotic use has become commonplace [42, 43]. Antibiotic overuse has resulted in the emergence of multidrug-resistant bacteria, contributing to a growing healthcare crisis as infections caused by these microorganisms have become increasingly difficult to treat [44]. The Infectious Diseases Society of America (IDSA) recently identified six antibiotic-resistant bacteria, acronymically labelled as the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), responsible for the most challenging infections to treat in the hospital setting [4]. Of the ESKAPE species, data collected by the Centers for Disease Control and Prevention (CDC) reported a significant resistance rate increase for methicillin-resistant *S. aureus* (MRSA) and multidrug-resistant *P. aeruginosa* [45], highlighting the significance of this dissertation’s focus on the detection of *P. aeruginosa* infections.

Despite the clear need to combat these pathogens, there have been challenges in gaining widespread public and federal support for research in antibiotic resistance [46, 47]. Even more alarming is the decline in antibiotic development as evidenced by the declining number of new antibiotic drugs approved by the Federal Drug Administration (FDA) in recent years [48]. In response to this crisis, the IDSA launched a collaborative effort with allied health organizations to develop 10 new, safe, and effective antibiotics by 2020 titled the “10 × ’20” Initiative [49]. However, this process will require
researchers to revisit old classes of drugs in order to develop new antibiotics. This challenging effort would include examining drugs such as colistin, which has previously been disregarded due to its toxicity [50-52]. The growing prevalence of ESKAPE pathogens and consequent impact on treatment and healthcare costs require national and worldwide attention [53-55].

2.2  *Pseudomonas aeruginosa*

*Pseudomonas aeruginosa* is a Gram-negative, rod-shaped aerobic bacterium measuring 0.5-0.8 µm wide and 1.5-3.0 µm long [56]. The majority of strains use a single polar flagellum as means for motility [57]. A widespread bacterium, *P. aeruginosa* can be found in a variety of environments including natural surroundings such as soil [58] and water [59]. Of greater concern, however, is its affinity for colonizing artificial settings. In the medical environment, *P. aeruginosa* can be found on hospital food, sinks, taps, and respiratory equipment [5, 56]. Due to its ability to persist in this setting, *P. aeruginosa* is the fourth most commonly-isolated nosocomial pathogen and accounts for 10.1% of all hospital-acquired infections [12]. The growing prevalence of *P. aeruginosa* infections indicates a significant need for further research in bacterial detection methods [60]. Pseudomonal infections can involve any part of the body [61]. The most common sources of *P. aeruginosa* infection are highlighted in Figure 2-1.
Figure 2-1: Possible sources of *P. aeruginosa* infections.

Unique to *P. aeruginosa* is its production of pyocyanin, a member of a large family of tricyclic compounds known as phenazines [62]. Pyocyanin, characterized by its blue-green color, is both a virulence factor and quorum sensing molecule for *P. aeruginosa*. Other pigmented molecules produced by *P. aeruginosa* include pyoverdin (yellow, green, and fluorescent) [63, 64], pyomelanin (light brown) [65], and pyorubrin (red-brown) [66, 67]. Because no other Gram-negative bacterium produces pyocyanin, however, it serves as a useful biomarker for *P. aeruginosa* identification and detection [68]. Over 90% of isolated *P. aeruginosa* strains produce pyocyanin [56]. In 1981, Reyes *et al.* tested 835 strains of *P. aeruginosa*, all clinical isolates, and found that 98% of the strains produced pyocyanin [69].
Colonies of *P. aeruginosa* grown on blood agar plates are shown in Figure 2-2. Plate cultures remain the gold standard of bacterial identification in clinical healthcare where bacteria are grown on a plate and identified manually or using automated instrumentation. While using selective plate cultures to identify bacteria is a well-established technique, it requires pure isolated colonies for successful identification, a challenge for polymicrobial infections as this may require several selective culture iterations before a pure colony can be isolated. Chloroform extraction paired with UV-Vis spectroscopy, another established technique, also presents its own challenges as it is not sensitive enough to detect low concentration levels of pyocyanin [70, 71]. More efficient methods for detecting *P. aeruginosa*, based on the electrochemical detection of pyocyanin, will be presented in the later sections of this dissertation.

2.2.1 Human Infections

The fourth leading cause of bacterial infections in the hospital setting is *Pseudomonas aeruginosa* [5, 6]. This opportunistic pathogen is frequently linked to
patients with cystic fibrosis [7-9], severe burn victims [11, 72, 73], and immunocompromised hosts such as patients with AIDS [12, 74, 75]. Although it is seldom responsible for infections in healthy individuals, several factors contribute to the success of *P. aeruginosa* in the compromised including its ability to thrive in the hospital setting and increasing resistance to antibiotics [5]. *P. aeruginosa* infections pose serious concerns for this patient population, making it an important bacterium to study in the scientific and medical community.

![Figure 2-3: The five stages of biofilm development.](image)

*P. aeruginosa* infections are linked to the bacteria’s formation of biofilms, hydrated polymeric matrices formed through bacterial aggregation [76]. Biofilm formation allows *P. aeruginosa* to survive in hostile environments and plays a key role in its survival in artificial settings such as the hospital [77]. Bacteria within biofilms are 10–1,000 times more resistant to antibiotics than in their planktonic form, making them remarkably difficult to treat [78]. The five stages of biofilm formation are shown in Figure 2-3. In the first stage of initial attachment, free-floating, planktonic bacteria adhere to the surface. This is followed by irreversible attachment where cells aggregate,
form colonies, and excrete extracellular polymeric substances (i.e., slime). The subsequent two stages are characterized by the formation and maturation of the biofilm. In the final stage, the biofilm reaches critical mass and disperses planktonic bacteria to colonize other surfaces. *P. aeruginosa* is renowned for its environmental persistence due to its biofilm, surviving for up to 16 months on inanimate surfaces [79]. Because these biofilms are also highly resistant to antimicrobial agents, they are thought to be responsible for chronic and persistent bacterial infections. Scanning electron microscopy images of biofilm formation of *P. aeruginosa* and *S. aureus* are shown in Figure 2-4.

![Figure 2-4: Scanning electron microscopy images of biofilm formation of A) *P. aeruginosa* and B) *S. aureus.*](image)

The study of *P. aeruginosa*'s biofilm formation pathway exposes important insights into this bacteria’s pathogenesis. Examples from literature are reported below.

Sauer *et al.* investigated *P. aeruginosa*’s biofilm development using direct observation and whole-cell protein analysis [80]. In this study, biofilms of *P. aeruginosa* (strain PAO1) were grown in continuous culture in a flow cell attached to a microscope stage, allowing for constant monitoring over the course of 12 days. Through this process,
the five distinct stages of biofilm development were identified. Each stage was characterized by a unique expression of proteins that would allow them to be exploited as regulation pathways. The authors found that the changes in protein patterns between each biofilm developmental stage was greater than expected [81], suggesting that the *P. aeruginosa* biofilm growth cycle is both complex and highly regulated. More importantly, identifying these protein regulation pathways holds important implications for designing effective methods for biofilm detection and control.

Singh *et al.* investigated biofilm development in patients diagnosed with cystic fibrosis [82]. *P. aeruginosa* colonizes the lungs of cystic fibrosis patients where it can persist for years despite the use of antibiotics [76, 83, 84], which further suggests the existence of a biofilm. As cited in the study, the authors isolated three strains from cystic fibrosis sputum that exhibited biofilm formation.

### 2.2.2 Wounds

*Pseudomonas aeruginosa* is well-recognized as a major organism contributing to healing delay in chronic wounds [85-88]. For patients undergoing split thickness skin grafting of chronic venous leg ulcers, the presence of *P. aeruginosa* is a predictor of skin graft outcome [89]. In clinical environments, bacterial culture methods are often inadequate for fully analyzing the microbial content of biofilm [90]. One of the challenges faced by the wound care community is the ability to distinguish between colonized and infected wounds [91, 92]. Current culturing and genetic methods do not provide quantitative bacterial counts, making it challenging for clinicians to determine when and how to treat wounds that are not healing appropriately and may be infected.
Notably, surgical site infections rank highest in healthcare-associated infections [93]. Giacometti et al. investigated 676 surgery patients with signs and symptoms indicative of wound infections. The second highest percentage of isolated bacteria from these wounds was *P. aeruginosa* [94].

All chronic wounds are colonized by bacteria, with low levels of bacteria considered beneficial to the wound healing process [95]. James et al. investigated biofilms in chronic wound specimens and found the presence of diverse polymicrobial communities including bacteria not revealed by culture [96]. Notably, the *Pseudomonas* species demonstrated the third highest prevalence in both chronic and acute wounds. Chronic non-healing wounds, such as leg ulcers, affect up to 2% of the population and continue to pose treatment challenges to clinicians [97, 98]. Clinical assessment of the wound bed for infection is based on subjective interpretation with little opportunity for objective analysis. However, there is evidence that monitoring surface pH may provide a means for measuring the condition of the wound and aid in determining the wound’s response to treatment [99]. The pH environment of chronic wounds has been recorded to be within the range of 7.15-8.19. The pH of the wound moves from neutral to more acidic as it progresses towards healing [100]. Bacteria that colonize the wounds produce ammonia at the wound site, which is toxic to the wound tissue, and also increases the pH of the surrounding environment [101]. This is significant because chronic wounds can harbor up to four different types of bacteria at any one time [102]. Wound healing is a complex physiological process. Monitoring pH may aid in assessing the condition of the wound and early colonization of bacteria. While diagnostic instrumentation for wound
healing and monitoring is still developing, there is a need for rapid tools that can be easily incorporated into the clinical setting.

2.2.3 Animal Infections

In veterinary care, *Pseudomonas aeruginosa* is also known to cause infections in a variety of animals. [103]. Bugden *et al.* found that *P. aeruginosa* was one of the most frequently isolated bacteria in samples taken from dogs with otitis externa [104]. Similar studies done by Adler *et al.* also found that cats are impacted by this bacterium, most notably in upper respiratory tract infections [105]. Reptiles, including snakes [106], and dairy herds infected with mastitis [107] are associated with this bacterium. However, there remain limited diagnostic tools for detecting *P. aeruginosa* in animals, with plate cultures obtained from swabs of animals remaining the gold standard for infection identification. Rapid identification using molecular diagnostics continues to be a viable opportunity; however, there remains a need to develop alternative rapid diagnostics for detecting bacterial infections in animals.

2.2.4 Quorum Sensing

It is well known that bacteria do not function as individual cells, but instead utilize a network of communication pathways to regulate many important physiological functions including gene expression, virulence, and biofilm formation [108-112]. This process, known as quorum sensing, involves the production and release of small molecules known as autoinducers that allow bacteria to monitor their environment and
alter their behavior in response to other bacteria, reflecting the behavior of multicellular organisms [113]. This is shown in Figure 2-5.

![Figure 2-5: Quorum sensing: A form of communication utilized by bacteria to regulate gene expression and the behavior of the cell community.](image)

For Gram-negative bacteria, the most common signaling molecules are N-acyl derivatives of homoserine lactone (acyl HSLs) [109, 114, 115]. Studies have shown that inhibiting these quorum sensing systems can reduce the virulence of *Pseudomonas aeruginosa* [116]. For *P. aeruginosa*, phenazine and pyocyanin production are highly regulated. Their production is controlled by two quorum sensing systems (Las and Rhl), a novel extracellular quinolone signal (PQS), and other components of the quorum sensing regulatory network [117]. These findings highlight the importance of studying quorum sensing as it can be exploited to control bacterial virulence.

### 2.2.5 Phenazines and Pyocyanin

*Pseudomonas* species produce a wide variety of pigments as secondary metabolites, which play an important role in the bacteria’s pathogenesis [15]. The
secondary metabolites include a variety of redox-active phenazine compounds (nitrogen-containing heterocyclic molecules). The structure and standard redox potential of the most common phenazines are listed in Table 2-1 [118].

**Table 2-1: Characteristics of phenazines excreted by *Pseudomonas* species.**

<table>
<thead>
<tr>
<th>Name</th>
<th>$R_1$</th>
<th>$R_2$</th>
<th>$R_3$</th>
<th>$R_4$</th>
<th>$E^0$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aeruginosin A</td>
<td>COOH</td>
<td>CH$_3$</td>
<td>NH$_2$</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Phenazine-1-carboxylic acid (PCA)</td>
<td>COOH</td>
<td></td>
<td></td>
<td></td>
<td>-177</td>
</tr>
<tr>
<td>Pyocyanin</td>
<td>OH</td>
<td>CH$_3$</td>
<td></td>
<td></td>
<td>-172</td>
</tr>
<tr>
<td>2-Hydroxyphenazine-1-carboxylic acid</td>
<td>COOH</td>
<td>OH</td>
<td></td>
<td></td>
<td>-34</td>
</tr>
<tr>
<td>Phenazine-1-carboxamide (PCN)</td>
<td>CONH$_2$</td>
<td></td>
<td></td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>1-Hydroxyphenazine (1-OHPHZ)</td>
<td>OH</td>
<td></td>
<td></td>
<td></td>
<td>-115</td>
</tr>
</tbody>
</table>

Phenazine compounds are produced through biosynthesis. The most common phenazine compounds secreted by *P. aeruginosa* are phenazine-1-carboxylic acid (PCA), pyocyanin, 1-hydroxyphenazine, and phenazine-1-carboxamide (PCN) [119]. The biosynthesis of these phenazines has been shown to be up-regulated by nutrient depletion, high bacterial cell density, and through the formation of a biofilm [120, 121].
While phenazines are produced by a variety of bacteria, pyocyanin (1-hydroxy-5-methylphenazine) is unique to *P. aeruginosa* [119]. Pyocyanin, characterized by its blue-green color, is considered both a virulence factor and quorum sensing molecule for *P. aeruginosa* [68]. Pyocyanin is naturally toxic to eukaryotic cells and has been shown to disrupt the beating of human cilia [122] and inhibit mammalian cellular respiration [123] and human lymphocyte proliferation [124]. Figure 2-6 shows a dilution series of pyocyanin from 0-100 µM in phosphate buffer solution. Notably, the characteristic blue color of pyocyanin decreases sharply at concentrations lower than 50 µM.

![Figure 2-6: Pyocyanin solutions (left to right: 100, 50, 10, 5, 1, 0 µM).](image)

The biosynthesis of pyocyanin has been previously explored [120, 125, 126]. Mavrodi *et al.* was the first to describe a biosynthetic pathway for pyocyanin as seen in Figure 2-7 [26, 120].
Figure 2-7: Phenazine biosynthesis pathway to synthesize pyocyanin, a redox-active biomarker for *P. aeruginosa*.

The first step in the proposed pathway involves the conversion of phenazine-1-carboxylic acid, a common phenazine precursor, to 5-methylphenazine-1-carboxylic acid catalyzed by the enzyme PhzM. The second step is then catalyzed by the enzyme PhzS, resulting in the formation of pyocyanin. A more thorough evaluation of this biosynthetic pathway was recently proposed, however, where the shikimic acid pathway is suggested as the primary metabolic pathway that branches into phenazine biosynthesis [127-129]. From this research, seven genes have been identified in the synthesis pathway of pyocyanin by *P. aeruginosa*. They include protein-encoding genes C, D, E, F, G, M, and S. Of these, only PhzM and PhzS are responsible for directly converting phenazine-1-carboxylic acid to pyocyanin [130-132]. Notably, *P. aeruginosa* is the only species that carries the two protein-encoding genes necessary to synthesize pyocyanin. Therefore, while phenazines are produced by a variety of bacteria, pyocyanin production is unique to *P. aeruginosa* [119].
A modified version of this biosynthetic pathway proposed by Pierson et al. shows the side products of chorismic acid as siderophores and three aromatic amino acids (phenylalanine, tryptophan, and tyrosine) [133]. Understanding the role of chorismic acid in phenazine biosynthesis is important because the compound plays a significant role as a branch-point in aromatic amino acid biosynthesis and iron-chelation [134]. This biosynthetic pathway leads to the possibility of using amino acids and iron as regulatory molecules towards phenazine biosynthesis.

### 2.2.6 Biofilm and Pyocyanin Up-Regulation

To better understand the role of biofilm in the pathogenesis of *Pseudomonas aeruginosa*, it is important to recognize the various regulatory pathways that lead to biofilm formation [135]. By identifying these pathways and regulatory molecules, it is possible to up-regulate the biofilm formation of *P. aeruginosa* (and subsequently pyocyanin production) as a means for faster bacterial detection. Previously identified up-regulatory compounds include select amino acids, iron, and *Pseudomonas* agar, which will be delineated in the subsequent sections.

#### 2.2.6.1 Amino Acids

Amino acids are the building blocks for protein synthesis and thus serve as key components for bacterial growth, such as the role of peptidoglycan in cell wall formation [136]. Amino acids have been shown to be growth substrates for *Pseudomonas aeruginosa* in sputum of cystic fibrosis patients, with higher amino acid concentrations reported in individuals with more severe cases of the disease as demonstrated by Thomas
et al. [121, 137]. In another study of cystic fibrosis sputum, Palmer et al. observed an increased production of *Pseudomonas* quinolone signal (PQS), an integral part of quorum sensing molecule production, in the presence of aromatic amino acids [121]. To further test this theory, the authors studied *P. aeruginosa* growth in three media types containing different amino acid constituents. The first media only contained serine, a non-aromatic amino acid, to serve as the control. The second media contained the aromatic amino acid, tryptophan, and the third had a combination of three different aromatic amino acids: tryptophan, tyrosine, and phenylalanine. While the addition of tryptophan and the three aromatic amino acid combination significantly increased the production of PQS by *P. aeruginosa*, the addition of serine had little effect on PQS production. The increase in PQS production was not due to an increase in *P. aeruginosa* cell growth count as the cellular densities between the three experiments were identical, which suggests that the aromatic amino acids were solely responsible for triggering PQS production. PQS is involved in the circuitry surrounding quorum sensing molecule production of *P. aeruginosa* and as a result, controls many genes that produce the proteins required for pyocyanin production [16, 138].

Previous work in the 1950’s began to explore the influence of amino acids on biofilm growth and pyocyanin production of *P. aeruginosa* [31, 32]. More recent work by Caiazza et al. found that arginine boosted biofilm growth [139]. Expanding upon this work, Bernier et al. looked at other amino acids to observe their effect on *P. aeruginosa* [29]. The authors used previously identified amino acids in cystic fibrosis sputum at concentrations typically found in this population as reported by Palmer et al. [140]. They tested concentrations at 2-, 4-, 8-, and 16-fold above typical concentration levels. A total
of 18 amino acids were tested to evaluate their ability to promote biofilm formation of *P. aeruginosa*. These were compared against a control, which only contained glucose with no amino acids. Each sample was grown in a microtiter well plate and stained with crystal violet solution. The quantification of biofilm biomass was taken by absorbance measurements at 550 nm. Each amino acid had varying regulatory effects on biofilm growth.

Bernier *et al.* further investigated the role of amino acid addition on the planktonic growth of *P. aeruginosa* using the top 11 biofilm-promoting amino acids. With the exception of proline, their results showed that the other 10 amino acids reduced planktonic growth when compared to the glucose control. After comparing and normalizing these two experiments, the authors identified seven amino acids that significantly increased the extent of biofilm formation. These amino acids (in order of increasing biofilm-inducing effect) were: isoleucine, arginine, leucine, phenylalanine, ornithine, tyrosine, and valine.

In the next phase of the study, the authors investigated the role of these seven amino acids on the swarming motility of *P. aeruginosa* and found that every amino acid demonstrated increased swarming motility with the exception of arginine. Overall, their results highlight the importance of amino acids in regulating *P. aeruginosa*’s biofilm formation and support their use as up-regulatory molecules.

### 2.2.6.2 Iron

Iron is an important molecule in bacterial growth. For bacterial pathogens such as *Pseudomonas aeruginosa*, the ability to obtain iron from transferrins, hemoglobin, and
other iron-containing proteins in their host is central to their survival [141, 142]. Iron acquisition systems in bacteria can be classified into two categories. The first mechanism involves direct contact between the bacteria and iron source. The second mechanism relies on compounds released by the bacteria into the extracellular medium to scavenge iron from various sources. These iron-chelating compounds are known as siderophores [143]. Recent work has shown that iron levels can serve as a signal for biofilm formation [144, 145]. Singh et al. demonstrated that lactoferrin, a mammalian secretion protein and iron-chelator, blocks *P. aeruginosa* biofilm formation. In the presence of lactoferrin, *P. aeruginosa* forms a thin layer of cells on glass surfaces and its individual cells exhibit incessant twitching, a form of surface motility, causing the bacteria to wander across the surface instead of forming cell clusters and biofilms [145]. While the mechanism for this phenomenon remains unknown, these findings further stress the importance of iron as a signaling molecule for biofilm formation of *P. aeruginosa*.

Pyocyanin plays an important role in iron metabolism where it is produced abundantly in media with low iron content [68, 146]. It has been shown that pyocyanin can generate free iron through a reduction mechanism from transferrin [147]. In addition, *P. aeruginosa* iron acquisition systems have been previously studied [148, 149]. *P. aeruginosa* synthesizes two iron-chelating siderophores, pyoverdine and pyochelin, which bind extracellular iron (Fe$^{3+}$) and transports them into the cell. *P. aeruginosa* also has a ferric uptake repressor (Fur) protein that functions as a regulator of iron-responsive genes [33, 150]. To better understand the role of iron in *P. aeruginosa* biofilm formation, Banin et al. studied a variety of *P. aeruginosa* mutants with defects in iron-uptake and regulatory functions [146]. Their findings showed that in the absence of lactoferrin and a
functional iron uptake system, *P. aeruginosa* forms thin, flat biofilms. *P. aeruginosa* was able to acquire iron using the siderophores, pyoverdine (at low iron concentrations) or pyochelin (at higher iron levels), but the mutant strains that lacked the ability to produce these siderophores could not form biofilms even in the presence of high levels of iron. A better understanding of biofilm regulation can be achieved by identifying and exploring these iron-regulated steps in biofilm formation.

Various studies using iron as a regulatory molecule for *P. aeruginosa* biofilm formation and pyocyanin production have been conducted. Musk *et al.* investigated the effect of iron salts on disrupting biofilm formation of *P. aeruginosa* [151]. They screened 4,509 compounds for biofilm inhibitory characteristics and found that ferric ammonium citrate inhibited biofilm formation in a dose-dependent fashion and disrupted previously formed biofilms in flow-channel experiments. As further evidence of this, *P. aeruginosa* strains from the sputum of cystic fibrosis patients showed a similar response to elevated iron levels. These studies found that both iron starvation (less than 1 µM) and high concentrations of iron (greater than 100 µM) inhibit biofilm formation, indicating that the optimal conditions for *P. aeruginosa* biofilm formation is somewhere between 1-100 µM of iron. Cai *et al.* investigated the influence of iron-depletion on twitching motility and quorum sensing in *P. aeruginosa*, finding that iron-depletion slows biofilm formation and increases twitching motility and expression of quorum sensing genes [152]. Yang *et al.* also showed that DNA release in *P. aeruginosa* biofilms is regulated by iron and lower levels of iron (5 µM) induces DNA release and biofilm formation [34].
2.2.7 Culture Parameters and Growth Media

Bacterial growth can be modeled with four different phases: lag phase, exponential or log phase, stationary phase, and death phase [153]. During the initial lag phase, bacteria adapt to their new environment and enter the exponential phase when they are ready to divide and grow. The exponential phase marks the beginning of bacterial growth, which varies considerably depending on the availability of nutrients in the environment. Bacteria eventually enter the stationary phase when growth is halted and upon the complete depletion of nutrients, they enter a phase of decline and die. Despite the model’s apparent simplicity, bacterial growth is not always linear as different bacteria can re-enter the growth phase from the stationary phase [154]. Bacterial growth is still not fully understood and varies from species to species [155]. The optimization of bacterial growth conditions is very important for the sustainability of *Pseudomonas aeruginosa* and its production of phenazines such as pyocyanin. Important factors affecting phenazine production by *P. aeruginosa* include cultivation temperature, pH, and the nutrients contained in the growth medium.

2.2.7.1 Temperature

Temperature plays a major role in the growth, metabolism, and enzymatic activity of microorganisms. Optimal growth temperatures can vary considerably. Slenting et al. found that *Pseudomonas* phenazine production, specifically phenazine-1-carboxylic acid (PCA), was very sensitive to culture temperature [156]. While high cell accumulations were achieved over a broad 25-34 °C range, varying PCA production was observed in specific temperature regimes. High, moderate, or low PCA production was observed at
25-27 °C, 29-32.5 °C, or 34 °C, respectively. Similarly, Knight et al. found that 50% of clinical *Pseudomonas aeruginosa* isolates produced pyocyanin at 37 °C [157].

### 2.2.7.2 pH

pH plays a major role in microbial enzyme production where even minor changes in pH can alter the metabolic pathways of microorganisms. In phenazine production, Slininger et al. found that phenazine-1-carboxylic acid production is sensitive to culture pH [156]. High and low phenazine production was observed at a culture pH of 7 or 8, respectively [156]. Optimum pH for phenazine production was observed at approximately 7, which is in agreement with the preparation of *Pseudomonas* agar (King’s A media), a specialized media that selectively identifies *Pseudomonas* species from other clinical specimens based on the bacterium’s production of blue-green pyocyanin observed on the agar [158].

### 2.2.7.3 Trypticase Soy Media

Trypticase soy is a bacterial growth medium and can be prepared as either a broth (TSB) or an agar. It contains glucose as an energy source as well as a variety of bacterial nutrients such as enzymatic digests of casein and soybean that provide the necessary amino acids for bacterial growth. Trypticase soy media largely supports the growth of common aerobic and facultative anaerobic bacteria, making it an ideal choice for growing bacterial pathogens such as *Escherichia coli* and *Pseudomonas* species [159, 160].
2.2.7.4 M63 Minimal Media

M63 is a minimal media used for bacterial growth of species such as Escherichia coli and Pseudomonas [29, 161, 162]. This media contains glycerol and glucose, providing both a carbon and energy source. It also has a variety of salts such as ammonium sulfate, potassium phosphate, and ferrous sulfate which supply bacteria the nitrogen, phosphorous, and trace metals needed for growth [163]. This media contains magnesium sulfate, which provides the magnesium ions essential for the enzymatic reaction of DNA replication and processing [164].

2.3 Electrochemical Sensors

Electrochemical sensors are increasingly being used for studying biological processes [165]. These well-established sensors demonstrate high sensitivity and can provide real-time monitoring of chemical processes through in situ measurements without sampling. Recent advances in sensor technologies in this field include: miniaturization of sensors to study biological systems in cell cultures, cell tissues, and living organisms; application of multi-sensor setups for measuring pH and dissolved oxygen in lab-on-a-chip systems; and the development of improved electrochemical measuring techniques.

2.3.1 Miniaturization of Sensors

Many commercially available electrochemical sensors have dimensions that do not allow for the direct measurement of small volume media such as blood, sweat, and other biofluids [165]. Recent advances in micro- and nanofabrication techniques, however, have led to the replacement of traditional bulky electrodes and electronic
equipment with miniaturized electrochemical systems [166]. Through this more portable approach, analytical information is obtained using a faster and cheaper method than traditional laboratory-based assays.

2.3.1.1 **Thick-Film Technology**

Miniaturization has led to the development of thick-film sensors that allow for integrated circuits to be hybridized into miniaturized packages with structural stability [167]. One common thick-film sensor technology is screen-printing, which has evolved into a promising technology for fabricating miniaturized planar electrochemical sensors [168]. One of the most well-known healthcare applications of screen-printing technology has been the development of blood glucose sensors for individuals with diabetes [169]. These sensors incorporate glucose oxidase on a microelectrode array to yield effective amperometric test strips for glucose. Screen-printing methods have also led to the development of miniaturized devices for continuous monitoring. Lam *et al.* designed a disposable screen-printed oxygen biosensor based on an amperometric Clark cell [170]. This configuration utilizes a three-electrode setup with gold working and counter electrodes and a silver/silver chloride reference electrode. This design was screen-printed onto an alumina substrate and then tested for its response to different oxygen levels. The performance of the sensor using different screen-printed membrane materials was also evaluated using cyclic voltammetry. The sensor was able to detect changes in oxygen levels with a relatively short delay in response time, making this a viable option for continuous oxygen monitoring with consideration to the sudden variations in oxygen levels that can occur almost instantaneously.
Other applications of screen-printed sensors are reported. Prasad et al. drop-coated a *Shewanella* bacterial suspension onto a disposable screen-printed carbon electrode [171]. Upon evaluation with cyclic voltammetry, the authors demonstrated the formation of an electroactive bacterial layer with direct electron transfer properties. Applications of this functionalized bacterial-modified electrode include the electrocatalytic reduction of arsenite, hydrogen peroxide, and nitrite. Ho et al. developed an electrochemical immunoassay for the detection of carcinoembryonic antigen, a protein tumor marker, using carbon nanoparticle polyethylene imine modified screen-printed graphite electrodes [172]. By using CdS nanocrystals as biotracers and carbon nanoparticles to enhance electron transfer, the authors were able to amplify the sensor’s electrochemical signal and create a novel system for the early detection of urothelial cell carcinoma. Liao et al. utilized gold-electrodeposited screen-printed electrodes modified with a self-assembled monolayer of single-stranded DNA for the detection of the rfbE gene, a gene specific to *Escherichia coli* O157:H7 [173]. The authors report the detection of the rfbE gene at an ultratrace level in the attomolar range, which was achieved through electrochemical signal amplification by using a redox-active hexaammineruthenium(III) (RuHex) as a competitive assay. This highly sensitive and rapid sensor is promising towards monitoring the food pathogenic bacterium *E. coli* O157:H7.

2.3.1.2 *Thin-Film Technology*

Thin-film technology is another field developed to target the miniaturization of sensors. Thin-films are utilized in a variety of biosensor applications, such as in biological recognition and amplification as well as electronic signal processing [174].
Using these techniques, it is possible to produce layers of different materials with a thickness of 10-20 µm [175]. A wide range of techniques can utilize thin-film technology such as electrochemistry, optical spectroscopy, surface plasmon resonance, and quartz crystal microbalance measurements [176].

One of the most established technologies for thin-film patterning is optical lithography, or more commonly known as photolithography [177]. This process patterns a photosensitive film from a mask to a substrate. Common substrate materials include silicon, silicon dioxide, silicon nitride, and polycrystalline silicon [178]. Many approaches towards utilizing thin-film technology in biosensors have recently been reported. Pfeiffer et al. developed an amperometric lactate oxidase catheter for in vivo application to real-time lactate monitoring [179]. In this study, the authors deposited silicon dioxide onto 0.7 mm thick glass slides. On the substrate, they created a two-electrode configuration system where titanium was used as the adhesion layer. The metals deposited to form the electrodes were processed under high vacuum systems using standard metal evaporation deposition techniques. This produced a platinum working electrode with an area of 0.2 mm² and a thickness of 1.5 µm. The working electrode was surrounded by a silver/silver chloride counter electrode with an area of 1 mm² and a thickness of 15 µm. Lactase oxidase was immobilized onto the electrode setup by mixing it with a polyurethane matrix and depositing it on the working electrode. The authors state that the electrochemical behavior of the platinum electrode is not significantly influenced by the lactate oxidase-polyurethane covering. The performance of the sensor was tested in vivo by inserting it into the skin of a dog, demonstrating the applicability towards real-time analysis.
Thin-film potentiometric pH sensors for clinical and biological applications have additionally been developed by Lakard et al. [180]. These sensors are based on films of linear polyethylenimine and polypropyleneimine, two synthetic enzymes and biocompatible polymers. These polymers have been shown to be good candidates as pH biosensors due to their sensitivity to proton gradients and relatively easy immobilization onto an electrode surface [181]. The sensors were fabricated using photolithography to pattern platinum electrodes on a silicon dioxide substrate. One electrode was covered with a polymeric film while the other was coated with silver to act as a reference electrode. By using an interdigitated electrode array, the authors were able to achieve higher sensitivity by increasing the exchange surface between the polymer coated electrode and the analyte in solution. The authors tested the buffered solutions and found that the sensors showed a linear relationship between the measured potentiometric response and pH. Given this linear potentiometric response to pH gradients, the chemically modified electrodes show potential for clinical applications such as monitoring pH in bacterial infections [182].

Additional commercial applications for thin-film sensors are demonstrated by the work of Revzin et al. who fabricated gold microelectrode arrays to detect glucose, lactate, and pyruvate [183]. The authors fabricated the electrodes on silicon dioxide and Mylar substrates using standard photolithographic techniques combined with metal deposition. The electrodes were functionalized with a negatively charged monolayer of 11-mercaptoundecanoic acid followed by a complex structure of redox polymers and anionic enzymes that consisted of either glucose oxidase, lactate oxidase, or pyruvate oxidase. The resulting sensors demonstrated high sensitivity and were tested for reproducibility
with standard deviation in measurements ranging between 18-20% depending on the target analyte. In addition, the sensors gave a linear range for physiologically relevant concentrations for glucose (0-20 mM), lactate (0-10 mM), and pyruvate (0-2 mM). To validate their sensor design, the authors further investigated the possibility for crosstalk between adjacent array members and found minimal signal interference. This sensor design illustrates the potential use of fabricating cost-effective multianalyte sensors to detect a wide range of biological molecules for clinical and industrial applications.

2.3.1.3 Microelectrodes

Miniaturization has also led to the investigation of different sizes and shapes of microelectrode fabrication [184]. Microelectrodes are attractive due to their fast establishment of steady-state mass transfer and small capacitive charging currents [185]. Generally, microelectrodes are defined as having one dimension smaller than 20 µm [186]. At this size, radial diffusion cannot be ignored as with larger electrodes. This is advantageous because it provides the device with a high current density value and rapid steady-state [187]. Due to this high current density, the device is able to test high resistance media (little to no supporting electrolyte) with high sensitivity [188].

The use of interdigitated electrode arrays has also gained interest. In this design, the electrodes are formed in a series of interconnected parallel microbands with a spacing of only a few microns [175]. This electrode design is advantageous because of its applicability for detecting electrochemically reversible materials. In addition, the design leads to a significant decrease in detection limits for reversible redox species such as ferrocene derivatives by redox cycling, a feedback effect process in which species
oxidized at one electrode will diffuse back to the other electrode and become reduced [185, 189]. Niwa et al. investigated redox cycling with interdigitated electrode arrays with different geometries and found that collection efficiency of the electrodes depended on the band electrode width and the gap between adjacent electrodes [185]. As the width and gap decreased, the number of redox cycles rapidly increased. Reported measurements were highly sensitive (10 nmol/dm$^3$) with a wide dynamic range (10 nmol/dm$^3$ to 1 mmol/dm$^3$).

A common application of interdigitated electrodes is in the construction of enzymatic biosensors. Niwa et al. investigated small volume voltammetric detection of 4-aminophenol using interdigitated electrode arrays as an application towards an electrochemical enzyme immunoassay [190]. The gold electrode array was fabricated using photolithography and dry etching techniques on a silicon substrate. The array consisted of 50 pairs of microbands, each with a width of 3 to 5 microns and a spacing of 2 to 5 microns. With this sensor, the authors established a linear relationship between 4-aminophenol concentration and the cathodic limiting current. This relationship was reproducible in the concentration range of 1 to 100 µM. This setup was also used to detect mouse IgG, illustrating its applications as an electrochemical immunoassay.

Interdigitated electrodes have also been utilized as electrochemical DNA biosensors as seen in the work of Finot et al. [191]. The authors described the fabrication of interdigitated nanoelectrode arrays with an area of 100 × 50 microns, electrode band width of 100 nm, and inter-electrode spacing of 200 nm. When the sensitivity and response time of these nanoelectrodes were compared to larger designs, the interdigitated design gave faster and more sensitive measurements in most cases, especially when
paired with square-wave voltammetry. By modifying the electrode surface with DNA and coupling it with a redox mediator (hexaammineruthenium(III) chloride), the setup was able to detect nucleotide bases from single- and double-stranded DNA. There is potential for using this sensor as a label-free method to study DNA interactions.

2.3.1.4 Lab-on-a-Chip Systems

Recent advances in thick- and thin-film technologies as well as the development of microelectromechanical systems (MEMS) have resulted in the development of sophisticated electrochemical lab-on-a-chip systems [165, 192]. MEMS bring together silicon-based microelectronics and micromachining technology by integrating mechanical elements such as valves and pumps with sensors on a common silicon substrate [193]. Lab-on-a-chip systems were developed as tools for high-throughput analysis and cost reduction. These systems, defined by their miniaturization of biological assays as well as the parallelization of analysis, have recently opened the possibility of expensive scientific undertakings such as mapping the complete human genome [194].

In 1990, Manz et al. first discussed the concept of lab-on-a-chip devices for integrated chemical and biochemical analysis, a term they called a total analysis system (TAS) [195]. TAS devices are all-in-one sensors where sampling, sample transport, any necessary chemical reactions, detection, and signal evaluation are carried out automatically. A series of miniaturized total analysis systems (µTAS) have been envisioned for commercial and industrial applications, including small flow injection analysis [196], open-tubular column chromatography [197], and gas chromatography [198]. In one of the first attempts at designing a µTAS device, Manz et al. used
micromachining to fabricate a chip-like structure to run capillary electrophoresis in what the authors state as a first step towards an integrated microflow system [199]. Since this effort, the concept of lab-on-a-chip and µTAS devices has grown considerably where applications now include proteomics, drug and chemical development, and point-of-care analysis [200, 201]. By incorporating processes such as injection, transport, separation, reaction, and detection in a single miniaturized sensor, the applications for miniaturized electrochemical sensors are nearly limitless.

2.3.2 Improved Electrochemical Measuring Techniques

With the emergence of small, cheap electrochemical equipment, dynamic electrochemical techniques such as electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV), and square-wave voltammetry (SWV) have gained more interest [165].

2.3.2.1 Electrochemical Impedance Spectroscopy

Electrochemical impedance sensors measure the resistance that a circuit presents when a current or voltage is applied as the frequency is varied. This means it measures the dielectric properties of a medium as a function of frequency. Similarly, electrical impedance spectroscopy (EIS) tracks changes in magnitude and phase versus frequency between small sinusoidal input voltages measured across a medium. This method was developed and discussed by McDonald et al. to capture small changes in coatings on electrodes [202]. One potential application of this method is the development of tissue impedance measurements using miniaturized electrodes as in vivo sensors to monitor
wound healing by means of EIS [203, 204]. Karp et al. investigated the foreign body response to implanted biosensors using in situ EIS [205]. As various biological structures have predictable frequency-dependent effects on electrical impedance [206], the authors tracked changes in electrical signal over time between micro-electrode arrays implanted in a chick embryo. As the needle array became encapsulated in collagen by the foreign body response, a time-dependent degradation of the current signal was observed providing valuable information on the physiological response to the implantation of a biosensor device. These results suggest this sensor can potentially be used to assess the biocompatibility of various coatings and surface treatments.

Impedance-based biosensors for bacterial detection have also been reported. This application involves the investigation of electrical properties of bacterial cells when they are attached to electrodes [207]. With the miniaturization of electrochemical sensors, impedance biosensors for rapid bacterial detection have gained further interest. Yang et al. reviewed impedance techniques as a means to detect and quantify foodborne pathogenic bacteria with interdigitated microelectrode arrays and microfluidic-based impedance biosensors [208].

2.3.2.2 Cyclic Voltammetry

Cyclic voltammetry (CV) is a well-established electrochemical technique that can determine oxidizable and reducible species in solution [165]. With this technique, the potential is changed linearly in time and a current response is measured where the current density correlates to the concentration of the redox species. However, this current response is highly dependent on the scan rate (V/s) of the measurements and electrode
A variety of different electrode materials have been explored, including glassy carbon, carbon fibers, carbon nanotubes, and conducting polymers [209-211]. Stamford et al. used fast cyclic voltammetry (scan rates of 100 V/s or greater) on carbon fiber microelectrodes in brain slices to measure neurotransmitters in real time [212]. This *in vivo* measurement demonstrated that it is possible to selectively identify neurotransmitters, such as dopamine and serotonin, from other biomolecules. Similarly, Jackson *et al.* used fast scan cyclic voltammetry to detect 5-hydroxytryptamine (serotonin) both *in vitro* and *in vivo* [209]. Akiyama *et al.* and Kumar *et al.* utilized cyclic voltammetry to detect dopamine concentrations in the brain [210, 211].

Applications towards using cyclic voltammetry for bacterial detection have also been reported. Kumar *et al.* developed a microbial biosensor using a screen-printed carbon electrode and cyclic voltammetry to detect methyl parathion, an organophosphorus hydrolase enzyme that can be hydrolyzed into the redox-active product p-nitrophenol [213, 214]. Using this sensor, the authors immobilized whole cells of recombinant *Escherichia coli* on screen-printed carbon electrodes using glutaraldehyde. The sensor demonstrated a wide linear detection range for methyl parathion (2-80 µM) and high specificity with minimal to no interference from other biological molecules, such as glucose and sucrose.

### 2.3.2.3 Square-Wave Voltammetry

Square-wave voltammetry (SWV) is a high-speed scanning technique characterized by its excellent sensitivity and rejection of background currents [215]. Compared to cyclic voltammetry, square-wave voltammetry has a significantly lower
limit of detection and broader dynamic range because of its efficient discrimination of capacitance current. As such, the observed peak height is directly proportional to the concentration of the electroactive species and detection limits of redox species as low as 10 nM are possible. Applications of square-wave voltammetry include the study of electrode kinetics and determining chemical species at trace levels [216, 217]. Lidong et al. developed an aptamer biosensor to detect angiogenin using square-wave voltammetry [218]. Angiogenin is related to the growth and metastasis of numerous tumors and thus is an important molecule to detect and monitor in cancer treatment. An anti-angiogenin aptamer was used as a molecular recognition element and immobilized onto the surface of a gold electrode. A ferro/ferricyanide redox probe was used as it elicits a very strong signal from SWV. Introducing angiogenin into the system results in less available binding sites for redox to occur, and correspondingly a decreased current. The authors introduced varying levels of angiogenin into the system and found that increasing concentrations led to a decreased current signal. They reported a linear range over 0.01 nM to 30 nM with a detection limit of 1 pM. To test the validity of the sensor in complex media, the authors looked at the sensor’s response to other proteins in serum and found that it exhibited high selectivity for angiogenin. As a rapid, selective, and label-free method for angiogenin detection, this sensor is a promising strategy for monitoring cancer development.

Other applications of square-wave voltammetry include detecting bacteria in water-polluted samples [219] and other areas such as medical diagnostics [220], environmental monitoring [221], food monitoring [222], and enzyme kinetics [223].
Chen et al. present a broad overview of biosensors utilizing SWV and its potential towards next generation point-of-care diagnostic devices [224].

### 2.3.2.4 AC Voltammetry

AC voltammetry (ACV) is similar to square-wave voltammetry. Instead of a linear staircase waveform, ACV applies a sinusoidal oscillating voltage to the electrochemical cell. While not as commonly used as the differential pulse voltammetry techniques, the sensitivity and detection limits of AC voltammetry are similar [225, 226]. AC voltammetry has been previously used as a highly sensitive detection method. Swisher et al. detected the redox signal of ferrocene using nanoelectrode arrays fabricated with vertically aligned carbon nanofibers [227].

### 2.3.3 Reference Electrodes

Reference electrodes are useful in electrochemical systems due to their ability to provide a stable potential during testing. Two reference electrodes used in this dissertation will be discussed: the common silver/silver chloride reference electrode and the palladium hydride reference electrode.

#### 2.3.3.1 Silver/Silver Chloride (Ag/AgCl)

One of the most commonly used reference electrodes for aqueous solutions is the silver/silver chloride (Ag/AgCl) electrode [215]. The following reaction is used to characterize the Ag/AgCl electrode:

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

\[(2.1)\]
According to equation 2.1, the activity and chemical potential of this electrode are dependent on the concentration of the chlorine ions in the solution [228]. With further evaluation using the Nernst equation, a relationship between the chemical potential of the Ag/AgCl electrode and the activity of chloride ions is derived. Due to this relationship, the Ag/AgCl electrode is able to hold a stable potential when it sits in a rich chloride filling solution, which causes a change in chloride content but prevents any deviations in the electrode’s chemical potential.

The miniaturization of Ag/AgCl electrodes has led to a wide range of applications such as a long-term stable reference in human serum samples [229] and soil salt measurements [230]. However, one of the key problems with microfabricated Ag/AgCl reference electrodes is the depletion of silver or silver chloride ions in the solution, which results in the degradation of the electrode [228]. These microfabricated Ag/AgCl reference electrodes without a filling solution, a type of quasi-reference electrode, have been gaining popularity due to their simplicity of fabrication and relatively good performance. By definition, quasi-reference or pseudo-reference electrodes do not contain an internal electrolyte. While this makes them easier to manufacture, it causes the chemical potential to drift when inserted into solution. To address this, miniaturized systems utilize integrated electronic circuits to compensate or cancel out the reference electrode drift. Another commonly used method to improve its stability has been to coat the Ag/AgCl electrode with a polymer or gel such as agar or polyurethane [231, 232]. These coatings act as a diffusion barrier to slow the rate of silver chloride degradation while also providing a relatively constant concentration of chloride ions at the Ag/AgCl surface. Zhou et al. adapted this technique by using nanofluidic channels as a salt bridge.
to fabricate microfluidic Ag/AgCl reference electrodes on a disposable microchip [233]. Another simple solution is to increase the amount of chloride on the surface of the electrode. Polk et al. created micrometer-sized quasi-reference electrodes by electroplating silver onto the setup [234]. The electroplated silver layer provides a larger electrochemically active surface due to its rough surface morphology. It also forms a quasi-bulk phase, which allows for a much thicker layer of silver chloride. Altogether, this device demonstrated improved stability and a longer lifetime.

2.3.3.2 Palladium Hydride (Pd/H₂)

Another useful reference electrode is the palladium hydride electrode, which acts like a reversible hydrogen electrode. The associated reaction is as follows:

\[
2H^+(aq) + 2e^- \rightleftharpoons H_2(g)
\]  

(2.2)

While it is similar to the standard hydrogen electrode, the palladium hydride electrode has the ability to absorb molecular hydrogen [235]. Because of this, electrochemical systems that utilize a palladium hydride reference are sensitive to changes in pH where increasing the pH decreases the electrode potential [19]. Imokawa et al. fabricated nanostructured palladium hydride pH microelectrodes by electrodepositing palladium films onto platinum microdisks [236]. The palladium metal is capable of storing 900 times its volume of hydrogen in its lattice due to the formation of the \( \alpha + \beta \) Pd hydride phase. This phase is known to behave as a hydrogen electrode and thus yields a Nernstian dependence of potential on pH. The authors characterized their sensor and found that the potentiometric pH response was rapid, stable, and reproducible over the pH range of 2-12. This device design is also advantageous because
it does not require calibration before taking measurements, a requirement of most other potentiometric pH sensors.

2.3.4 Electrochemical Detection of Bacterial Biomarkers

The use of electrochemical sensors to study bacterial pathogens continues to be a developing field. Direct detection methods include monitoring redox-active metabolites uniquely produced by a bacterial species [17]. Monitoring pH at the site of infection is also a useful biomarker for bacterial infection [237]. These two markers, as they relate to electrochemical detection, are discussed below.

2.3.4.1 Pseudomonas aeruginosa

Multiple approaches towards detecting *Pseudomonas aeruginosa* have been reported. Yoon *et al.* showed that *P. aeruginosa* biofilms can be monitored via electrochemical impedance spectroscopy (EIS) [238] and cyclic voltammetry [239]. In these reports, EIS measured changes in impedance as the cells attached to the electrode surfaces. Cyclic voltammetry was also used to differentially monitor bacterial attachment and biofilm formation on platinum electrodes, deriving quantitative information about the level of bacterial attachment to the surface coverage of the electrode.

Sharp *et al.* were the first to report an inexpensive approach to detecting *P. aeruginosa* using carbon. They fabricated a carbon fiber sensor to detect the presence of pyocyanin, a unique quorum sensing molecule secreted by *P. aeruginosa* [17]. As pyocyanin is redox-active, it can be observed using standard electrochemical techniques.
such as cyclic and square-wave voltammetry. Figure 2-8 shows the redox-active structure of pyocyanin.

![Chemical structure of pyocyanin](image)

**Figure 2-8:** Chemical structure of pyocyanin (reduced and oxidized forms).

**Oxidation of pyocyanin occurs around -0.25 V vs. a Ag/AgCl reference electrode.**

With the carbon sensor developed by Sharp *et al*., the authors achieved high sensitivity (detection limit of 0.030 µM) and a linear detection range for pyocyanin across a biomedically relevant concentration range of 1-100 µM. Webster *et al*., built upon this work and utilized nanofluidic electrode assemblies to amperometrically monitor changes in pyocyanin concentration [20]. By employing a closely-spaced electrode geometry, the authors were able to achieve a low detection limit (1.07 µM) through redox cycling. These electrochemical approaches are much faster than previously established techniques including chloroform extraction, a common liquid-liquid phase extraction technique that separates the pyocyanin supernatant into an organic phase [70, 71] and using selective growth media such as *Pseudomonas* agar, developed by King *et al*., to selectively isolate *Pseudomonas* species from other clinical specimens [24, 158]. Overall, this work demonstrates the potential of using electrochemistry to monitor *P. aeruginosa* infections.
2.3.4.2 pH

Sensors that utilize potentiometric pH indicators have been reported [19]. pH at the site of infection plays a pivotal role in the biochemical reactions taking place, inherently affecting the pathogenesis of a bacterial infection. While the natural pH of skin varies from person to person, it normally falls within a narrow acidic range between pH 4 and pH 6 which impedes bacterial proliferation [240]. However, a wound exposes the underlying tissue, which is normally regulated at pH 7.4, a relatively neutral pH that encourages bacterial growth and promotes infection. At the onset of infection, bacteria will attempt to increase the pH of their surroundings in order to support their growth [182, 241]. In an effort to detect pH as a means for monitoring bacterial growth, Phair et al. developed a disposable electrochemical pH sensor for point-of-care applications [237]. The authors modified commercially available screen-printed electrodes utilizing the three-electrode configuration with a carbon working and counter electrode as well as a Ag/AgCl pseudo-reference. Using uric acid as a biomarker for the measurement of wound pH, the authors used square-wave voltammetry to measure the electrochemical oxidation of urate to allantoin under various pH regimes. The oxidation peak of urate occurs at 0.3 V at a pH of 7.2 and shifts to more positive potentials as the pH becomes more acidic. The authors also validated their results in whole blood, further illustrating the applicability of using sensors as means for wound monitoring.

More recently, Phair et al. developed a carbon fiber weave as a modified material for electrochemical pH wound monitoring [242]. Both of these approaches use pseudo-Ag/AgCl reference electrodes that assume that the chloride concentration at the site of infection remains relatively constant, but this is not always true given the variability of
wound infections. A more novel approach was developed that measured the potential difference between two redox peaks to determine pH, providing a calibrationless pH sensor that is not dependent on the stability of the reference electrode. These approaches utilize pH sensitive redox indicators such as hydroquinone alkylthiol [243], anthracene [244], or a nitrophenyl group [245] versus the pH insensitive redox species alkylferrocene. While these techniques are novel, they have yet to be tested in complex media, such as human biofluids, which contain an assortment of electroactive compounds that may interfere with the redox indicators.

The ability to monitor bacterial biomarkers such as pyocyanin and pH simultaneously in a single device is promising towards applications in the hospital setting, specifically in the form of a smart bandage containing a miniaturized electrochemical sensor that can rapidly detect the onset of bacterial infections [39, 40]. The development and optimization of these sensors will allow for a cheap, rapid detection method for *Pseudomonas aeruginosa* and other bacterial pathogens in the hospital setting.

### 2.4 Alternative Electrochemical Sensing Strategies

While the direct electrochemical detection of redox-active quorum sensing molecules is a straightforward method to monitor bacteria, this option is not always available. One alternative is the coupling of electrochemistry with naturally existing biomolecules for molecular recognition techniques that are sensitive and selective [246]. One such example is the use of an electroactive species as a label or tag where its electrochemical properties would change following molecular recognition. While
electrochemical labeling provides an alternative way to detect a biomolecule, labeling may change the properties of the host which may not be immediately known or predicted.

2.4.1 Aptamer Sensors

Aptamers are short, single-stranded nucleic acids (DNA or RNA) that selectively bind to low-molecular-weight substrates or macromolecules such as proteins [247]. Derived from the Latin word “aptus,” meaning “to fit” [248], these oligonucleotide ligands fulfill molecular recognition needs such as found in diagnostics and therapeutics [249, 250]. Aptamer-based detection is a relatively new field that attempts to address the limitations of using antibodies as molecular recognition elements. The use of antibodies originated in the 1950s and has since become wide-spread and well-established, but one of the challenges with developing antibody recognition elements is that it starts within an animal, a complex in vivo requirement that entails a laborious and expensive identification process [247, 251]. In contrast, aptamers are identified through an in vitro process that allows for greater ease and control over their molecular recognition properties. This process, known as SELEX (Systematic Evolution of Ligands by Exponential Enrichment), was first developed in the 1990s as a means of screening very large combinatorial libraries of oligonucleotides by iterative in vitro selection and amplification [248, 252, 253]. In this process, a target molecule (a small molecule, protein, or supramolecular structure) is identified and exposed to a library of random oligonucleotides (approximately $10^{15}$ oligonucleotides). The oligonucleotides that bind to the target are considered aptamers, which are amplified and put through several additional selection steps while non-binding oligonucleotides are separated. Finally, the
leftover high affinity aptamers are isolated and sequenced. This process makes it possible to determine aptamers faster and cheaper than it would be to develop antibodies. As a result, aptamers for hundreds of targets have been developed [254].

Known for their high affinity and remarkable specificity, aptamers have contributed to tremendous advances in electronic biosensor design, particularly in detection schemes with integrated aptamer devices known as aptasensors [37, 255, 256]. Nucleic acid aptamers fold their flexible, single-stranded chains into well-defined three-dimensional structures upon binding to their target molecules [37]. By tethering the aptamer with a redox-active molecule, it is possible to probe the aptamer’s binding interactions electrochemically. Various electrochemical detection methods based on this strategy have been developed, including electrochemical impedance spectroscopy [257], electrogenerated chemiluminescence [258], cyclic voltammetry [259], and amperometry [260]. Common redox-active reporter molecules for electrochemical signal transduction include methylene blue [261], ferrocene [262], ferrocene-bearing polymers [263], and ruthenium complexes [258].

One possible use of aptasensors is in the detection of *Acinetobacter baumannii*, a primary cause of ventilator-associated pneumonia in patients in intensive care units [264, 265]. *A. baumannii* is another ESKAPE pathogen of concern among the growing pool of antibiotic-resistant bacteria in the hospital setting [4]. *Acinetobacter* species are aerobic, Gram-negative, non-motile coccobacilli (short plump rods) measuring 1.0-1.5 by 1.5-2.5 μm [265]. They display high environmental persistence, surviving for up to 5 months on inanimate surfaces [79]. The National Nosocomial Infections Surveillance System has shown that the rate of antimicrobial resistance has increased primarily among
Acinetobacter species and Pseudomonas aeruginosa [266]. Due to their inherent resistance to antibiotics and opportunistic behavior, these ubiquitous organisms plague the nosocomial environment where they infect those with compromised immune systems causing pneumonia, meningitis, septicemia, and urinary and respiratory tract infections [264]. In this respect, A. baumannii is very similar to P. aeruginosa and both have caused serious concern for the future of antibiotic healthcare.

Previous work done by Norton et al. has identified one gene (A1S_1389) conserved by all strains of A. baumannii [267, 268]. A1S_1389 has been linked as a DNA damage response gene where it plays the role of a regulatory molecule [269, 270]. More importantly, the protein encoded from this gene presents a novel sensing strategy to apply towards detecting A. baumannii infections with aptamers specific to A1S_1389. A relatively simple detection strategy that can be applied to this scenario was demonstrated by Cash et al., who used small molecule recognition elements as a means of electrochemically detecting the presence of macromolecules in solution [271]. The development of a sensor that can detect A. baumannii would be a major step towards point-of-care diagnostics in the hospital setting and more importantly, address the multi-facet ordeal that antibiotic-resistant ESKAPE pathogens present for healthcare.
3.0 Research Aims

This dissertation proposes the following four aims for its completion:

Aim 1: The Up-Regulation of Pyocyanin Production by *Pseudomonas aeruginosa* for Rapid Bacterial Identification

Aim 2: Screening Other Bacterial Pathogens for Redox-Active Biomarkers for Electrochemical Detection

Aim 3: Developing Alternative Electrochemical Sensing Strategies for Bacterial Detection

Aim 4: Applications of Electrochemical Sensors towards the Hospital Setting

3.1 Aim 1: The Up-Regulation of Pyocyanin Production by *Pseudomonas aeruginosa* for Rapid Bacterial Identification

The following lists the sub-aims to reach completion:

- Identify a series of molecules that can up-regulate biofilm formation and pyocyanin production of *P. aeruginosa*.
- Spike molecules into samples containing *P. aeruginosa* while electrochemically monitoring the production of pyocyanin.
- Investigate the role of compound concentration and initial bacterial concentration on detection times.
3.2 **Aim 2: Screening Other Bacterial Pathogens for Redox-Active Biomarkers for Electrochemical Detection**

The following lists the sub-aims to reach completion:

- Screen other bacterial pathogens for possible redox-active biomarkers.
- Determine how conserved pyocyanin is in all strains of *Pseudomonas aeruginosa* and if other *Pseudomonas* species produce it.

3.3 **Aim 3: Developing Alternative Electrochemical Sensing Strategies for Bacterial Detection**

The following lists the sub-aims to reach completion:

- Develop an aptamer-based electrochemical sensor to detect bacterial pathogens.
- Test a positive and negative control for this aptamer.

3.4 **Aim 4: Applications of Electrochemical Sensors towards the Hospital Setting**

The following lists the sub-aims to reach completion:

- Develop a sensor that measures both pH and pyocyanin for *P. aeruginosa* detection.
- Explore the relationship between drug resistance and pyocyanin production for *P. aeruginosa*.
- Validate electrochemical platform for detecting pyocyanin in human and animal samples containing *P. aeruginosa*. 
## 4.0 Methodology

### 4.1 Materials

#### 4.1.1 Instruments, Equipment, and Fabrication Tools

**Table 4-1: Instruments and Equipment**

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<th>Model</th>
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<td></td>
<td></td>
<td>CHI111 (Ag/AgCl Reference Electrode w/ porous Teflon Tip)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CHI115 (Platinum Wire Counter Electrode)</td>
</tr>
<tr>
<td>Micropipette (2.5, 20, 200, and 1000 µL)</td>
<td>Eppendorf (Hamburg, Germany)</td>
<td>Research and Reference</td>
</tr>
<tr>
<td>Microscope Camera</td>
<td>Zeiss (Jena, Germany)</td>
<td>AxioCam MRm</td>
</tr>
<tr>
<td>Oven</td>
<td>Precision Scientific Instruments (NY, USA)</td>
<td>Model 19</td>
</tr>
<tr>
<td>Petri Dish</td>
<td>Fisher Scientific (NH, USA)</td>
<td>Fisherbrand Slippable Lid</td>
</tr>
<tr>
<td>pH Electrode</td>
<td>Fisher Scientific (NH, USA)</td>
<td>Accumet</td>
</tr>
<tr>
<td>pH Meter</td>
<td>Fisher Scientific (NH, USA)</td>
<td>Model AB15</td>
</tr>
<tr>
<td>Photomask</td>
<td>Front Range Photomask (CO, USA)</td>
<td>Chrome/Glass 4” High Resolution</td>
</tr>
<tr>
<td>Potentiostat</td>
<td>CH Instruments (TX, USA)</td>
<td>Models 842C, 1040C</td>
</tr>
<tr>
<td>Equipment</td>
<td>Manufacturer</td>
<td>Model/Type</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>--------------------------------------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>Potentiostat (Portable)</td>
<td>DropSens (Asturias, Spain)</td>
<td>Portable Multi Potentiostat μStat 4000P, μStat 200</td>
</tr>
<tr>
<td>Refrigerator/Freezer</td>
<td>Fisher Scientific (NH, USA)</td>
<td>Isotemp General-Purpose Model 13-986-106A</td>
</tr>
<tr>
<td>Scanning Electron Microscope</td>
<td>Hitachi (Tokyo, Japan)</td>
<td>Model S-4800 (Field Emission)</td>
</tr>
<tr>
<td>Screen-Printed Electrode</td>
<td>Zensor (Taichung City, Taiwan)</td>
<td>TE100</td>
</tr>
<tr>
<td>Shaker</td>
<td>VWR (PA, USA)</td>
<td>Advanced Orbital Shaker, Model 3500 (Speed Range 15-500 rpm, Orbit Size 19 mm)</td>
</tr>
<tr>
<td>Silicon Wafer</td>
<td>University Wafer (MA, USA)</td>
<td>Model 447 3” P-type</td>
</tr>
<tr>
<td>Stirring Hotplate</td>
<td>Fisher Scientific (NH, USA)</td>
<td>Isotemp Ceramic Top</td>
</tr>
<tr>
<td>Swab</td>
<td>BD Diagnostics (MD, USA)</td>
<td>BBL CultureSwab 108C Amies Gel without Charcoal</td>
</tr>
<tr>
<td>Syringe</td>
<td>Fisher Scientific (NH, USA)</td>
<td>BD Disposable with Luer-Lok Tips 3 mL</td>
</tr>
<tr>
<td>Syringe Filter</td>
<td>Sartorius (Goettingen, Germany)</td>
<td>Minisart RC 0.2 µm</td>
</tr>
<tr>
<td>Test Tube</td>
<td>Thermo Scientific (MA, USA)</td>
<td>14 mL Round Bottom</td>
</tr>
<tr>
<td>Apparatus</td>
<td>Manufacturer (Origin)</td>
<td>Model</td>
</tr>
<tr>
<td>---------------------------------</td>
<td>----------------------------------------</td>
<td>-------------------------------</td>
</tr>
<tr>
<td>Ultra-Low Freezer</td>
<td>Revco Technologies (NC, USA)</td>
<td>ULT 2586-5-D35</td>
</tr>
<tr>
<td>Vacuum Desiccator</td>
<td>Bel-Art Products (NJ, USA)</td>
<td>Scienceware Space Saver 42022000</td>
</tr>
<tr>
<td>Vibration Isolation Platform</td>
<td>Minus K Technology, Inc. (CA, USA)</td>
<td>100 lb Load</td>
</tr>
<tr>
<td>Vortexer</td>
<td>Fisher Scientific (NH, USA)</td>
<td>MiniRoto Vortexer</td>
</tr>
<tr>
<td>Water Purification System</td>
<td>Millipore (MA, USA)</td>
<td>Direct 8</td>
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</table>

**Table 4-2: Fabrication Tools**

<table>
<thead>
<tr>
<th>Apparatus</th>
<th>Manufacturer (Origin)</th>
<th>Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion Furnace</td>
<td>Bruce Technologies, Inc. (MA, USA)</td>
<td>7355B</td>
</tr>
<tr>
<td>E-Beam Metal Deposition System</td>
<td>Kostas Research Center (MA, USA)</td>
<td>Custom</td>
</tr>
<tr>
<td>Inductively Coupled Plasma</td>
<td>Unaxis (Pfaffikon, Switzerland)</td>
<td>Unaxis Shuttleline ICP/RIE</td>
</tr>
<tr>
<td>Reactive Ion Etcher System</td>
<td></td>
<td>PlasmaTherm 790</td>
</tr>
<tr>
<td>Mask Aligner</td>
<td>Neutronix Quintel (CA, USA)</td>
<td>Quintel 4000</td>
</tr>
<tr>
<td>Plasma Bonder System</td>
<td>Anatech (CA, USA)</td>
<td>SP-100</td>
</tr>
<tr>
<td>Plasma-Enhanced Chemical Vapor</td>
<td>Nexx Systems (MA, USA)</td>
<td>Cirrus 150</td>
</tr>
<tr>
<td>Deposition System</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spinner</td>
<td>Laurell Technologies Corporation (PA, USA)</td>
<td>WS-400 Lite Series</td>
</tr>
</tbody>
</table>
4.1.2 Chemical and Biological Materials

Table 4-3: Chemical Materials

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Manufacturer (Origin)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Propanol</td>
<td>Fisher Scientific (NH, USA)</td>
<td>Certified ACS Plus</td>
</tr>
<tr>
<td>6-Mercaptohexanol</td>
<td>Sigma-Aldrich (MO, USA)</td>
<td>N/A</td>
</tr>
<tr>
<td>Acetone</td>
<td>Fisher Scientific (NH, USA)</td>
<td>Certified ACS</td>
</tr>
<tr>
<td>Ammonium Hydroxide</td>
<td>Acros Organics (MA, USA)</td>
<td>Certified ACS</td>
</tr>
<tr>
<td>Chloroform</td>
<td>Acros Organics (MA, USA)</td>
<td>99.9%</td>
</tr>
<tr>
<td>Chlorotrimethylsilane</td>
<td>Acros Organics (MA, USA)</td>
<td>98%</td>
</tr>
<tr>
<td>Ethanol</td>
<td>Decon Labs, Inc. (PA, USA)</td>
<td>N/A</td>
</tr>
<tr>
<td>Ferrous Sulfate</td>
<td>Fisher Scientific (NH, USA)</td>
<td>N/A</td>
</tr>
<tr>
<td>Glycerol</td>
<td>Fisher Scientific (NH, USA)</td>
<td>Certified ACS</td>
</tr>
<tr>
<td>Hydrochloric Acid</td>
<td>Sigma-Aldrich (MO, USA)</td>
<td>Certified ACS</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>Sigma-Aldrich (MO, USA)</td>
<td>Reagent Grade, ≥98%</td>
</tr>
<tr>
<td>Chemical</td>
<td>Manufacturer (Origin)</td>
<td>Notes</td>
</tr>
<tr>
<td>----------------------</td>
<td>-------------------------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>Sigma-Aldrich (MO, USA) ReagentPlus, ≥99%</td>
<td></td>
</tr>
<tr>
<td>L-Leucine</td>
<td>Sigma-Aldrich (MO, USA) Reagent Grade, ≥98%</td>
<td></td>
</tr>
<tr>
<td>L-Proline</td>
<td>Sigma-Aldrich (MO, USA) ReagentPlus, ≥99%</td>
<td></td>
</tr>
<tr>
<td>L-Tyrosine</td>
<td>Sigma-Aldrich (MO, USA) Reagent Grade, ≥98%</td>
<td></td>
</tr>
<tr>
<td>L-Valine</td>
<td>Sigma-Aldrich (MO, USA) Reagent Grade, ≥98%</td>
<td></td>
</tr>
<tr>
<td>Magnesium Chloride</td>
<td>Fisher Scientific (NH, USA)</td>
<td>N/A</td>
</tr>
<tr>
<td>Magnesium Sulfate</td>
<td>MP Biomedicals (CA, USA)</td>
<td>N/A</td>
</tr>
<tr>
<td>Polydimethylsiloxane</td>
<td>Dow Corning Corporation (MI, USA)</td>
<td>Sylgard 184 Base and Curing Agent</td>
</tr>
<tr>
<td>Potassium Sulfate</td>
<td>EMD Chemicals (PA, USA)</td>
<td>N/A</td>
</tr>
<tr>
<td>Sodium Hydroxide</td>
<td>Fisher Scientific (NH, USA)</td>
<td>N/A</td>
</tr>
<tr>
<td>Sodium Hypochlorite</td>
<td>Ricca Chemical Company (TX, USA)</td>
<td>6% Cl₂</td>
</tr>
<tr>
<td>Sulfuric Acid</td>
<td>Fisher Scientific (NH, USA)</td>
<td>Certified ACS Plus</td>
</tr>
</tbody>
</table>

**Table 4-4: Biological Materials**

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Manufacturer (Origin)</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-hydroxyphenazine</td>
<td>Tokyo Chemical Industry (Tokyo, Japan)</td>
<td>528-71-2</td>
</tr>
<tr>
<td>Agar Technical Solidifying Agent</td>
<td>BD Diagnostics (MD, USA)</td>
<td>Difco</td>
</tr>
<tr>
<td>Media Description</td>
<td>Supplier</td>
<td>Catalog Number</td>
</tr>
<tr>
<td>-------------------------</td>
<td>---------------------------</td>
<td>----------------</td>
</tr>
<tr>
<td>LB Broth</td>
<td>BD Diagnostics (MD, USA)</td>
<td>Difco</td>
</tr>
<tr>
<td>M63 Minimal Media</td>
<td>Amresco (OH, USA)</td>
<td>N/A</td>
</tr>
<tr>
<td>Thioglycollate Media</td>
<td>BD Diagnostics (MD, USA)</td>
<td>Difco</td>
</tr>
<tr>
<td>Trypticase Soy Broth</td>
<td>BD Diagnostics (MD, USA)</td>
<td>BBL</td>
</tr>
<tr>
<td>Pyocyanin</td>
<td>Sigma-Aldrich (MO, USA)</td>
<td>85-66-5</td>
</tr>
<tr>
<td>Bronchial Lavage</td>
<td>Bioreclamation (NY, USA)</td>
<td>Lot #BRH723769</td>
</tr>
<tr>
<td>Human Whole Blood</td>
<td>Bioreclamation (NY, USA)</td>
<td>Lot #BRH724097</td>
</tr>
<tr>
<td>(Sodium Heparin)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human Whole Blood</td>
<td>Bioreclamation (NY, USA)</td>
<td>Lot #BRH724098</td>
</tr>
<tr>
<td>(Anti-Coagulant Free)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sputum</td>
<td>Bioreclamation (NY, USA)</td>
<td>Lot #BRH723767</td>
</tr>
<tr>
<td>Urine</td>
<td>Bioreclamation (NY, USA)</td>
<td>Lot #BRH723768</td>
</tr>
<tr>
<td>N-(3-oxododecanoyl)</td>
<td>Sigma-Aldrich (MO, USA)</td>
<td>168982-69-2</td>
</tr>
<tr>
<td>homoserine lactone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-butyryl homoserine</td>
<td>Cayman Chemicals (MI, USA)</td>
<td>67605-85-0</td>
</tr>
<tr>
<td>lactone</td>
<td></td>
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### 4.1.3 Bacterial Isolates

#### Table 4-5: Bacterial Isolates

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Strain/Identifier</th>
<th>Biosafety Level</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>PA01, PA14</td>
<td>2</td>
<td>Brigham and Women’s Hospital (Boston, MA)</td>
</tr>
<tr>
<td>Organism</td>
<td>Strain Number</td>
<td>Source</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------------</td>
<td>--------------------------------</td>
<td></td>
</tr>
<tr>
<td><em>Acinetobacter baumannii</em></td>
<td>ATCC 17978</td>
<td>Northeastern University (Boston, MA)</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus cereus</em></td>
<td>AR156</td>
<td>Northeastern University (Boston, MA)</td>
<td></td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>NCIB 3610</td>
<td>Angell Animal Medical Center (Boston, MA)</td>
<td></td>
</tr>
<tr>
<td><em>Enterococcus faecalis</em></td>
<td>ATCC 29212</td>
<td>Northeastern University (Boston, MA)</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>DH5α GFP, DH5α m-cherry, K-12</td>
<td>Northeastern University (Boston, MA)</td>
<td></td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>ATCC BAA-2470</td>
<td>Northeastern University (Boston, MA)</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 25923, 43300</td>
<td>Northeastern University (Boston, MA)</td>
<td></td>
</tr>
<tr>
<td><em>Staphylococcus epidermidis</em></td>
<td>ATCC 35984</td>
<td>Northeastern University (Boston, MA)</td>
<td></td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>ATCC 6303</td>
<td>Northeastern University (Boston, MA)</td>
<td></td>
</tr>
</tbody>
</table>
4.1.4 Culture Media

Trypticase Soy Broth

Per liter of distilled water:

- Pancreatic Digest of Casein 17.0 g
- Papaic Digest of Soybean 3.0 g
- Sodium Chloride 5.0 g
- Dipotassium Phosphate 2.5 g
- Dextrose 2.5 g

Autoclave at 121 °C for 15 min
Final pH 7.3 ± 0.2

Trypticase Soy Agar

Per liter of distilled water:

- Pancreatic Digest of Casein 15.0 g
- Papaic Digest of Soybean 5.0 g
- Sodium Chloride 5.0 g
- Agar 15.0 g

Autoclave at 121 °C for 15 min
Final pH 7.3 ± 0.2
Lysogeny Broth (Luria-Bertani)

Per liter of distilled water:

- Tryptone 10.0 g
- Yeast Extract 5.0 g
- Sodium Chloride 10.0 g

Autoclave at 121 °C for 15 min
Final pH 7.0 ± 0.2

Lysogeny Broth Agar (Luria-Bertani)

Per liter of distilled water:

- Tryptone 10.0 g
- Yeast Extract 5.0 g
- Sodium Chloride 10.0 g
- Agar 15.0 g

Autoclave at 121 °C for 15 min
Final pH 7.0 ± 0.2

M63 Minimal Media

Per liter of distilled water:

- Ammonium Sulfate 2.0 g
- Potassium Phosphate, Monobasic 13.6 g
- Ferrous Sulfate × 7H₂O 0.5 mg
Adjust pH to 7.0 ± 0.2 with KOH

Autoclave at 121 °C for 15 min

After medium cooled to 50 °C, the following components were added:

20% Glycerol 10 mL
1 M Magnesium Sulfate 1 mL

4.2 Methods

4.2.1 Bacterial Culturing

All bacterial isolates used in this dissertation were incubated under aerobic conditions on nutrient agar at 37 °C on a rotary shaker at 150 rpm (unless otherwise noted).

4.2.2 Bacterial Sample Collection

*Pseudomonas aeruginosa* strains PAO1 and PA14 were obtained from the Channing Laboratory at Brigham and Women’s Hospital in Boston, MA. Other strains and bacterial isolates were obtained in accordance with state and federal environmental health and safety guidelines and are reported in Table 4-5.

4.2.3 Storage

Maintenance of *Pseudomonas aeruginosa* and other bacterial isolates were performed according to Maniatis *et al.* [272].
4.2.3.1 Short-Term Storage

Bacterial isolates were cultured on a nutrient-containing agar medium and incubated at 37 °C for 24 hours on a rotary shaker at 150 rpm. They were stored in a refrigerator at 4 °C for a period of up to 2 weeks.

4.2.3.2 Long-Term Storage

Bacterial isolates were stored in small, capped vials of nutrient broth containing 15% glycerol in an ultra-low freezer at -80 °C.

4.2.4 Bacterial Cell Count

Bacterial isolates from agar medium were sampled and placed into a test tube containing nutrient broth. The sample was diluted 1000:1 and loaded in a hemocytometer (Hausser Scientific, Model 3500) for cell counting using a light microscope.

4.2.5 Solution Preparation

All solutions were prepared using ultra-purified water from a Milli-Q system (Millipore) with a resistance of less than 18 Mohms. Bacterial solutions were prepared by centrifugation (10,000 rpm × 3 min) and filtration of the supernatant through 0.2 μm nylon filters.
4.2.6 Device Fabrication

Photomasks for photolithography were designed using Tanner L-Edit (version 15.1) and purchased from Front Range Photomask (CO, USA). All fabrication was completed in either Northeastern University’s Kostas Research Center clean room or Harvard’s Center for Nanoscale Systems.

4.2.7 Data Analysis

Electrochemistry measurements were obtained using potentiostats purchased from CH Instruments (TX, USA) or DropSens (Asturias, Spain) with bundled user software. Experimental data and figures were analyzed or created using OriginPro 9.1 (OriginLab Corporation), with baselines created for each measurement using spline interpolation with multiple base points. The resulting baseline-subtracted data was used to identify peaks in current due to pyocyanin oxidation. These peaks in current can be correlated to the pyocyanin concentration in the sample using a calibration curve obtained from testing different pyocyanin standards. Figures were cropped, combined, and annotated using Adobe Photoshop CC (Adobe Systems, Inc.). For bar graphs, unless otherwise stated, error bars are reported as standard error of the mean (SEM), with statistical analysis performed by one-way ANOVA. The 95% confidence level was considered significant. All software programs were installed on Dell computers (TX, USA) running either Intel i5 or i7 processors with 8 or 16 GB of RAM.
4.3 **Electrochemistry**

Electrochemistry studies chemical phenomena through the transport of electrons and applies this knowledge to a wide array of applications. This dissertation is heavily based on electrochemical techniques and electrochemical-based sensors, which will be delineated in the subsequent sections.

4.3.1 **Three-Electrode Cell Configuration**

Electrochemistry involves the use of electrodes, a conductive material that interfaces with an electrolyte solution. There are a number of electrode configurations for electrochemical testing with the most common setup being the three-cell electrode. The three-electrode cell configuration consists of a working, counter, and reference electrode. The working electrode is the designation for the electrode where the redox reaction occurs. This electrode is often made out of an inert material such as gold, platinum, or carbon. These inert materials allow electrons to exchange with redox species without being affected by the current passing through the system. The counter (or auxiliary) electrode is the electrode that completes the path of the current and also the electrical circuit, as all electrochemistry experiments require a working and counter pair. This electrode acts as a current source/sink and is typically made out of an inert material such as gold, platinum, or carbon. The main function of the counter electrode is to prevent current from entering the reference electrode. Finally, the reference electrode serves as the experimental reference point. It is designed to hold a constant, stable potential during testing. Ideally, no current should flow through the reference electrode, and the reference should be placed relatively close to the working electrode to minimize the potential drop.
across the solution. The most common and commercially available reference electrodes are the silver/silver chloride (Ag/AgCl) and saturated calomel electrode (SCE).

The value of the three-electrode setup is evident when comparing it to the two-electrode configuration, the simplest cell setup in which the counter and reference constitute a single electrode. This system is useful in only a limited number of cases, most notably in systems with low currents or short timescale experiments, as this configuration is plagued by current drift. Current drift occurs because of the reference electrode’s difficulty to maintain a constant potential while simultaneously passing current as the counter electrode. The three-electrode cell bypasses this problem by splitting up the reference and counter electrodes. Additionally, there are a number of configurations that utilize more than three electrodes, but they are all considered more complex versions of the three-electrode setup. As it is the most accurate and common electrode configuration, the three-electrode cell was chosen for all electrochemical measurements. The basic setup is shown in Figure 4-1.

**Figure 4-1:** Three-electrode cell configuration.
A potentiostat is used to control the potentials that are being applied to the electrode cell. The two potentiostats (models CHI842C and CHI1040C) used for these experiments were purchased from CH Instruments. Portable potentiostats from DropSens (μStat 4000P and μStat 200) were purchased to use for clinical testing.

4.3.2 Electrochemical Techniques

The electrochemical techniques used in this dissertation were cyclic voltammetry (CV), square-wave voltammetry (SWV), and alternating current voltammetry (ACV) where the current is measured as the potential is varied [215].

4.3.2.1 Cyclic Voltammetry

For cyclic voltammetry, the working electrode potential is increased linearly versus time until it reaches a set potential, at which time the potential is inverted. The waveform for this technique can be seen in Figure 4-2.

![Cyclic voltammetry waveform](image)

**Figure 4-2:** Cyclic voltammetry waveform.
Cyclic voltammetry (CV) is a broad technique that can be used to understand redox processes and reaction intermediates. Due to its versatility, it is one of the most widely used electrochemical techniques. However, it is rarely used for quantitative analyses due to its lower sensitivity. To address this, pulse techniques were introduced, which presents the second technique used in this dissertation: square-wave voltammetry. Unless otherwise stated, the CV scans ranged from -0.7 to 0.0 V at a scan rate of 0.1 V/s and a sample interval of 0.001 V.

4.3.2.2 Square-Wave Voltammetry

As the name suggests, square-wave voltammetry (SWV) incrementally increases the potential in a stair-case step pattern. The waveform for this technique is shown in Figure 4-3.

Figure 4-3: Square-wave voltammetry waveform.

Advantages of using square-wave voltammetry include its excellent sensitivity and rejection of background currents. It is also a very fast technique that, when paired with computer control, allows for rapid experimental testing. For all quantitative
measurements, square-wave voltammetry was used. Unless otherwise stated, the SWV scans ranged from a potential window of -0.7 to 0.0 V at a step potential of 0.004 V, amplitude of 0.05 V, and frequency of 15 Hz.

4.3.2.3 AC Voltammetry

As the name suggests, AC voltammetry applies a sinusoidal oscillating voltage to the electrochemical cell. While not as commonly used as the differential pulse voltammetry techniques, the sensitivity and detection limits of AC voltammetry are similar. The waveform for this technique is shown in Figure 4-4.

Figure 4-4: AC voltammetry waveform.

Unless otherwise stated, the ACV scans ranged from -0.7 to 0.0 V at a step potential of 0.004 V, amplitude of 0.05 V, and frequency of 100 Hz.
4.3.3 Electrochemical Sensors

Two different electrode sensors were utilized in this dissertation: commercially available screen-printed electrodes and in-house nano- and microfabricated electrode sensors.

4.3.3.1 Screen-Printed Electrodes

Some of the electrochemical measurements performed in this dissertation used commercially available Zensor TE100 (Taichung City, Taiwan) screen-printed electrodes, which feature carbon working (3 mm diameter) and counter electrodes as well as a silver/silver chloride (Ag/AgCl) pseudo-reference electrode. In some cases, a 3 M NaCl Ag/AgCl reference electrode (CHI111, CH Instruments) was used in lieu of the pseudo-reference built in the sensor. The electrode design can be seen in Figure 4-5. Scanning electron microscopy images of the sensor’s carbon working electrode and Ag/AgCl reference electrode are shown in Figure 4-6. The surface morphology of the working electrode indicates carbon is in its crystalline form, graphite [273]. The Ag/AgCl reference electrode surface is consistent with previously reported literature [274]. For small volume analysis (minimum 7.5 µL), a polymeric membrane (DRP-MEMB, DropSens) was placed on top of the sensing surface (Figure 4-7). Without this membrane, a minimum of 100 µL of fluid is needed to cover the electrodes.
Figure 4-5: Disposable screen-printed three-electrode sensors (Zensor TE100).

Figure 4-6: Scanning electron microscopy images of Zensor TE100’s A) carbon working electrode and B) Ag/AgCl reference electrode.
4.3.3.2 Nano- and Microfabricated Electrodes

In addition to commercially screen-printed electrodes, multiple variations of nano- and microfabricated sensors were designed for use at Northeastern University’s Kostas Research Center clean room and Harvard’s Center for Nanoscale Systems. Sensor designs ranged from interdigitated electrodes to electrodes employing a nanofluidic channel. One such sensor utilized a pH-sensitive palladium hydride reference electrode. The fabrication scheme is shown in Figure 4-8.
This sensor is fabricated using a series of optical photolithography and physical deposition steps to pattern a bottom gold electrode (a), a chromium sacrificial layer (b), a top gold electrode (c), and a palladium hydride reference electrode (d). A silicon dioxide insulating layer is added (e) with access holes etched down to the chromium layer (f). By selectively removing the chromium layer, a nanochannel is exposed (200 µm × 15 µm × 60 nm) (g), which allows for small redox-active molecules to diffuse down to the electrode setup (Figure 4-9). A finalized sensor is shown in Figure 4-10 and a top view optical micrograph of the sensor is shown in Figure 4-11.
Figure 4-9: Small molecules produced by bacteria diffuse into the nanochannel where they are oxidized, generating current which can be measured. Front view (left), side view (right).

Figure 4-10: Finalized nanofabricated sensor containing a palladium hydride reference electrode (razor blade included as a size reference).
This sensor is used in Aim 4 when pH detection is used as a biomarker for bacterial infection.

4.4 Aim 1: The Up-Regulation of Pyocyanin Production by *Pseudomonas aeruginosa* for Rapid Bacterial Identification

4.4.1 Materials and Methods

The following work using amino acids to up-regulate pyocyanin production by *Pseudomonas aeruginosa* was published in the journal Analyst (2014) (DOI: 10.1039/c4an00756e) [22].

All bacterial tests were completed using wild-type *P. aeruginosa* strain PA14. Cell cultures were routinely grown in trypticase soy broth (TSB) (BD Biosciences 211768) at 37 °C and stored on TSB agar plates at 4 °C when not in use. All amino acids were purchased from Sigma-Aldrich (St. Louis, MO) and dissolved in solution either using TSB or M63 minimal salts medium (Fisher Scientific, 50-751-6740) [((NH₄)₂SO₄ (15 mM), KH₂PO₄ (100 mM)] supplemented with MgSO₄ (1 mM) and glycerol (0.027
mM). Electrochemical measurements were performed using commercially available Zensor TE100 (Taichung City, Taiwan) screen-printed electrodes featuring carbon working and counter electrodes. Although the Zensor TE100 electrodes contained a silver paste reference, a separate 1 M KCl Ag/AgCl reference electrode (CHI111, CH Instruments) was employed to minimize the chances of drift in reference potential during measurements lasting several hours, which can occur when the reference electrode is in direct contact with the sample solution. All electrochemical measurements were recorded using a potentiostat (CHI842C, CH Instruments).

Amino acids were selected based on their ability to up-regulate biofilm formation of *P. aeruginosa* [29]. The six amino acids chosen were proline, histidine, arginine, leucine, tyrosine, and valine. The following detection strategy is envisioned.

![Figure 4-12](image)

**Figure 4-12:** Detection scheme for amino acid up-regulation of pyocyanin production by *P. aeruginosa*.

Individual amino acids were dissolved at specific concentrations in either 10 mL of TSB or M63 media. Each solution was inoculated with a specific concentration of *P. aeruginosa* and incubated at 37 °C. 100 µL of each solution was removed at designated time points and loaded onto a screen-printed electrode sensor for electrochemical testing.
Square-wave voltammetric scans were performed at potentials ranging from -0.4 to -0.1 V at an amplitude voltage of 0.050 V and a frequency of 15 Hz. Scans were performed for each sample three times with three replicates for each tested condition. The data was analyzed using OriginPro 9.1 (OriginLab Corporation). Baselines were created for each data set using spline interpolation with 8 base points. The resulting baseline-subtracted data set was used to determine the max currents observed from pyocyanin production.

For experiments utilizing iron as an up-regulatory molecule for pyocyanin production by *P. aeruginosa*, ferrous sulfate was dissolved in TSB broth to create liquid samples of iron at various concentrations. TSB already contains a certain amount of iron in its formula, which was considered when the solution’s final iron concentration was calculated. The more virulent wild-type strain of *P. aeruginosa*, PA14 was used. A liquid culture of PA14 was grown overnight in TSB at 37 °C on a rotary shaker at 150 rpm. Using a hemocytometer (Hauser Scientific, Model 3500), a cell count was taken to approximate the cell density of the bacterial culture. PA14 cells were inoculated into each of the iron samples with approximately $10^6$ bacterial cells per mL. This is a biologically relevant concentration and previous research has shown that *P. aeruginosa* often grows to high cell densities in cystic fibrosis sputum ($>10^9$ cells per mL sputum) [140]. After the samples were inoculated with bacteria, they were stored at 37 °C on a rotary shaker. Every 2 hours, 100 µL of sample was pipetted onto a disposable screen-printed carbon electrode connected to an external Ag/AgCl reference. Square-wave voltammograms were taken from a potential range of -0.4 to -0.1 V at an amplitude voltage of 0.050 V and a frequency of 15 Hz. A similar set of experiments were done with simple sugars, glucose and sucrose, as additives instead of iron.
4.5 Aim 2: Screening Other Bacterial Pathogens for Redox-Active Biomarkers for Electrochemical Detection

4.5.1 Materials and Methods

The electrochemical setup was miniaturized to increase its portability for off-site use. A portable potentiostat, with the ability to administer all necessary electrochemical techniques while also maintaining a small-form factor for easy transportation, was purchased. A handheld potentiostat was also designed so that it could be more easily implemented at off-site testing locations. These different potentiostat form factors are shown in Figure 4-13:

![Different potentiostats for electrochemical testing, with increasing portability from left to right.](image)

<table>
<thead>
<tr>
<th>Potentiostat from CH Instruments</th>
<th>Portable potentiostat from DropSens</th>
<th>Handheld potentiostat from QSM Diagnostics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimensions: 12.5 x 11 x 4.75”</td>
<td>8.7 x 7.9 x 2.8”</td>
<td>4.7 x 4.7 x 2.3”</td>
</tr>
<tr>
<td>Weight: 12 lb</td>
<td>3.5 lb</td>
<td>0.5 lb</td>
</tr>
</tbody>
</table>

The following work detecting pyocyanin production in clinical *Pseudomonas aeruginosa* isolates was published in the journal Biosensors and Bioelectronics (2017) (DOI: 10.1016/j.bios.2017.05.042) [26].

A series of clinically-relevant bacteria were obtained from various sources including Brigham and Women’s Hospital. Samples were grown overnight in liquid batch...
cultures and electrochemically scanned using square-wave voltammetry at 15 Hz from a potential window of -0.7 to 0.0 V.

Clinical isolates of *Pseudomonas aeruginosa* were obtained from patients with cystic fibrosis or patients with a hospital-acquired infection. Samples obtained from these patients were stored frozen (-80 °C) at the Channing Division of Network Medicine at Brigham and Women's Hospital. All patient records and information was anonymized and de-identified prior to analysis. Sterile, media-free swabs (Becton Dickinson, 220115) were used to collect 94 different *P. aeruginosa* clinical isolates for electrochemical testing. Each swab was placed into a labeled, round-bottom tube for transport. A total of 34 hospital strains, 31 mucoidal strains, and 29 cystic fibrosis *P. aeruginosa* strains were collected for analysis.

Swab samples were inoculated with trypticase soy broth (BD 211768) and incubated at 37 °C while shaking at 150 rpm. Inoculates were grown over the course of 2 days under these conditions, with electrochemical testing completed after 1 and 2 days. For each sample, a disposable, screen-printed electrode (Zensor TE100) was used to determine the presence of pyocyanin. The sensing surface was covered with a polymeric membrane (DropSens, DRP-MEMB) to reduce the amount of sample volume required for analysis.

For each sample, the swab was directly placed onto the sensor to cover the sensing region with fluid. A minimum sample volume of 7.5 µL was needed to make electrical contact with all three electrodes and successfully perform a measurement. For all electrochemical measurements, square-wave voltammetry was used at potentials ranging from -0.7 to 0.0 V at an amplitude voltage of 0.05 V, step voltage of 0.004 V,
and a frequency of 100 Hz. Pyocyanin (Sigma-Aldrich, 85-66-5) and 1-hydroxyphenazine (Tokyo Chemical Industry, 528-71-2) were purchased commercially and readily used to create standards for electrochemical testing.

To determine pyocyanin production rates, three different isolates were randomly chosen from each of the hospital, mucoidal, and cystic fibrosis *P. aeruginosa* strains. The samples were grown for 18-24 hours at 37 °C while shaking at 150 rpm. The samples were then centrifuged at 10,000 rpm for 3 minutes to separate the cells from the supernatant. The supernatant was removed and the cells were reconstituted in fresh growth media. The samples were then inoculated into individual test tubes containing fresh trypticase soy broth at a starting concentration of $4 \times 10^6$ cells/mL. Electrochemical measurements to monitor pyocyanin production were taken every 2 hours for 24 hours, and then every 6 hours for an additional 24 hours. Using a hemocytometer (Hausser Scientific, Model 3500), cell density measurements of the samples were taken every 4 hours for 24 hours, and then every 6 hours for an additional 24 hours. Pyocyanin production rates per bacterial cell were determined as the ratio between the electrochemically measured pyocyanin and the number of cells.

Complete (N=199) and draft (N=2380) genomes within the genus *Pseudomonas* were downloaded by GenBank [275] on 06/01/2016. These included 1651 complete and draft genomes for *P. aeruginosa* with the remaining data from other *Pseudomonas* species. The downloaded genomes/draft genomes were subject to gene calling using Prodigal (Prokaryotic Dynamic Programming Genefinding Algorithm) [276] with default parameters using the –c flag (i.e., closed ends setting) only for complete genomes followed by matching against the Kyoto Encyclopedia of Genes and Genomes (KEGG)
database [277] using RAPSearch2 (Reduced Alphabet based Protein similarity Search) [278] with default parameters. A total of 2579 genomes from the genus *Pseudomonas* were tested for the absence or presence of these two genes, based on annotation results against the KEGG database.

Electrochemical measurements were analyzed using OriginPro 9.1 (OriginLab), with baselines created for each measurement using spline interpolation with 32 base points. The resulting baseline-subtracted data was used to identify peaks in current due to pyocyanin oxidation. These peaks in current can be correlated to the pyocyanin concentration in the sample using a calibration curve obtained from testing different pyocyanin standards (Figure 4-14). Results are presented as means ± SEM, with statistical analysis performed by one-way ANOVA. The 95% confidence level was considered significant.

![Figure 4-14: A) Baseline-subtracted square-wave voltammograms at 100 Hz and B) linear fit of 0-100 µM pyocyanin in trypticase soy broth.](image)
4.6 **Aim 3: Developing Alternative Electrochemical Sensing Strategies for Bacterial Detection**

4.6.1 **Materials and Methods**

The signaling molecule, N-(3-oxododecanoyl) homoserine lactone (3O-C12-HSL) was purchased from Sigma Aldrich (MO, USA). The aptamer sequence that binds strongly to this target (CGGGGCCCCTTCTGTTGCGGTGTACTAGTGACCG) was purchased from Biosearch Technologies (CA, USA). The aptamer was modified to have a reactive thiol (sulfhydryl) group at the 5’ end and methylene blue at the 3’ end. It is well known that sulfur binds strongly to gold, making it easy to incorporate onto a gold working electrode. The methylene blue acts a redox-active label for analyzing binding events of the aptamer to the target molecule of interest (3O-C12-HSL). A negative control, N-butyryl homoserine lactone (C4-HSL), was purchased from Cayman Chemicals (MI, USA). For electrochemical testing, 2 mm diameter gold working electrodes were purchased from CH Instruments (TX, USA) along with a platinum wire counter electrode and a Ag/AgCl reference electrode (CHI111, CH Instruments). A polishing kit to clean the gold electrodes contained 0.3 and 0.05 micron alumina powder and a microcloth polishing pad (CHI120, CH Instruments). For electrochemical cleaning, the gold electrodes were immersed in a 50 mM sulfuric acid solution and scanned from -400 to 1400 mV vs. Ag/AgCl at a rate of 100 mV/s for 12 cycles using cyclic voltammetry. Cycling the electrode potential in a weak sulfuric acid solution until a stable CV scan is achieved is a common electrochemical cleaning technique [279].

The preparation of the electrodes for aptamer immobilization and procedure for testing the aptamer sensor was based on protocols established by Plaxco et al. [280]. The
gold working electrodes were polished with a microcloth using 0.3 and 0.05 micron alumina powder, followed by acid cleaning using cyclic voltammetry in a dilute sulfuric acid. The gold working electrodes were immersed in a 1 μM aptamer solution for 2 hours at room temperature in the dark. Afterwards, any excess probe DNA physically adsorbed onto the electrode’s surface was removed using deionized water. The probe was then transferred to a 2 mM 6-mercaptohexanol solution for 3 hours at room temperature in the dark. Afterwards, any excess 6-mercaptohexanol was rinsed off of the probe using deionized water. The probe was then stored in phosphate-buffered saline (PBS) until testing.

For electrochemical testing of the aptamer-modified working electrode, a three-electrode cell was formed with a platinum wire counter electrode and Ag/AgCl (saturated with 3 M NaCl) reference electrode. Background scans used target-free PBS (10 mM phosphate with 1.0 M NaCl supplemented with 1 mM Mg²⁺). A concentration series of 3O-C12-HSL target (0, 0.1, 1, 10 µM) was made in PBS and tested in order of increasing concentration. The following detection strategy is envisioned.
Figure 4-15: Aptamer-based detection scheme for detecting other bacterial pathogens. Binding of the aptamer to the target causes a conformational change, increasing the distance between the redox label and electrochemical surface, leading to a decrease in electrochemical signal.

4.7 Aim 4: Applications of Electrochemical Sensors towards the Hospital Setting

4.7.1 Materials and Methods

For all experiments towards the development of a sensor to detect pH, the sensor fabrication is shown in Figure 4-8. To test the device’s dual ability to monitor pH and pyocyanin, samples of 100 µM pyocyanin in 100 mM phosphate buffer were created at different pH values (4.28, 6.94, and 10.4) through the addition of HCl acid or NaOH base. Square-wave voltammograms ranged from -0.75 to 0.00 V vs. an integrated palladium hydride reference electrode. With this setup, square-wave voltammetry was used at a frequency of 15 Hz to detect the presence of pyocyanin in these samples.

For the electrochemical detection of clinical *Pseudomonas aeruginosa* isolates using AC voltammetry, 31 different clinical isolates of *P. aeruginosa* were obtained for testing. 12 of these isolates were multidrug-resistant strains (defined as being non-
susceptible to at least 1 antibiotic in at least 3 classes of antibiotics). The applied potentials swept from -0.7 to 0.0 V at an amplitude of 0.05 V and a frequency of 100 Hz.

The following work detecting *Pseudomonas* in human wound exudate was published in the journal *Wound Repair and Regeneration* (2016) (DOI: 10.1111/wrr.12414) [25].

All patient samples were obtained through the Wound Etiology and Healing (WE-HEAL) Study, a biospecimen and data repository designed for studying chronic wounds approved by the George Washington University Institutional Review Board (041408). Subjects are eligible for this study if they have an open wound at the time of evaluation and are older than 18 years of age. All subjects gave written informed consent for collection of specimens and data. 14 paired wound fluid and biofilm samples from 12 patients were selected for analysis (Table 4-6). This was a convenience sample selected based on availability of wound fluid and wound microbiome samples from the same collection date.
Table 4-6: Demographic and clinical characteristics of patients (n=12) from whom wound fluid samples were tested. Wound size (mean ± SD) of all wounds with specimens collected (n=14).

<table>
<thead>
<tr>
<th></th>
<th>All patients n = 12</th>
<th>Pseudomonas spp. positive on 16S rRNA n = 6</th>
<th>Pseudomonas spp. negative on 16S rRNA n = 6</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years, mean ± SD)</td>
<td>50.76 (±17.14)</td>
<td>49.86 (±11.57)</td>
<td>51.67 (±22.55)</td>
<td>0.8642</td>
</tr>
<tr>
<td>Male sex (n, %)</td>
<td>8 (66%)</td>
<td>4 (66%)</td>
<td>4 (66%)</td>
<td>1.00</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>African American (n, %)</td>
<td>8 (66%)</td>
<td>5 (83%)</td>
<td>3 (50%)</td>
<td>0.3998</td>
</tr>
<tr>
<td>Caucasian (n, %)</td>
<td>3 (25%)</td>
<td>1 (16.7%)</td>
<td>2 (33.3%)</td>
<td></td>
</tr>
<tr>
<td>Asian (n, %)</td>
<td>1 (8.3%)</td>
<td>1 (16.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Past</td>
<td>5</td>
<td>2</td>
<td>3</td>
<td>1.00</td>
</tr>
<tr>
<td>Never</td>
<td>7</td>
<td>4</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Current</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>1.00</td>
</tr>
<tr>
<td>Renal disease</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1.00</td>
</tr>
<tr>
<td>Wound surface area</td>
<td>88.41 (±177.3)</td>
<td>14.13 (±12.77)</td>
<td>146.5 (±230.9)</td>
<td>0.19</td>
</tr>
</tbody>
</table>

Wound size (mean ± SD) of all wounds with specimens collected (n = 14).

Wound effluent specimens were collected using the Levine technique [281]. This technique has been well validated to ensure standardization throughout all specimens collected in the WE-HEAL Study. After collection, the swabs were immediately placed in 0.65 μm pore size centrifugal filters (Ultrafree-MC DV, Merck Millipore, MA, USA). Samples were centrifuged at 12000 rpm for 4 minutes to extract wound exudate and remove cellular and fibrinous debris. Samples were stored at -80 °C until analysis. Wound biofilm specimens were collected by swabbing the wound with a cotton swab also using the Levine technique [281, 282]. Samples were then stored at -80 °C until analysis.

For 16S rRNA profiling by 454 pyrosequencing, bacterial DNA was isolated from wound swabs. Wound swabs were resuspended in 1,200 μL of lysis buffer (20mM Tris-Cl, pH 8.0, 2mM EDTA, 1.2% Triton X-100) and vortexed thoroughly for 1 minute. Lysate (1,000 μL) was transferred into a lysing Matrix B tube (MP Biomedicals, 6911-
(500) where it was vortexed and centrifuged. Lysate was subsequently incubated at 75 °C for 10 minutes and treated with 200 mg/mL lysozyme and 20 mg/mL Proteinase K. DNA from the lysate was extracted twice using phenol-chloroform-isoamyl alcohol followed by ethanol precipitation.

DNA extracted from individual chronic wound swab samples was amplified using PCR primers that target the V1-V3 regions of the 16S rRNA gene (27) and carry a unique 12 bp barcode. 16S amplicons were generated using 100 ng of extracted DNA, Platinum Taq polymerase (Life Technologies, CA) in the following cycling conditions: first cycle of 95 °C 5 min; 35 cycles of 95°C/30s, 55°C/30s, 72°C/30s; last cycle of 72 °C 7 min. Amplicons were purified (QIAquick PCR purification kit, Qiagen, Valencia, CA), quantified fluorometrically (Tecan Group Mannedorf, Switzerland), normalized, and pooled. 454 sequencing was completed using Titanium chemistry (Roche, Branford, CT) following the manufacturer’s protocol.

Taxonomic classification was performed using the YAP package [283] which implements the mothur software [284] based on 16S rRNA gene reference sequences from the Ribosomal Database Project [285]. Biofilm specimens were considered to be positive for Pseudomonas spp. if any Pseudomonas reads were detected in the specimen regardless of relative abundance.

For electrochemical detection, disposable, screen-printed electrode sensors (TE100, Zensor, Taichung City, Taiwan) were used to detect the presence of pyocyanin in clinical samples (Figure 4-16). The sensors utilized a 3-electrode setup containing carbon-based working (3 mm diameter disk) and counter electrodes along with a Ag/AgCl reference electrode. All electrochemical measurements were recorded using a
portable potentiostat (μStat 200, DropSens, Parque Tecnológico de Asturias, Spain). The sensing surface was covered with a polymeric membrane (DRP-MEMB, DropSens, Parque Tecnológico de Asturias, Spain) to reduce the amount of sample volume required for analysis.

![Figure 4-16: Disposable, screen-printed electrode sensor with mesh modification for small-volume analysis.](image)

For each test, 7.5 µL of wound exudate was pipetted into the detector well. 7.5 µL was determined to be the minimum fluid volume required for the sensor to successfully perform a measurement. The minimum sample volume was ascertained by placing increasing amounts of wound exudate collected from a patient that was diagnosed with a *Pseudomonas* infection on a sensor and running the instrument, starting from 5 µL. When less than 7.5 µL is used for analysis, there is insufficient fluid to electrically connect all three electrodes on the sensor. A lack of connection between the three electrodes during measurement results in voltammograms that have numerous, randomly distributed, sharp current spikes. For all of the experiments, square-wave voltammetric scans were
performed at potentials ranging from -0.7 to 0.0 V at an amplitude voltage of 0.05 V, step voltage of 0.004 V, and a frequency of 15 Hz.

Each clinical sample was tested in duplicate with a new sensor used each time. The investigators were blinded to the microbiome 16S rRNA results at the time of the sensor detection experiment. The data was analyzed by two independent investigators using OriginPro 9.1 (OriginLab Corporation, Northampton, MA). To quantify the amplitude of the peaks measured, a baseline was subtracted from the data to remove background signals observed in the measurements. Baselines were created for each data set using spline interpolation with 32 base points. The resulting baseline-subtracted data set was used to identify peaks in the current and to determine the maximum currents of those peaks. From these maximum current values, using a cutoff of 0.030 µA for the average of the two measurements, a binary determination was made for whether the probe was detecting pyocyanin (positive) or not (negative) (Table 5-3).

Data was analyzed using GraphPad Prism 5.03 (for Windows, GraphPad Software, San Diego California, USA). Fisher’s exact test and Chi-squared tests were used for categorical variables and Student’s t-test was used for continuous variables. Results are represented as mean ± SD. A p-value less than 0.05 indicates statistical significance; all significance tests were performed and interpreted in a two-sided manner.

Results obtained from the microbiome profile generated by 16S ribosomal RNA sequencing were reviewed and samples with any positive *Pseudomonas* reads were considered to test positive for *Pseudomonas*. These results were compared to the results from the pyocyanin detector. The sensitivity and specificity of the sensor were calculated. The following detection strategy is envisioned.
Figure 4-17: Detection scheme for pyocyanin production by *P. aeruginosa* in clinical wound exudate samples.

For the electrochemical testing of animal samples for the presence of *P. aeruginosa*, samples were obtained from Angell Animal Medical Center (Boston, MA). All positive aerobic cultures submitted to Angell Animal Medical Center’s clinical laboratory during a 36 week period between November 2015 and July 2016 were included in this study. Culture samples were plated upon submission and inoculated in thioglycollate (thio) broth as part of the initial phases of microbiologic identification. Inoculated thio broth was incubated until turbidimetry (using the Sensititre AutoInoculator, TREK Diagnostic Systems, Thermo Fisher Scientific, Oakwood Village, Ohio) indicated the sample had reached the 0.5 McFarland standard (approximately 1.5×10^8 CFU/mL) and then sub-cultured. Automated portions of the identification process were performed using the TREK Sensititre. In addition to standard microbiologic identification and susceptibility testing, 10 microliters of the thio broth was pipetted onto a pyocyanin-detecting electrode (Zensor TE100, Taiwan). The samples were scanned using square-wave voltammetry from -0.5 to 0 V at a frequency of 15 Hz and an amplitude voltage of 50 mV to screen for the presence or absence of pyocyanin.
Detectable current in this range served to indicate the presence (positive result) or absence (negative result) of pyocyanin. Results were compared to the gold standard of microbiologic identification and susceptibility testing when they became available roughly 24 to 48 hours later. Due to the delay in microbiologic identification through traditional methods, the investigators were effectively blinded to the gold standard results when pyocyanin data was obtained. An additional five aliquots of 200 microliters of thio broth was pipetted into each of 5 microtainer tubes, stored with their anonymized microbiologic identification and susceptibility results, and frozen to -80 °C for future investigations.

Sensitivity and specificity were calculated using standard microbiologic identification and susceptibility testing as the gold standard. 95% confidence intervals were generated for each calculation.
5.0 Results and Discussion

5.1 Aim 1: The Up-Regulation of Pyocyanin Production by *Pseudomonas aeruginosa* for Rapid Bacterial Identification

5.1.1 Calibration Curves for Pyocyanin Detection in M63 and TSB Media

The following work using amino acids to up-regulate pyocyanin production by *Pseudomonas aeruginosa* was published in the journal Analyst (2014) (DOI: 10.1039/c4an00756e) [22].

Known concentrations of pyocyanin (1-hydroxy-5-methylphenazine, Sigma Aldrich, 85-66-5) were spiked in either M63 minimal media or trypticase soy broth (TSB) to create a calibration curve. Concentrations of pyocyanin ranged from 0-50 µM. For each concentration, 100 µL of sample was spotted onto a disposable screen-printed carbon electrode connected to an external Ag/AgCl reference. Square-wave voltammetry was used to measure the electrochemical response of pyocyanin, scanning from -0.4 to 0.0 V at an amplitude voltage of 0.050 V and a frequency of 15 Hz (Figures 5-1 and 5-3). The sensitivity and limit of detection (LOD) were calculated for each media type and are reported in Table 5-1. The sensitivity is the slope of the line calculated from the plot of the maximum current vs. concentration (Figures 5-2 and 5-4) while the LOD was calculated as $3\sigma$/sensitivity, where $\sigma$ is the standard deviation of the blank solution current at the pyocyanin oxidation potential.
Figure 5-1: Square-wave voltammograms of 0-50 µM pyocyanin in M63 minimal media.

Figure 5-2: Linear fit of 0-50 µM pyocyanin in M63 minimal media.
Figure 5-3: Square-wave voltammograms of 0-50 µM pyocyanin in trypticase soy broth.

Figure 5-4: Linear fit of 0-50 µM pyocyanin in trypticase soy broth.
Table 5-1: Pyocyanin sensitivity and limit of detection (LOD) for each media type.

<table>
<thead>
<tr>
<th>Media Type</th>
<th>Sensitivity (µA/µM)</th>
<th>LOD (µM)</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>M63 Minimal Media</td>
<td>0.36</td>
<td>0.042</td>
<td>0.99</td>
</tr>
<tr>
<td>Trypticase Soy Broth</td>
<td>0.25</td>
<td>0.048</td>
<td>0.99</td>
</tr>
</tbody>
</table>

5.1.2 Electrochemical Detection of *P. aeruginosa* in M63 and TSB Media

Liquid cultures of *Pseudomonas aeruginosa* were grown in either M63 minimal media or TSB to observe the effect of amino acid addition on the bacteria’s production of pyocyanin. 10 µL of stock PA14 culture was loaded into 10 mL liquid cultures containing one of six different amino acids (final concentration of 4 million cells/mL). To maximize the effect that individual amino acids may have on pyocyanin production, the amino acid concentrations chosen were 16-times higher than the concentrations typically found in patients with cystic fibrosis with the exception of tyrosine which was only increased 4-fold due to its lower solubility [29, 30]. Electrochemical scans were taken roughly every 2 hours over the course of a 24-hour period.

Figure 5-5A and B shows scans taken after 10 hours of growth in M63 minimal media and trypticase soy broth, respectively. A control sample with no added amino acids was also tested. Pyocyanin is expected to produce an electrochemical signal around -0.25 V versus a Ag/AgCl reference and this result is observed for all samples grown in the TSB media. However, no peak is observed in any of the samples in M63 minimal media after 10 hours.
Figure 5-5: Square-wave voltammograms of *P. aeruginosa* grown for 10 hours in the presence of individual amino acids in A) M63 minimal media and B) trypticase soy broth. Amino acids were used at the following concentrations (mM): proline (27.2), histidine (8), arginine (4.8), leucine (25.6), tyrosine (3.2), and valine (17.6).

Consistent among all tests was that samples grown in TSB produced a pyocyanin signal faster than those grown in M63 media. Cells can grow and divide normally in both media; however, TSB contains a series of additional nutrients not found in the M63 minimal media, such as casein and soybean lysate, which accelerate bacterial growth rate. However, of greater importance is that select amino acids had an up-regulatory effect on pyocyanin production as demonstrated by the samples containing tyrosine and valine. The results show that the addition of tyrosine to minimal media lowers the amount of time needed to detect the presence of *P. aeruginosa* in a sample via detection of current produced by pyocyanin.
Figure 5-6: Monitoring *P. aeruginosa*’s production of pyocyanin, by plotting the maximum current at -0.25 V vs. Ag/AgCl, over the course of 1 day in the presence of individual amino acids in A) M63 minimal media and B) trypticase soy broth. Amino acids were used at the following concentrations (mM): proline (27.2), histidine (8), arginine (4.8), leucine (25.6), tyrosine (3.2), and valine (17.6).

Scans were taken over the course of 1 day and the maximum currents are reported in Figure 5-6. The amino acids had varying effects on *P. aeruginosa*’s production of pyocyanin with tyrosine having the greatest up-regulatory effect in both media. These results correlate up-regulation of pyocyanin production with increased biofilm formation induced by the addition of individual amino acids [29]. Without the addition of amino acids, it took nearly 24 hours for a pyocyanin peak to be observed in M63 media, while a peak was seen within 10 hours for the control experiment using TSB. In M63 minimal media, addition of tyrosine and histidine resulted in the appearance of a pyocyanin peak in significantly less time. Addition of amino acid to TSB, however, did not appreciably change the amount of time needed to observe a pyocyanin peak. In all cases, the signal appeared 6 to 8 hours after the start of the experiment. However, the amount of pyocyanin produced after that point varied significantly. A small shift towards the
positive potential (< 0.10 V) was observed for samples grown in the M63 media, which can be attributed to minor differences in the salt and pH concentration of the surrounding media. The error bars, shown in the figure, increase with time because of heterogeneity in the bacterial population. As the number of cells increases over time, they do not all divide or produce pyocyanin at exactly the same rate. This results in greater variability in the pyocyanin concentration over time, for each experiment. However, as a sensing mechanism, the primary concern is inducing the production of pyocyanin and the actual variance is secondary.

5.1.3 Electrochemical Detection of P. aeruginosa by Varying Tyrosine and Valine Concentration

The next set of experiments investigated the optimal concentration at which tyrosine and valine should be present in the growth media to maximize pyocyanin production. Tyrosine and valine were selected as the target amino acids to add in the TSB media having demonstrated the largest up-regulatory effect of the six amino acids tested. Because cells grown in TSB growth media gave the fastest pyocyanin response, TSB was chosen as the growth media for the next phase of the study.
Tyrosine and valine were prepared at concentrations ranging from those quantified in typical cystic fibrosis infection levels (tyrosine: 0.2 mM, valine: 1.1 mM) [140] to an 80-fold increase, which reached the solubility limit of the amino acids in TSB. The initial concentration of *Pseudomonas aeruginosa* loaded in each sample was maintained at a constant of roughly 4 million cells/mL and electrochemical scans were taken every 2 hours over the course of 10 hours. Figure 5-7 shows scans taken after 8 hours of growth in addition to a control with no amino acid additives. Again, consistent among all scans was the observance of a pyocyanin peak around -0.25 V versus a Ag/AgCl reference. An interesting observation is that the largest pyocyanin concentration for the experiments with valine was recorded for the 17.6 mM concentration, but pyocyanin production decreased when the valine concentration was raised further to 88 mM. It is possible that valine has an inhibitory effect on pyocyanin production at high concentrations. While such inhibition has been observed for other molecules [151], *P.
*P. aeruginosa* has not been previously studied at valine concentrations that are over 100 times greater than what is typically found in a pseudomonal infection.

**Figure 5-8:** Monitoring *P. aeruginosa*’s production of pyocyanin over the course of 10 hours in TSB cultures containing A) tyrosine at various concentrations and B) valine at various concentrations.

Figure 5-8 shows the increase in pyocyanin over time for each of the concentrations of tyrosine and valine tested. From the data presented in Figure 5-8A, a statistically significant increase in current output between 6 and 8 hours is observed, marking the minimum amount of time necessary to electrochemically detect a *P. aeruginosa* infection in a processed sample. More importantly, varying the concentration of tyrosine and valine added to the solution has a minimal effect on the amount of time needed for cells to up-regulate pyocyanin production, but has a significant effect on the amounts produced after this critical time point. The pyocyanin production rates by the cells in 16 mM tyrosine and 17.6 mM valine are nearly identical.

To observe the effect these two amino acids have on the bacterial cell concentration in the samples, a hemocytometer was used to determine cell density every
2 hours for up to 10 hours during cell growth. These results are reported in Figure 5-9 and highlight that both tyrosine and valine increase *P. aeruginosa*’s growth and thus contribute to increased pyocyanin production rates.

![Graph showing bacterial cell growth](image)

**Figure 5-9:** Monitoring bacterial cell growth of *P. aeruginosa* over the course of 10 hours in trypticase soy broth. A hemocytometer was used to determine cell density.

### 5.1.4 Electrochemical Detection of *P. aeruginosa* by Varying Initial Bacterial Concentration

The next set of experiments studied how varying the initial *Pseudomonas aeruginosa* concentration would affect the production of pyocyanin in the presence of amino acids. TSB media was used as the growth media to which tyrosine and valine were added. Tyrosine (16 mM) and valine (17.6 mM) concentrations were held constant while varying amounts of *P. aeruginosa* were added into the samples (4, 20, 40, 400 million...
cells/mL). Two control experiments were also included: one with an initial *P. aeruginosa* concentration of 4 million cells/mL without additional amino acids and the second with the amino acids added but no bacteria. Electrochemical scans were taken every 2 hours over the course of 10 hours. Figure 5-10 shows scans taken after 8 hours of growth.

![Square-wave voltammograms of *P. aeruginosa* grown in TSB for 8 hours in A) tyrosine (16 mM) and B) valine (17.6 mM). *P. aeruginosa* was used at the following concentrations (million cells/mL): 4, 20, 40, and 400, respectively. Control experiments contained 4 million cells/mL without additional amino acids added and sterile growth media with added amino acids.](image)

There is a clear correlation between the increasing starting concentration of bacteria and the amount of pyocyanin produced over a constant amount of time. For initial cell concentrations above 4 million cells/mL, 16 mM tyrosine causes *P. aeruginosa* to produce more pyocyanin than 17.6 mM valine. The minimum incubation time needed to detect a *P. aeruginosa* infection increases with decreasing initial cell concentration (Figure 5-11). From the data presented in Figure 5-11A, a statistically significant increase in current output is obtained for the highest initial cell concentration (400 million...
cells/mL) between 4 and 6 hours after the start of the experiment. These results indicate that the amount of time necessary to detect a current change due to pyocyanin production can be used to quantify the number of initial cells present in the sample.

![Image](image_url)

**Figure 5-11:** Monitoring *P. aeruginosa*’s production of pyocyanin over the course of 10 hours in TSB cultures containing A) tyrosine (16 mM) and B) valine (17.6 mM). *P. aeruginosa* was used at the following concentrations (million cells/mL): 4, 20, 40, and 400, respectively. Control experiments contained 4 million cells/mL without additional amino acids added and sterile growth media with added amino acids.

The theoretical cellular limit of detection for this approach, without sample pre-concentration, is 0.1 cells/mL, since a 10 mL sample volume is used in the analysis. It is expected to take approximately 1 day to observe a pyocyanin peak for this minimal concentration. Previous work, where fresh growth media was inoculated with only a few cells from a *P. aeruginosa* colony on a plate, showed that it took approximately 24 hours to produce a 5 µM concentration of pyocyanin [19], which supports the data from this study.
To determine if complexing samples with multiple amino acids would cause an additive up-regulatory effect, both tyrosine and valine were inserted into samples as previously described. A decrease in pyocyanin output is observed when both amino acids are present, as shown in Figures 5-12 and 5-13. Therefore, the effect of adding multiple amino acids on the pyocyanin production of *P. aeruginosa* is more complex and warrants further investigation.

**Figure 5-12:** Square-wave voltammograms of *P. aeruginosa* grown in trypticase soy broth for 10 hours in the presence of tyrosine (16 mM) and valine (17.6 mM). *P. aeruginosa* was used at the following concentrations (million cells/mL): 4, 20, 40, and 400, respectively. Control experiments contained 4 million cells/mL without additional amino acids added and sterile growth media with added amino acids.
Figure 5-13: Monitoring *P. aeruginosa*’s production of pyocyanin over the course of 10 hours in TSB cultures containing both tyrosine (16 mM) and valine (17.6 mM). *P. aeruginosa* was used at the following concentrations (million cells/mL): 4, 20, 40, and 400, respectively. Control experiments contained 4 million cells/mL without additional amino acids added and sterile growth media with added amino acids.

5.1.5 Iron and Sugar Up-Regulation

*Pseudomonas aeruginosa* was grown in TSB liquid cultures in the presence of various iron concentrations. Ferrous sulfate was used as the iron supplement as it is the most commonly used medical treatment for iron deficiency [286]. Final concentrations of iron in solution varied from 0-10 mM. Previous literature has shown that both iron-depletion (less than 0.001 mM) and iron-repletion (greater than 0.1 mM) slows biofilm formation [151, 152]. The upper and intermediate regimes were explored. Figure 5-14 shows square-wave voltammetry scans from -0.4 to -0.1 V vs. a Ag/AgCl reference electrode after 8 hours of growth. From this data, it is clear that iron plays a role in *P.*
*P. aeruginosa*’s production of pyocyanin. For the upper regime concentrations (greater than 0.1 mM), a decrease in the current output is observed. For the intermediate concentrations (0.01 and 0.1 mM), an increase in the current output is observed, showing that iron at these concentrations increases pyocyanin production which can lead to faster electrochemical detection of *P. aeruginosa*.

![Figure 5-14](image_url)

**Figure 5-14**: Square-wave voltammograms of *P. aeruginosa* grown in trypticase soy broth for 8 hours in the presence of iron at various concentrations.

Similar experiments were run to test the effectiveness of sugars as up-regulatory molecules for pyocyanin production in samples containing *P. aeruginosa*. Two simple sugars were chosen for testing, glucose and sucrose, with concentrations based on literature values for supplementing growth media for *P. aeruginosa* growth [36]. The results are reported in Figure 5-15. After 10 hours of growth, both sugars have some observable effect on regulating pyocyanin production. Overall, further experiments are
required to fully understand the effect of iron and simple sugars on the production of pyocyanin by \textit{P. aeruginosa}.

\textbf{Figure 5-15:} Square-wave voltammograms of \textit{P. aeruginosa} grown in trypticase soy broth for 10 hours in the presence of simple sugars at various concentrations.

It was also shown that selective media can be used to up-regulate the production of pyocyanin as a means for faster identification. \textit{Pseudomonas} agar is a media developed by King \textit{et al.} to selectively isolate \textit{Pseudomonas} species from other clinical specimens \cite{158}. Most notably, it contains the magnesium chloride and potassium sulfate needed to promote the production of pyocyanin. As a result, this media allows for the easy identification of \textit{Pseudomonas} species due to its enhancement of the blue-green pyocyanin molecule. While it is a solid agar medium, if a sensor is embedded into the plate, it can be used to detect pyocyanin production faster than visual inspection based on...
the blue-green coloration of pyocyanin [24]. The setup of this platform is shown in Figure 5-16.

Figure 5-16: Disposable screen-printed electrodes embedded into plastic culture plates filled with *Pseudomonas* agar.

5.2 Aim 2: Screening Other Bacterial Pathogens for Redox-Active Biomarkers for Electrochemical Detection

5.2.1 Screening Hospital Samples and Human Biofluids

A series of clinically-relevant bacteria obtained from various sources including Brigham and Women’s Hospital were electrochemically scanned using square-wave voltammetry at 15 Hz. These scans are shown in Figure 5-17.
Notably, *Pseudomonas aeruginosa* is the only bacteria to produce the redox-active molecule, pyocyanin. No other redox-active peaks appear in the voltammograms from the other clinically-relevant bacteria. The bacteria scanned included: *Acinetobacter baumannii*, *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Enterococcus faecalis*, *Klebsiella pneumoniae*, *Staphylococcus aureus* (including a MRSA strain), *Staphylococcus epidermidis*, and *Streptococcus pneumoniae*. Two liquid growth mediums were also scanned: lysogeny broth (LB) and trypticase soy broth (TSB).

One concern that may arise is the nature of complex media, such as human biofluids, which contain a plethora of molecules that may elicit an electrochemical response. Redox-active molecules from these samples could potentially interfere in the potential window that pyocyanin is observed. To address this concern, 5 µM pyocyanin was spiked into five different human samples containing bronchial lavage, sputum, urine,
whole blood, or whole blood with sodium heparin [21]. These human biofluids were obtained from Bioreclamation (NY, USA) from 20 healthy human donors. Figure 5-18 shows square-wave voltammograms from -0.5 to 0.0 V. As evident in the figure, there are no interfering peak signals in the area where pyocyanin is detected, demonstrating the potential for translating this technology into the clinic. Figure 5-19 gives the linear range for pyocyanin detection in the human biofluid samples. These results further illustrate this technique’s applicability towards real-world detection scenarios.

**Figure 5-18:** Square-wave voltammograms of 5 µM pyocyanin spiked in A) bronchial lavage, B) sputum, C) urine, D) whole blood, and E) whole blood with sodium heparin.
5.2.2 Screening Clinical Strains of *P. aeruginosa* for Pyocyanin

The following work detecting pyocyanin production in clinical *Pseudomonas aeruginosa* isolates was published in the journal Biosensors and Bioelectronics (2017) (DOI: 10.1016/j.bios.2017.05.042) [26].

Although additional redox-active molecules were not identified, the results are important as they validate that pyocyanin is the only redox-active molecule to be produced by *Pseudomonas aeruginosa* in this potential window. To extend this further, even greater validity can be obtained by screening many different strains of *P. aeruginosa* to verify that all clinically-relevant strains produce pyocyanin.

To achieve this goal, a total of 94 different clinical isolates of *P. aeruginosa* were obtained from Brigham and Women’s Hospital and electrochemically scanned. A total of 94 electrochemical scans were taken (34 hospital strains, 31 mucoidal strains, and 29...
cystic fibrosis strains). A typical electrochemical square-wave voltammogram is shown in Figure 5-20 where the larger current peak observed at -0.25 V is a result of pyocyanin oxidation while the smaller, secondary peak can be attributed to 5-methylphenazine-1-carboxylic acid, a precursor to pyocyanin synthesis [287].

![Square-wave voltammogram of a clinical isolate of *P. aeruginosa* obtained from a patient with cystic fibrosis.](image)

**Figure 5-20:** Square-wave voltammogram of a clinical isolate of *P. aeruginosa* obtained from a patient with cystic fibrosis.

Table 5-2 shows the electrochemical results for all clinical isolates of *P. aeruginosa*. After 24 hours of growth, 75.6% of isolates tested positive for pyocyanin, with all isolates producing the redox-active biomarker after 48 hours of growth.
Table 5-2: Positive predictive values for whether clinical isolates of *P. aeruginosa* produce the redox-active, biomarker pyocyanin. Experimental determinations were based on the presence or absence of peak currents associated with pyocyanin oxidation, obtained from electrochemical square-wave voltammograms.

<table>
<thead>
<tr>
<th>Clinical <em>Pseudomonas aeruginosa</em> isolates</th>
<th>After 24 hours of growth</th>
<th>After 48 hours of growth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hospital strains (N=34)</td>
<td>0.618</td>
<td>1.00</td>
</tr>
<tr>
<td>Mucoidal strains (N=31)</td>
<td>0.774</td>
<td>1.00</td>
</tr>
<tr>
<td>Cystic fibrosis strains (N=29)</td>
<td>0.897</td>
<td>1.00</td>
</tr>
</tbody>
</table>

To investigate whether other phenazine molecules interfere with the pyocyanin oxidation peak, Figure 5-21 shows a square-wave voltammogram from a clinical *P. aeruginosa* isolate after 1 day of growth at 37 °C, with additional scans taken after spiking the culture with pyocyanin and subsequently 1-hydroxyphenazine, another phenazine side product in the pyocyanin biosynthesis pathway (Figure 2-6). Results show that all three redox-active phenazines are readily resolved from each other and the electrochemical peak observed around -0.25 V is confirmed to be pyocyanin.
Figure 5-21: Square-wave voltammograms of a clinical *P. aeruginosa* isolate added with other redox-active phenazines (pyocyanin, 1-hydroxyphenazine). The *P. aeruginosa* culture was grown at 37 °C for 18-24 hours and placed onto an electrochemical sensor. Additional scans were taken after spiking the sample with a pyocyanin standard and subsequently 1-hydroxyphenazine. Phenazine standards were at 100 µM prior to addition. Square-wave voltammograms are shown after baseline-subtraction.

Bioinformatics analysis of currently available genomic data in GenBank [275] was completed to determine if all *P. aeruginosa* strains have genes encoding for the PhzM and PhzS enzymes necessary to produce pyocyanin. Complete (N=199) and draft (N=2380) genomes within the genus *Pseudomonas* were analyzed by gene calling using Prodigal [276], followed by annotation of predicted genes against the KEGG database [277] using RAPSearch2 [278]. A total of 2579 genomes from the genus *Pseudomonas* were tested for the absence or presence of these two genes (i.e., PhzS: Kegg Orthology
It was determined that only the species *Pseudomonas aeruginosa* possessed both genes, with 1649 of 1651 different strains capable of producing pyocyanin, with two scaffold-level draft genomes lacking the two genes likely as a result of incomplete genome assembly.

Figure 5-22 shows the electrochemically determined pyocyanin concentrations for the clinical isolates after 24 and 48 hours of growth. From this data, it was observed that the hospital strains of *P. aeruginosa* produced the least amount of pyocyanin (0.6-1.9 µM), followed by mucoidal (2.7-20.1 µM), and cystic fibrosis strains (4.2-41.3 µM).
Figure 5-22: Electrochemically measured pyocyanin concentrations from different clinical isolates of *P. aeruginosa* after 24 and 48 hours of growth. Hospital (N=34), mucoidal (N=31), and cystic fibrosis (N=29) strains of *P. aeruginosa* were grown at 37 °C over the course of 2 days and electrochemically scanned after each day. The change in current observed in the pyocyanin oxidation peak was correlated with the pyocyanin concentration in each of the samples using a calibration curve. Error bars represent the standard error of the mean. * denotes *P* < 0.05.

To better understand the amount of pyocyanin produced by clinical isolates of *P. aeruginosa*, three isolates for each of the three strain categories were randomly selected and electrochemical measurements were taken concurrently to cell density measurements. Electrochemical measurements are shown in Figure 5-23.
Figure 5-23: Monitoring pyocyanin produced over time of different clinical isolates of *P. aeruginosa*. For each strain type, three isolates were tested. The samples were grown for 18-24 hours at 37 °C and 150 rpm and inoculated into individual test tubes containing fresh trypticase soy broth at a starting concentration of $4 \times 10^6$ cells/mL. Electrochemical measurements to monitor pyocyanin production were taken every 2 hours for 24 hours, and then every 6 hours for an additional 24 hours. Error bars represent the standard error of the mean.

From this data and monitoring cell counts over time (Figure 5-24), it is possible to determine the number of pyocyanin molecules produced per *P. aeruginosa* cell over time (Figure 5-25A). Average pyocyanin production rates over 24 and 48 hours are reported in Figure 5-25B. The highest pyocyanin production rate over the course of 48 hours of growth is observed by the cystic fibrosis strains ($1.7 \times 10^4$ molecules/cell/h), followed by mucoidal strains ($1.4 \times 10^4$), and finally hospital strains ($1.0 \times 10^4$).
Figure 5-24: Monitoring bacterial cell growth over time of different clinical isolates of *P. aeruginosa*. For each strain type, three isolates were tested. Error bars represent the standard error of the mean. The samples were grown for 18-24 hours at 37 °C and 150 rpm and inoculated into individual test tubes containing fresh trypticase soy broth at a starting concentration of $4 \times 10^6$ cells/mL. Using a hemocytometer (Hausser Scientific, Model 3500), cell density measurements of the samples were taken every 4 hours for 24 hours, and then every 6 hours for an additional 24 hours.
Figure 5-25: Monitoring pyocyanin production rates in clinical isolates of \textit{P. aeruginosa}. A) Electrochemically monitoring pyocyanin molecules produced per \textit{P. aeruginosa} bacterial cell over the course of 48 hours. B) Average pyocyanin production rates for the different clinical isolates of \textit{P. aeruginosa}. Error bars represent the standard error of the mean. * denotes $P < 0.05$.

With this electrochemical detection method, it is important to be able to resolve the electrochemical signal caused by pyocyanin oxidation from other interferents. The data from Figure 5-20 highlights another redox-active molecule that \textit{P. aeruginosa} secretes in its environment, 5-methylphenazine-1-carboxylic acid, which oxidizes at more positive potentials than pyocyanin [18]. While both PhzM and PhzS enzymes are required to catalyze the formation of pyocyanin, 5-methylphenazine-1-carboxylic acid synthesis only requires PhzM (Figure 2-6) and thus may not be a unique biomarker for \textit{P. aeruginosa}. In addition, 1-hydroxyphenazine, another side product in pyocyanin biosynthesis, is also shown to have redox-active properties, but it oxidizes at more negative potentials than pyocyanin and thus can be readily distinguished (Figure 5-21). Electrochemical scans of other bacterial species have also demonstrated that no other
redox-active interferents are observed in the potential range of pyocyanin oxidation, highlighting the selectivity of this detection scheme (Figure 5-17).

The positive predictive values in Table 5-2 show that all 94 clinical isolates tested positive for pyocyanin and validates the hypothesis that all clinically-relevant strains of *P. aeruginosa* produce pyocyanin. This claim is also supported by the bioinformatics analysis. While some samples took longer than 1 day before testing positive for pyocyanin, many factors are known to impact when pyocyanin production is observed. These factors can include growth conditions, differences in starting concentration of bacterial cells obtained from swabs, and level of infection at the time of patient sampling. Interestingly, the clinical isolates obtained from patients with hospital-acquired infections had the lowest positive predictive value, which may be attributed to the low abundances of pyocyanin that these strains produce (Figure 5-22). Figure 5-22 highlights different pyocyanin production rates between the different strains and as expected, *P. aeruginosa* obtained from patients with cystic fibrosis produced the most amount of pyocyanin as these isolates are likely more virulent and have been shown to be in the more advanced biofilm stage of infection in sputum obtained from patients with this disease [82]. The relatively low pyocyanin concentrations found in the hospital strains are consistent with previous work that measured pyocyanin concentrations in infected chronic wound patients [25].

The pyocyanin production rates in Figure 5-25 show that the amount of pyocyanin produced over time is fairly consistent (Figure 5-25A) with some small deviations observed by the cystic fibrosis strains, which can be attributed to their higher virulence. However, averages calculated over the course of 24 and/or 48 hours indicate that all three
P. aeruginosa strain types produce pyocyanin at statistically distinct rates. Increased production rates are observed over the first 24 hours of growth (Figure 5-25B), which can be likely attributed to the bacteria’s formation of biofilm and increased intercellular communication during this exponential phase of bacterial growth [288]. The decreased pyocyanin production rates after 24 hours is in agreement with previous literature, which measured pyocyanin in mucoidal strains of P. aeruginosa and found that the regulated quorum sensing system led to decreased virulence factor production upon entering the stationary phase after prolonged growth [289]. These lowered production rates are supported by another study, which measured pyocyanin across a panel of 42 different strains of P. aeruginosa [290]. These approaches utilized chloroform extraction along with UV-Vis spectroscopy to measure pyocyanin. This technique, while proven, is not sufficiently sensitive and requires lengthy sample processing. The data from this study is the first attempt to quantify the amount of pyocyanin produced in clinical isolates of P. aeruginosa with a statistically significant difference observed in pyocyanin production between cystic fibrosis and non-cystic fibrosis strains. More importantly, the data shows that P. aeruginosa produces pyocyanin even at low bacterial densities that can be detected at early stages of infection with this electrochemical platform. Early detection of clinically-relevant pathogens is a highly desirable goal in clinical care, providing time-sensitive information that can improve patient outcomes.

One of the barriers to achieving this goal is the inherent limitations of the sensor itself. While the data from this study illustrates that current screen-printing sensor technologies are sensitive enough to measure pyocyanin concentrations in these samples, it is important to note that the detection limit of the electrochemical sensors can be
greatly refined (low nM sensitivity) by switching to micro- and nanofabricated electrodes [20, 291]. Given the high costs of photolithography fabrication, screen-printing and laser etching remain as some of the less expensive alternatives for sensing applications [292, 293]. Cost concerns will likely be further mitigated with the ongoing advancement of technology and recent shift in focus towards developing inexpensive sensor platforms. Lastly, as an exploit for early detection of *P. aeruginosa* infections, the biosynthesis of pyocyanin and other phenazines has been shown to be up-regulated in the presence of amino acid supplements, nutrient depletion, and through the formation of a biofilm [22, 29, 120], factors which are known to impact organism pathogenicity.

5.3 **Aim 3: Developing Alternative Electrochemical Sensing Strategies for Bacterial Detection**

5.3.1 **Aptamer-Based Electrochemical Sensor**

To study the use of an aptamer-based electrochemical sensor for detection of clinically-relevant bacteria, a previously synthesized aptamer for a known target was chosen and incorporated in the current electrochemical platform for testing. The aptamer for detecting 3O-C12-HSL, a quorum sensing molecule unique to *Pseudomonas aeruginosa*, was tested as a proof-of-concept [294].

Square-wave voltammograms (15 Hz frequency) of the aptamer sensor in a phosphate-buffered saline (PBS) blank and in the presence of the 3O-C12-HSL target are shown in Figure 5-26. The oxidation of the methylene blue on the aptamer is being monitored in this electrochemical platform as an indicator of bound target. It is expected that in the presence of the target, the electrochemical response from methylene blue will
decrease due to a conformational change in the aptamer that occurs when it is bound to
the target. The conformational change increases the distance between the redox probe and
electrochemical surface, resulting in a decrease in electrochemical signal. It is important
to mention that there are aptamer structures that behave in the opposite way, where the
binding event to the target decreases the distance from the redox probe to the
electrochemical surface, resulting in an increase in electrochemical signal.

Figure 5-26: Square-wave voltammograms showing aptamer detection of 30-C12-
HSL, a unique quorum sensing molecule secreted by \textit{P. aeruginosa}.
The blank scan is in PBS and the decrease in current signal observed
in the presence of target correlates with the concentration of target in
the sample.

In Figure 5-26, there is a notable decrease in current observed after the addition of
the target. The signal of the blank is directly proportional to the surface coverage of the
aptamer on the electrochemical surface and the decrease in current correlates with the
concentration of the target [271, 280]. To illustrate this correlation, different target concentrations were tested on a single aptamer sensor in order of increasing concentration. The square-wave voltammograms are shown in Figure 5-27.

Figure 5-27: Square-wave voltammograms of the *P. aeruginosa* aptamer bound to different target concentrations of 3O-C12-HSL.

Figure 5-27 shows that as the target (3O-C12-HSL) concentration increases, the peak current decreases as expected. While the change in signal is not as large as observed in Figure 5-26, this could be due to several factors including differences in aptamer coverage on the electrode surface and binding kinetics of the aptamer. Nevertheless, the downward trend is readily observed. To better illustrate the relative differences between runs, three electrodes were functionalized with the aptamer and tested with increasing concentration of target. The results of the peak current vs. target concentration for each electrode are reported in Figure 5-28.
Figure 5-28: Measuring max peak currents over different target concentration ranges. Differences in starting currents are due to differences in electrode surface coverage of the aptamer.

Figure 5-28 illustrates a correlating decrease in current based on increasing concentrations of target present in the sample. Differences in starting currents are due to differences in electrode surface coverage of the aptamer. This can corrected for by determining the electrode density [280]. In addition, it can also be corrected by reporting current differences as a percentage of the initial peak current. The electrochemical platform was tested with the 3O-C12-HSL positive target (Figure 5-29) and the C4-HSL negative target (Figure 5-30) for this aptamer sequence [294].
Figure 5-29: Semi-log plot showing a linear correlation between the aptamer sensor’s percent signal change and 3O-C12-HSL target concentration.

![Semi-log plot showing a linear correlation between aptamer sensor's percent signal change and 3O-C12-HSL target concentration.]

\[ \Delta \text{Signal} = -3.54[3O-C12-HSL] - 10.02 \]
\[ R^2 = 0.98 \]

Figure 5-30: Semi-log plot showing that the aptamer sensor’s signal is not affected by C4-HSL target concentration.

![Semi-log plot showing that aptamer sensor's signal is not affected by C4-HSL target concentration.]

\[ \Delta \text{Signal} = -0.15[\text{C4-HSL}] - 1.27 \]
\[ R^2 = -0.20 \]
As seen in Figure 5-29, the aptamer sensor demonstrates a linear correlation with the concentration of the 3O-C12-HSL target when plotted on a semi-log scale. As expected, the sensor’s percent signal change becomes larger in response to increasing target concentration. When this aptamer is tested with the C4-HSL negative target, the percent signal change remains relatively unchanged in response to increasing concentration of target. The contrast in trend lines between the two figures confirms that this aptamer is selective to the 3O-C12-HSL target it was originally developed for [294].

The successful incorporation of this *P. aeruginosa* aptamer in the current electrochemical platform is the first step towards developing novel diagnostic tools for detecting other clinical pathogens. Further investigation would require the development of a novel aptamer to a new, unique target from another clinical pathogen. *Acinetobacter baumannii*, another ESKAPE pathogen, is primarily responsible for ventilator-associated pneumonia in patients in hospital intensive care units [4, 264, 265]. Previous work has identified one gene (A1S_1389) that is conserved by all strains of *A. baumannii* [267, 268]. A1S_1389 has been established as a DNA damage response gene that also plays the role of a regulatory molecule [269, 270]. This gene encodes for a protein that exists in multiple copies found only inside the cell. While it would require cell lysis to extract, it presents a novel sensing strategy for detecting *A. baumannii* infections. An aptamer can be used for the selective capture of the protein. The aptamer itself would need to be developed and selected using the SELEX (Systematic Evolution of Ligands by Exponential Enrichment) process [247, 252]. The development of a sensor that can detect *A. baumannii* and other ESKAPE pathogens would be a major step towards point-of-care diagnostics in the hospital setting.
5.4 Aim 4: Applications of Electrochemical Sensors towards the Hospital Setting

5.4.1 Dual *P. aeruginosa* and pH Sensor

Previous work on a nanofabricated electrochemical sensor utilizing a pH-sensitive hydride reference electrode was further investigated [19]. This sensor is unique as it is sensitive to both changes in pH and pyocyanin concentration. The device fabrication scheme and finalized device can be seen previously in Figure 4-8.

To demonstrate the device’s ability to measure pyocyanin vs. the screen-printed electrodes presented previously, a dilution series of pyocyanin was created and scanned using square-wave voltammetry at 15 Hz (Figure 5-31). Note that the current readings are in the nanoamp regime due to the smaller electrode surface area.

![Square-wave voltammograms at 15 Hz of 0-100 µM pyocyanin in 100 mM phosphate buffer using a microfabricated electrode sensor.](image)

**Figure 5-31:** Square-wave voltammograms at 15 Hz of 0-100 µM pyocyanin in 100 mM phosphate buffer using a microfabricated electrode sensor.
In another proof-of-concept, the fabricated sensor can also measure the oxidation of pyocyanin in real time. To demonstrate this, a polydimethylsiloxane (PDMS) microwell was aligned over the electrode area and solutions of pyocyanin were loaded into the well at different time points and the current response measured. The real-time electrochemical response is shown in Figure 5-32 [20].

![Figure 5-32: Amperometric measurements showing the real-time current response measured by a nanofluidic electrochemical assembly with an integrated Pd reference electrode. The current measured by the top working electrode as pyocyanin concentration is A) increased or B) decreased. The top electrode was set to -1.5 V and the bottom electrode to 0 V versus a Pd reference.](image)

This highlights the device’s ability to monitor pyocyanin in real time, an important characteristic if used to identify *Pseudomonas aeruginosa* in patients with ongoing infection.

To demonstrate the usefulness of the sensor to pH measurements, the measured current versus applied voltage was recorded for three different pH samples (Figure 5-33).
As the pH of the sample varied, a shift in the maximum peak potential was observed. Further analysis shows a linear correlation between pH and the peak potential with a change of 37 mV per pH unit (Figure 5-34). For ideal electrodes, a shift of 59 mV per pH unit is expected but palladium is known to undergo hydrogen-palladium phase changes and slow decomposition, and thus only exhibits ideal pH shifts for very short times after extensive cleaning and pretreatment [19].
The ability to simultaneously measure the concentration of pyocyanin and the pH of a fluid sample showcases the device’s future medical application as part of a smart bandage where pH is linked to general bacterial infection and the presence of pyocyanin indicates the presence of *P. aeruginosa* in the infection.

An updated sensor made from a glass wafer is shown in Figure 5-35, highlighting its potential use in optical microscopy for imaging bacterial cells on the device. Because this device requires wire bonds to an Ethernet cable to make electrical connection to a potentiostat, a redesigned sensor with larger contact pads for USB connection is shown in Figure 5-36. The USB connection allows the sensor to function like a plug-and-play device.
In addition, these sensors can be easily integrated into microfluidic systems to study biofilm formation in real time [295]. Shown in Figure 5-37 are microfabricated interdigitated electrodes that were irreversibly plasma bonded to PDMS microfluidic channels to monitor the presence of P. aeruginosa biofilms. Channels were
electrochemically monitored in real time via square-wave voltammetry to detect the
presence of pyocyanin by the biofilms growing in the channel (Figure 5-37A).

Microfabricated interdigitated electrode arrays were patterned on a glass wafer
with a single photolithography mask, followed by electron beam deposition and metal
lift-off. The electrode arrays consisted of a pair of gold interdigitated electrodes, a gold
counter, and quasi-reference electrodes. A finished device can be seen in Figure 5-37B.
Completed devices contained two parallel PDMS channels (100 µm wide) with
microfabricated electrodes underneath. One of the channels was filled with *P. aeruginosa*
in TSB while the other was filled with only TSB (control).

**Figure 5-37:** A) Monitoring peak currents due to pyocyanin oxidation of the two
microfluidic channels. B) Bright-field image of the two channels, one
with *P. aeruginosa* biofilm growing and the other with no bacteria
present.

In addition, the disposable, screen-printed sensors can also be integrated with a
microfluidic setup for biosensor applications. Figure 5-38 shows a screen-printed sensor
(Zensor TE100) with a PDMS microchannel irreversibly plasma bonded to the sensor.
This sensor platform was used to monitor *P. aeruginosa*’s production of pyocyanin during antibiotic exposure as a way to test for antibiotic susceptibility [23]. This was demonstrated using colistin sulfate [296], inserted at different minimum inhibitory concentrations (MIC) in a microfluidic chamber containing *P. aeruginosa* biofilm. The production of pyocyanin was monitored in real time and used as an indicator of the health of the biofilm. The results are reported in Figure 5-39.
As seen in Figure 5-39, higher concentrations of antibiotic lead to a decreased pyocyanin response. This is most evident at 45 hours (roughly 20 hours after exposure to antibiotic). At this time point, *P. aeruginosa* biofilm exposed to 4 mg/L colistin sulfate showed no significant difference when compared to *P. aeruginosa* biofilm exposed only to TSB, indicating that the lower MIC value was not significantly affecting the production of pyocyanin. However, pyocyanin production rates were statistically lower when the cells were exposed to 16 and 100 mg/L colistin sulfate. Live/dead cell viability assays were done post-exposure and supported the hypothesis that a reduction in the pyocyanin signal is correlated with a reduction in the number of living cells. This data highlights the microfluidic electrochemical platform’s ability to monitor bacterial antibiotic susceptibility for clinical point-of-care applications.
5.4.2 Detecting Multidrug-Resistance in Clinical \textit{P. aeruginosa} Isolates

In an effort to optimize the electrochemical platform for point-of-care applications, it is important to test the detection limits of square-wave voltammetry and explore the prospect of using other, possibly more sensitive electroanalytical techniques as a way to improve upon these detection limits.

In addition, to make the \textit{Pseudomonas aeruginosa} sensor more applicable to the hospital setting, it is important to understand the role that drug-resistance plays in \textit{P. aeruginosa}’s production of pyocyanin. To this end, AC voltammetry was used to electrochemically monitor pyocyanin production rates from multidrug-resistant strains of \textit{P. aeruginosa}. AC voltammetry has yet to be applied towards this detection scheme and can potentially provide higher sensitivities than previously demonstrated electroanalytical techniques, including differential pulse voltammetry and square-wave voltammetry.

As a proof-of-concept, a 1 µM pyocyanin standard was electrochemically tested against a negative control (growth media). Both square-wave voltammetry (15 Hz frequency) and AC voltammetry (100 Hz frequency) were used to measure the current response from the oxidation of pyocyanin. The results are shown in Figure 5-40.
Figure 5-40: Electrochemical scans of pyocyanin with either SWV or ACV.

The figure highlights the sensitivity differences between the two electroanalytical techniques. Due to the higher observed current for the same sample, ACV has theoretically higher detection limits than SWV for this specific sensor application. This is even more readily apparent when scanning clinical samples of *P. aeruginosa* as seen in Figure 5-41.
As seen in Figure 5-41, ACV demonstrates higher sensitivities than SWV, even resolving additional redox-active peaks (1-hydroxyphenazine) in the clinical scans not shown by SWV.

However, it is important to note that the two techniques were run at different frequencies. While these are typical frequencies for each respective technique, the role of frequency was explored to determine its effect on detection limits. Both SWV and ACV were run at 15 and 100 Hz frequency on a 1 and 100 μM sample of pyocyanin. The scans are shown in Figures 5-42 and 5-43.
Figure 5-42: Electrochemical scans of 1 µM pyocyanin with either SWV or ACV while varying the scan frequency.

Figure 5-43: Electrochemical scans of 100 µM pyocyanin with either SWV or ACV while varying the scan frequency.
For both 1 and 100 µM samples of pyocyanin, SWV and ACV show similar sensitivity at 15 Hz. However, a notable divergence is observed when both techniques are scanned at 100 Hz. For the higher concentration of pyocyanin, SWV demonstrates a significantly higher sensitivity than ACV. This disparity is less significant at the lower concentration of pyocyanin. This information is useful because it highlights the importance of optimizing the frequency parameters for these electroanalytical techniques for biosensor applications. In addition, slight variances in the oxidation potential of pyocyanin are observed, where the ACV scans are shifted to more positive potentials. This phenomenon is observed in both the low and high concentrations of pyocyanin and in both the 15 and 100 Hz scans, indicating that the actual electrochemical technique may be affecting the potential at which the redox reaction occurs. Interestingly, this shift in oxidation potential is not as apparent when running actual *P. aeruginosa* samples (Figure 5-41). Also, there is a higher measured current observed with the 100 Hz scans for both techniques, which may potentially be used to increase the sensitivity and detection limit of the sensor. Both techniques have higher and lower frequency limits that warrant further investigation.

As there are no other documented cases of using ACV to detect *P. aeruginosa*, this technique was chosen as a novel electrochemical method to investigate pyocyanin production in 31 clinical isolates of *P. aeruginosa*. 12 of these isolates were multidrug-resistant strains (defined as being non-susceptible to at least 1 antibiotic in at least 3 classes of antibiotics). Notably, all 31 clinical *P. aeruginosa* isolates produced measurable levels of pyocyanin using this electrochemical platform after 24 hours of
incubated growth at 37 °C. Calibration curves for pyocyanin detection using ACV at 100 Hz are shown in Figure 5-44.

Figure 5-44: A) Baseline-subtracted AC voltammograms at 100 Hz and B) linear fit of 0-100 µM pyocyanin in trypticase soy broth.

Similar to scans with SWV, ACV demonstrates excellent sensitivity for pyocyanin detection in the biologically relevant concentration range of 0-100 µM. Pyocyanin production rates were compared against non-MDR and MDR strains of *P. aeruginosa*. Scans after 24 hours of growth are reported in Figure 5-45.
After 24 hours of growth, the 12 multidrug-resistant strains of *P. aeruginosa* produced statistically less pyocyanin than the non-multidrug-resistant strains when cultured under the same conditions. This result means that it may be possible to distinguish between MDR and non-MDR strains by simply measuring the pyocyanin production with some optimization of the measurement protocol. This would be a substantial achievement in the field of diagnostics as there are limited methods for rapidly determining drug resistance at point-of-care [53].
5.4.3 Clinical Validation with Human Wound Exudate

The following work detecting *Pseudomonas* in human wound exudate was published in the journal Wound Repair and Regeneration (2016) (DOI: 10.1111/wrr.12414) [25].

Paired wound effluent and biofilm samples were analyzed from 14 unique samples obtained from 12 patients (2 patients with serial samples collected at different time points were available). The mean age of patients was 50.18 years. Of the 14 samples subjected to microbiome profiling by 16S rRNA sequencing, 7 had detectable *Pseudomonas* spp. (sequencing positive). All 14 wounds were recalcitrant at the time of specimen collection.

There were no significant differences in age, sex, race, or comorbidities in the patients whose samples were positive for *P. aeruginosa* using 16S rRNA sequencing compared to those that were negative (Tables 5-2 and 5-3). Wounds that were positive for *P. aeruginosa* using 16S rRNA sequencing tended to be larger but this did not reach statistical significance.
Table 5-3: Experimental determinations (Positive/Negative) for whether clinical samples contained *P. aeruginosa* based on peak currents obtained from electrochemical square-wave voltammograms.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak current (µA)</th>
<th>Determination using 0.030 µA average threshold cutoff</th>
<th>16S rRNA sequencing results</th>
<th>16S rRNA sequencing results (% relative abundance)</th>
</tr>
</thead>
<tbody>
<tr>
<td>01A</td>
<td>0.0000</td>
<td>Negative</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>01B</td>
<td>0.0000</td>
<td>Negative</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>02A</td>
<td>0.1792</td>
<td>Positive</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>02B</td>
<td>0.0000</td>
<td>Positive</td>
<td>Positive</td>
<td>0.0479</td>
</tr>
<tr>
<td>03A</td>
<td>0.0000</td>
<td>Positive</td>
<td>Positive</td>
<td>0.0558</td>
</tr>
<tr>
<td>03C</td>
<td>0.0094</td>
<td>Positive</td>
<td>Positive</td>
<td>0.0005</td>
</tr>
<tr>
<td>04A</td>
<td>0.1241</td>
<td>Positive</td>
<td>Positive</td>
<td>0.0027</td>
</tr>
<tr>
<td>04B</td>
<td>0.0517</td>
<td>Positive</td>
<td>Positive</td>
<td>0.0005</td>
</tr>
<tr>
<td>05A</td>
<td>0.2263</td>
<td>Positive</td>
<td>Positive</td>
<td>0.9779</td>
</tr>
<tr>
<td>06A</td>
<td>1.3491</td>
<td>Positive</td>
<td>Positive</td>
<td>0</td>
</tr>
<tr>
<td>06B</td>
<td>0.9303</td>
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<tr>
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</tr>
<tr>
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<td>0</td>
</tr>
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</tr>
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<td>Negative</td>
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</tr>
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</tr>
<tr>
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<td>Negative</td>
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<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>13B</td>
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<td>Negative</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>14A</td>
<td>0.6262</td>
<td>Positive</td>
<td>Positive</td>
<td>0.2478</td>
</tr>
</tbody>
</table>

A positive test on the pyocyanin detector was considered to be an oxidation peak around -0.25 V vs. a Ag/AgCl reference electrode with a cutoff of 0.030 µA [18, 22]. Data was analyzed as the mean of duplicates. Of the 14 samples, 8 tested positive using the pyocyanin detector cutoff of 0.030 µA.

Sensitivity and specificity of the pyocyanin probe for detecting the samples that contained *Pseudomonas* spp. based on microbiome 16S sequencing are reported in Table 5-4. The probe tested positive in 5 out of 7 samples that were positive for *Pseudomonas* on 16S rRNA sequencing and was negative on 4 out of 7 samples with negative 16S
rRNA results, giving a sensitivity of 71% (95% CI: 0.29-0.96) and specificity of 57% (95% CI: 0.18-0.90).

Table 5-4: Sensitivity and specificity of pyocyanin probe compared to 16S rRNA sequencing for *Pseudomonas* spp. Data analyzed using Fisher’s exact test.

<table>
<thead>
<tr>
<th></th>
<th>16S rRNA positive</th>
<th>16S rRNA negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyocyanin sensor</td>
<td>Pyocyanin sensor</td>
<td>Pyocyanin sensor</td>
</tr>
<tr>
<td>positive</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>negative</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sensitivity (95% CI)</th>
<th>Specificity (95% CI)</th>
<th>Positive predictive value (95% CI)</th>
<th>Negative predictive value (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.71 (0.29–0.96)</td>
<td>0.57 (0.18–0.90)</td>
<td>0.63 (0.24–0.91)</td>
<td>0.67 (0.22–0.96)</td>
</tr>
</tbody>
</table>

The pyocyanin probe was simple to use and had high inter-observer agreement regarding interpretation of a positive result. When compared with the diagnostic gold-standard of 16S rRNA sequencing, the pyocyanin probe had a sensitivity of 71% and specificity of 57% indicating that it may be useful as a point-of-care test in screening for presence of *Pseudomonas* in human wound fluid.

One of the concerns raised about the utility of electrochemical probes for testing human samples is that there may be other molecules which may interfere with probe performance. Human wound samples often contain polymicrobial flora and this was the case for the specimens reported here. The results reported showed no other redox peaks in the reference window for the pyocyanin probe. This indicates that despite the polymicrobial nature of human wound specimens, there do not appear to be other redox-active molecules that would impede the probe’s performance in a clinical setting. Previous work has shown that other bacteria such as *Escherichia coli* and *Staphylococcus aureus* also do not produce redox peaks in the reference window for pyocyanin [21].
Small potential shifts in the location of the pyocyanin peak were observed in some samples. This may be attributed to differences in the salt and pH concentration of the sample media and the limited stability of the Ag/AgCl quasi-reference electrode of the disposable sensor [21, 22].

It was found that most of the samples containing *P. aeruginosa* tested pyocyanin positive using this electrochemical approach. By lowering the threshold for current peak identification, this would have improved the sensor’s sensitivity to 85.7% while decreasing specificity to 42.9%. Although still unclear if it is necessary, the detection limit of electrochemical sensors can be improved by switching to micro- and nanofabricated electrodes, albeit with increased sensor cost [291]. Nevertheless, future technological advances will lead to reduced costs and more sensitive electrochemical sensors, making this approach a practical option.

Testing revealed some false negative results using the pyocyanin probe. While pyocyanin is a very specific molecule, produced only by *Pseudomonas* spp., the genes for producing the molecule are unique to *P. aeruginosa* [120]. Furthermore, there exist environmental conditions in which pyocyanin production by *P. aeruginosa* is low. It is possible that the wound microenvironment may have impacted pyocyanin production in some of the clinical cases studied here, and thus warrants further investigation using specimens from a larger cohort of patients with chronic wounds.

This study has several other limitations which merit discussion. The sample size is small because this was a pilot study designed to be hypothesis generating. Wound exudate is relatively challenging to collect at bedside, and so as this probe is developed as a point-of-care testing device, further refinement will be necessary to enhance probe
performance at low fluid volumes and improve clinical utility. Finally, while the 16S rRNA testing is the gold standard test for determination of bacterial presence in the wound and relative abundance of specific bacteria relative to the entire microbiome profile of the specimen, 16S rRNA testing alone does not give information regarding the quantitative abundance of a particular bacterium in one specimen relative to other specimens. It is possible that some of the false positives and false negatives in this study are attributable to very low *Pseudomonas* abundance in the sample and further testing is needed to understand the clinical relevance of false positive and false negative tests as this device is further refined and developed for clinical use. Future studies intend to assess quantitative PCR with a universal 16S probe to extrapolate the count number to an estimation of bacterial abundance.

5.4.4 Clinical Validation with Animals

A significant limitation to antibiotic stewardship is the delay between obtaining a microbiologic clinical sample and the availability of culture and susceptibility results [53]. In the presence of a life-threatening infection, a clinician must choose an antibiotic to which they have reasonable confidence the causative agent will be susceptible. During this time period, anywhere from 2 to as many as 5 days, it is increasingly common that veterinary patients are exposed to drugs used to treat life-threatening infections in humans such as imipenem and meropenem [297]. Since intimate contact, such as face licking and handling of urine and feces, between animals and their owners is commonplace, it is a reasonable assumption that flora resistant to these drugs could be exchanged from animal to human leading to drug-resistant infections in the pet-owning,
human population. Rapid identification, or exclusion, of highly-resistant pathogens would allow clinicians to select the most appropriate antibiotic for treatment during this waiting period. A review of currently available veterinary antibiograms [298] illustrates that the potential presence of *Pseudomonas* is in certain types of infections. Most notably, pneumonia and aural infections frequently force veterinarians to choose an antibiotic normally reserved for severe life-threatening infections, as it is known that all penicillins and cephalosporins (with the exception of those developed specifically for *Pseudomonas* such as ceftazidime), as well as chloramphenicol, azithromycin, clindamycin, potentiated sulfa drugs, and doxycycline, are ineffective at killing this organism. It takes over 24-48 hours to receive a positive identification using plate cultures, the gold standard in animal clinical care [299]. *P. aeruginosa* is a common bacterium that can cause skin infections and colonize in the ears of dogs, cats, and exotic animals [106, 300]. If a rapid test was available, one that could reliably detect the presence of *Pseudomonas* in a culture sample, it would allow the clinician to choose with confidence, drugs that are less likely to result in multidrug-resistance in veterinary or human patients.

To address this need, veterinary samples were electrochemically scanned for the presence of *P. aeruginosa*. A total of 48 animal samples were tested on a variety of species including dogs, cats, birds, and rabbits. Swab collection areas varied from skin lesions, ear canals, nasal, ulcers, and surgical sites. Two of the electrochemical scans are shown in Figures 5-46 and 5-47.
Figure 5-46: Square-wave voltammograms of thioglycollate broth (negative control) and nasal fluid obtained from a rabbit that had a *P. aeruginosa* infection.

Figure 5-47: Square-wave voltammograms of thioglycollate broth (negative control) and pericardial fluid obtained from a canine that had a *P. aeruginosa* infection.
Figures 5-46 and 5-47 highlight the successful detection of pyocyanin in these animal samples. Both of these animals tested positive for the presence of *P. aeruginosa* using secondary lab culture results, and more importantly, the bacteria produced measureable levels of pyocyanin at the time of the clinic visit. This means it would be possible to rapidly detect this infection as opposed to waiting for results from an automated identification system, which can take a few days to process.

Interestingly, some animal samples showed a redox-active peak in the initial scan that disappeared after subsequent scanning (Figure 5-48). This may be due to an unstable, redox-active molecule that is not pyocyanin. The current observed from pyocyanin oxidation is consistent between multiple scans with the same sample.

![Graph showing sequential square-wave voltammograms of nasal fluid from a bird that did not have a *P. aeruginosa* infection. An unstable redox-active molecule present in the first scan is removed after multiple electrochemical scans.](image)

**Figure 5-48:** Sequential square-wave voltammograms of nasal fluid from a bird that did not have a *P. aeruginosa* infection. An unstable redox-active molecule present in the first scan is removed after multiple electrochemical scans.
From a total of 48 animal samples, the sensor correctly identified 3 true positives and 45 true negatives and validates use of an electrochemical sensor for point-of-care applications in the clinical veterinary market. Further clinical data collection is ongoing, with a focus on testing animal samples presumed to be positive with *P. aeruginosa* infections.


6.0 Conclusions and Recommendations

This dissertation focuses on the development and optimization of electrochemical sensors to detect bacterial pathogens for point-of-care applications. This was achieved through the completion of four aims: up-regulating pyocyanin production by *Pseudomonas aeruginosa* for rapid bacterial identification, screening additional bacterial pathogens for redox-active biomarkers for electrochemical detection, developing alternative electrochemical sensing strategies for bacterial detection, and applying electrochemical sensors towards use in the hospital setting.

Aim 1 was completed after pyocyanin production by *P. aeruginosa* was successfully up-regulated using selective amino acids at varying concentrations. Of the six amino acids tested, the addition of tyrosine and valine had the largest up-regulatory effect on the production of pyocyanin. These up-regulation variables will prove useful when selecting a medium type and composition to provide the fastest electrochemical detection of a *P. aeruginosa* infection. Future work and recommendations for Aim 1 include further investigation of iron and sugar, explored briefly, as potential up-regulatory molecules for pyocyanin production.

Aim 2 was completed after the electrochemical setup was miniaturized for off-site hospital testing and screening of clinically-relevant bacteria. While no other bacteria were identified as having a redox-active biomarker, validation of the *P. aeruginosa* sensor was accomplished through extensive testing of clinical isolates which verified that all clinically-relevant strains of *P. aeruginosa* produce pyocyanin. Furthermore, complex human biofluid samples were screened electrochemically to validate this sensing strategy.
for real-world applications. Future work and recommendations include continued testing of additional clinical *P. aeruginosa* strains for further clinical validation.

Aim 3 was completed after an aptamer was successfully incorporated into the current electrochemical platform, providing an alternative detection strategy. This aptamer was previously identified as being highly selective and sensitive to an acyl homoserine lactone secreted by *P. aeruginosa*, but has not been previously used in an electrochemical setup for sensor applications. The successful demonstration of aptamer use in detecting molecules offers credibility and proof-of-concept for using these highly selective recognition elements for biosensor applications. Future work and suggestions include testing this aptamer with *P. aeruginosa* batch cultures and developing an aptamer through the SELEX process for a unique molecule for a different bacterial pathogen.

Aim 4 was completed after the sensor was functionalized and validated for medical applications. The miniaturized electrochemical device was fabricated and coupled with palladium, a pH-sensitive reference electrode. The ability to simultaneously measure pH and the concentration of pyocyanin of a fluid sample highlights the device’s future medical application as part of a smart bandage where pH is linked to general bacterial infection and the presence of pyocyanin indicates the presence of *P. aeruginosa*. As with any new diagnostic device, extensive clinical validation is required before it can be implemented into the market. The electrochemical platform was tested using human wound exudate samples to determine if this approach could be used as a rapid diagnostic. These experiments proved successful, in addition to preliminary data obtained from testing animal samples. Future work and recommendations include continued data collection for human and animal samples for even greater clinical validation.
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