DEVELOPMENT AND APPLICATION OF A NOVEL ORGANIC CRYSTAL FOR REAGENT-FREE, IN-SITU ELECTROCHEMICAL SENSING OF BACTERIAL VIABILITY

A Thesis in
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by
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ABSTRACT

Monitoring bacterial viability is important in clinical, commercial, and academic settings. One of the important areas for measuring bacterial viability is antibiotic susceptibility testing (AST), which is critical in determining bacterial resistance or susceptibility to a particular antibiotic. Simple-to-use, phenotype-based AST platforms can assist care-givers in timely prescription of the right antibiotic. Monitoring the change of bacterial viability by measuring electrochemical Faradaic current is a promising approach for rapid AST. However, the existing works require mixing redox-active reagents in the solution which can interfere with an antibiotic’s effect. In this thesis, we developed a facile electrodeposition process for creating redox-active crystalline layers (denoted as RZx) on various graphitic carbon electrodes, including pyrolytic graphite sheet (PGS), laser induced graphene (LIG) on polyimide sheets, and screen-printed carbon electrodes on paper, and utilized them as the sensing layer for reagent-free bacterial monitoring. Effects of the conductive substrate and composition of the deposition solution on elemental composition, crystallinity, and morphological properties of RZx were studied. Our studies show that phenylalanine plays a critical role in formation of stable RZx crystals. In addition, compared to common metals, graphene-based substrates lead to a much higher crystal surface coverage. The sensors enable detection of bacterial metabolic activity/respiration mainly due to the pH-sensitivity of RZx (~ 53 mV/pH) and also oxidation of excreted redox-active metabolites from cells. By monitoring the differential voltammetric signals, the sensors enable accurate prediction of the minimum inhibitory concentration (MIC) of two model antibiotics (ampicillin or kanamycin) for Escherichia coli (E. coli) K-12 in 60 minutes ($p < 0.03$). The sensors are stable after 60 days storage in ambient conditions and enable analysis of microbial viability in complex solutions, as demonstrated in spiked milk and human whole blood. We have also demonstrated a proof-of-principle integrated sensor and microfluidic system using RZx/LIG electrodes to monitor bacterial viability in real time.
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# Chapter 1

## Introduction to Electrochemical Biosensors for Microbial Monitoring

### Current and Future Uses of Biosensors

The term “biosensor” describes a device that probes biological samples/processes and thereby produces a measurable signal proportional to analyte concentration (Bhalla et al., 2016; Mehrotra, 2016; Vigneshvar et al., 2016). Biosensing is a relatively new field of science, but it has had far-reaching and important implications for life-sciences and medicine over the past century (Bhalla et al., 2016; Grand View Research, 2019; Mehrotra, 2016; Vigneshvar et al., 2016). Two important aspects of any biosensor are specificity and sensitivity. Specificity is generally achieved through functionalization with a biorecognition element (Bhalla et al., 2016); sensitivity can be enhanced by engineering high surface-area-to-volume ratio (SAVR) materials and optimizing device architecture (Holzinger et al., 2017).

The applications for biosensors are numerous and span a range of fields, including medical diagnostics, food/water quality monitoring, wearable health sensors, fundamental research, pharmaceuticals, prosthetic devices, environmental monitoring, and others (Bhalla et al., 2016). By far, the most successful commercial biosensors are glucose monitors (Bhalla et al., 2016; Grand View Research, 2019) which are electrochemical sensors, but other biosensing technologies are also advancing rapidly (Grand View Research, 2019).

### Classes of Biosensors by Mechanism of Transduction

Biosensors must be able to transduce analyte presence/concentration into a measurable signal. The transduction pathway can be multifaceted, such as a transduction from concentration to optical signal and from optical signal to electronic signal (Accelerate Diagnostics, 2020). Once collected, the end signal may require further post-processing for final utilization. Biosensors can generally be classified according to their transduction mechanism: these include (but are not
limited to) piezoelectric, optical, electronic, magnetic, and electrochemical biosensors. Among these various classes, electrochemical biosensors are attractive for portable and point-of-care (PoC) applications due to their capacity for: miniaturization, ease of utilization, sensitivity, fast response, low relative cost of equipment, small sample volume requirements, and versatility (Ahmed et al., 2008; Grieshaber et al., 2008a; Menon et al., 2020; Yoon et al., 2020).

The general architecture of any biosensor, including electrochemical, can be split into four components (Luka et al., 2015). The first is the sample; the sample is the medium that contains the bioanalyte. Samples can come in a range of forms, such as bodily fluids, environmental specimens, consumables, etc. The samples may or may not need to undergo some processing prior to interfacing with the biosensors. The second component is the biorecognition element. It must be able to recognize the target bioanalyte (through binding, catalysis, electrostatics, etc.) and provide selectivity by excluding interferants. The biorecognition element may be naturally derived (such as antibodies and enzymes) or engineered (such as artificial surfaces and aptamers). The third component is the transducer. It takes the changes (electrical, chemical, optical,
magnetic, etc.) induced by the interaction of the bioanalyte and biorecognition element and transforms them into a measurable signal (may include multiple steps). Examples of transducers include the electrode of an electrochemical biosensor, the fluorophore and photodetector of a fluorescence biosensor, and the piezoelectric material of a mass-based biosensor. The final component is the equipment used for signal amplification and processing. Figure 1.1, adapted from (Luka et al., 2015), shows a schematic of biosensor construction based on each of the four components.

**A Brief Introduction to Electrochemical Biosensing for Bacterial Monitoring**

Electrochemical biosensors measure the change of Faradaic current profile stemming from changes in the concentration of target analyte (Grieshaber et al., 2008b). This may occur by direct/indirect reaction of the analyte with a redox-active reagent, altered resistance to charge transfer, or the analyte may be redox-reactive on its own. There are various architectures for electrochemical sensors, although they often utilize a three-electrode setup. There is a reference electrode (RE) of known potential, such as commercial Ag/AgCl REs (Bard and Faulkner, 2001). An ideal RE is non-polarizable, meaning that the potential does not change with the passage of small currents. As an alternative to REs, pseudo-RE’s can be used. For pseudo-REs, which have the benefit of miniaturization, the reduction potential is tied to the exact electrolyte being utilized. Besides REs, there is a working electrode (WE) that is biased with respect to the RE by using a potentiostat (Bard and Faulkner, 2001). The WE electrode is engineered to have the specific electrochemical properties that fit the application. Finally, there is a counter electrode (CE) that is used to complete the electrochemical circuit (as opposed to passing it through the RE). In some cases though, when only small currents are being passed, the CE and RE are combined. Figure 1.2 shows a schematic of a typical 3-electrode setup.

Electrochemical biosensors used for studying and monitoring bacteria have various designs and target analytes. They may be used to probe metabolites, nucleic acids, proteins, and whole cells (Bolotsky et al., 2019). In general, they work by directly oxidizing the target molecule (this requires careful surface engineering of the WE) (Ebrahimi et al., 2019) or indirectly through the use of a redox reagent added to the solution (Besant et al., 2015; Chotinantakul et al., 2014; Ertl et al., 2000; Liu et al., 2014; Mishra et al., 2019; Rao R et al., 2020). For whole cell
electrochemical sensing, the usual approach is the indirect method by adding a redox reagent. One example comes from Besant et al.; they utilize a microwell capture design to perform electrochemical AST with resazurin (Besant et al., 2015). In their works, they create a series of electrodes inside of microwells on a chip, as shown in Figure 1.3a. The microwells are open on two sides, with one side having a filter on it, as shown in Figure 1.3b. The testing procedure (shown in Figure 1.3c) involves flowing the urine sample through the device, which traps bacteria in the wells since they cannot pass through the filter. Then, culture medium with resazurin and antibiotic is flowed through to replace the existing solution. Next, air is run through the device to separate the wells, which are then capped with FC-40 oil. Finally, the device is incubated at 37°C and electrochemical measurements taken. If bacteria are metabolizing, they would reduce resazurin to resorufin. This would mean a lower peak height in Differential Pulse Voltammetry (DPV) measurements. After 60 minutes of incubation with a starting concentration of 100 CFU/μL (1×10^5 CFU/mL), they were able to determine the susceptibility of either *E. coli* or *K. pneumoniae* to ampicillin or ciprofloxacin. The data is shown in Figure 1.3d, where a smaller normalized signal decrease indicates inhibition of growth.

![Figure 1.2. A typical 3-electrode setup for electrochemistry. A working electrode (WE) is immersed in sample liquid. The WE is biased with respect to a reference electrode (RE) whose reduction potential ideally remains constant. A counter electrode (CE) is used to complete a circuit with the working electrode. The current through the circuit is measured and recorded.](image)

Unfortunately, adding the redox probe to the medium can be problematic in that the redox reagent can interact with the cells to disrupt normal physiological processes. By disrupting normal physiology, the results of the sensing experiment may be misleading or incorrect. Additionally, the probe may interact with added drug molecules and alter their efficacy. In many applications,
such as in vivo testing or food monitoring, adding the redox mediator is not feasible. Thus, it is favorable to design reagent-free designs for monitoring cells electrochemically.

Figure 1.3. Example electrochemical device for monitoring whole cells. a) Device schematic. b) Zoomed in schematic of a single well device. Open on one side and a filter on the other. c) Testing procedure. Urine flowed through to trap bacteria. Culture medium with resazurin redox mediator and antibiotic replaces urine. The wells are capped with oil. d) Results from 60-minute incubation at 37°C. A smaller normalized signal decrease indicates hindered growth. The data can be used to determine the susceptibility of the bacteria. Reproduced from (Besant et al., 2015) with permission from The Royal Society of Chemistry.

One of the common methods for probing electrochemical processes is voltammetry. To produce a voltammogram, a time-dependent potential $E(t)$ is applied to the WE. In parallel, the current $I$ is measured. The voltammogram is the plot of $I$ vs. $E$. In this work, cyclic voltammetry (CV) is used for sensing. CV is one of the simpler voltametric techniques, where the $E(t)$ is cycled linearly from one extreme value to another (Elgrishi et al., 2017). If an electrochemical reaction occurs within the potential window, then peaks will show up when a sufficiently high scan speed is utilized (V/s). To explain why this occurs, consider the case of a completely reversible electrochemical reaction involving ferrocenium ($\text{Fc}^+$) $\rightleftharpoons$ ferrocene (Fc). A schematic of the process, reproduced from (Elgrishi et al., 2017), is shown in Figure 1.4. Concentrations are in mM and the scan rate is 100 mV/s. First, see Figure 1.4i which shows the time vs. potential graph. The CV curve associated with this sweep is shown in Figure 1.4h. To understand the voltammogram, it is useful to inspect the mechanisms occurring at the electrode-electrolyte
interface. Figure 1.4a-g shows the instantaneous concentration vs. distance plots of Fc⁺ and Fc at the specified points on the CV curve. At point A, there is only Fc⁺ in solution; the bulk concentrations of species are the same as the surface concentrations. At point B, the potential is at $E_{1/2}$ and the ratio of Fc⁺/Fc at the surface is unity. The reduction current is determined by the flux of Fc⁺ to the surface. At point C, the reduction current is maximized because the flux of Fc⁺ to the surface (determined by the slope of the concentration profile by Fick’s 1st Law) is maximized. At point D, the current relative to point C has decreased because of the buildup of Fc at the surface, which hinders diffusion of Fc⁺. A similar situation occurs during the oxidation portion of the scan (E-G), although the case is slightly complicated by the non-monotonic concentration profiles.

![Figure 1.4. Schematic of the evolution of a cyclic voltammogram. A-G) Concentration (mM) vs. distance (mm) profiles for Fc⁺ (blue) and Fc (green) at different point on the CV curve. H) CV curve generated by the potential sweep, with points corresponding to (A-G). I) Applied potential sweep that generates the CV curve. Reproduced from (Elgrishi et al., 2017) with permission from the American Chemical Society. Further permissions related to the material excerpted should be directed to the ACS.](https://pubs.acs.org/doi/10.1021/acs.jchemed.7b00361)

The peaks can be described by their height $i_{p,a}$ and $i_{p,c}$ and their potentials $E_{p,a}$ and $E_{p,c}$. The peak height of an ideal, electrochemically reversible electron transfer process involving freely diffusing species can be described by the Randles-Sevcik equation:

$$i_p = 0.446nFAC^* \left( \frac{nFvD}{RT} \right)^{0.5}.$$  

The $F \equiv$ Faraday’s constant, $n =$ number of electrons transferred, $A =$ area, $C^* =$ bulk concentration of analyte, $v =$ scan rate, $D =$ diffusion coefficient, $R =$ gas
constant, and \( T = \) temperature. All else equal, the \( E_p \) potentials will shift as \( E_{1/2} \) shifts. In general, \( E_{1/2} \approx E_0 + 2.303 \frac{RT}{nF} \log(Q_r) \) where \( Q_r \) is the reaction quotient. For a pH dependent reaction, \( \Delta E_{1/2} = -2.303 \frac{RT}{nF} \Delta pH \) at room temperature:

\[
\Delta E_{1/2} = -59.2 \text{ mV} \times \Delta pH
\]

Outline of This Thesis

RZx, which is the focus of this thesis, is a novel redox reactive crystalline material that can be applied to electrodes to provide functionality. The functionalized electrodes show redox activity in aqueous electrolytes without the need to add an external redox reagent. In voltametric measurements, the RZx-coated electrodes have a well-defined oxidation peak. The position of the peak can be used to investigate changes in the chemical makeup of the electrolyte. Here, we use RZx electrodes to monitor the viability/growth of bacterial cultures, with special attention paid to antibiotic susceptibility testing.

In chapter 2, we explore the synthesis, characterization, and optimization of RZx. We first discuss the procedures we utilized. Then, we discuss the synthesis of RZx and how it relates to its precursor materials, resazurin and Brain Heart Infusion (BHI). Subsequently, we explore how the electrodeposition substrate and the electrolyte composition affect RZx morphology. We conclude that carbon electrodes are a preferable conductive substrate for RZx electrodeposition and that RZx is a salt containing resazurin and L-phenylalanine molecules. We also show that RZx can be embedded into a polymer matrix for decreased surface roughness and an alternative synthesis strategy. Next, we discuss the microscopic, spectroscopic, diffraction, and behavioral characteristics of RZx. We verify that RZx is organic, crystalline, and chemically distinct from resazurin sodium salt. We then explore the sensing characteristics of RZx and show that it is sensitive to pH changes, with a near-Nernstian response of \(-53 \text{ mV/pH}\). Bioassays indicate that RZx does not induce any toxic effects in the surrounding medium.

In chapter 3, we utilize RZx-based sensors for rapid antibacterial susceptibility testing (RAST). Specifically, we developed a reagent-free electrochemical RAST method (ORACLE-AST) based on Nafion/RZx/PGS sensors. We demonstrated the performance of ORACLE-AST using \textit{Escherichia coli} (\textit{E. coli}) K-12 exposed to different concentrations of ampicillin (\( \rho_{\text{AMP}} \))
and kanamycin ($\rho_{KAN}$). The method successfully determined ampicillin and kanamycin minimum inhibitory concentrations (MICs) in 60 minutes without requiring any redox reagent. The devices can be used to probe complex and optically opaque media, such as milk and blood. Additionally, we demonstrate possible multi-use application and show the shelf life in ambient storage is long. Furthermore, RZx can be synthesized on LIG. The Nafion/RZx/LIG sensors similarly show sensitivity towards bacterial metabolic activity. RZx-based electrochemical sensors can thus provide a portable, low-cost, simple-to-use, and rapid phenotypic RAST approach for potential use in both traditional settings and resource-limited areas.

In chapter 4, we detail a proof-of-principle microfluidics device with self-contained pseudo-reference electrodes for bacterial monitoring using RZx/LIG. Such a device is useful as a portable and compact design for PoC bacterial monitoring. We detail the fabrication of the device and explore how device architecture and plasma treatment affect usability. We show that a simple design, when plasma treated for enhanced hydrophilicity, can be used to sensitively monitor bacteria. Specifically, we show that with a starting concentration of $1 \times 10^5$ CFU/mL and an incubation temperature of $37^\circ$C, the sensors can differentiate between live and dead cultures in 210 minutes post-inoculation. This proof-of-concept is promising for further utilization in point-of-use settings, especially in resource limited settings.

In chapter 5, we provide conclusions and outlook for this research.

List of Student’s Publications

Journals (*Equal Contribution)


Conferences


2. Bolotsky, A. and Ebrahimi, A. Toward Rapid Antibacterial Susceptibility Testing Using Electrochemical Biosensors Based on Organic-Inorganic Catalytic Complexes. 236th ECS Meeting, Atlanta, GA, October 2019 (Oral presentation)

Chapter 2

Synthesis, Characterization, and Optimization of RZx

In this chapter, we study RZx as a sensing material and evaluate how select parameters affect the synthesis and/or performance of RZx. The contents herein are reproduced from our paper in Biosensors and Bioelectronics (Bolotsky et al., 2021). We first discuss the materials and methods we employed in this chapter. Then, we explore the connections between RZx and one of its precursor materials, resazurin. Subsequently, we delve into the detailed synthesis studies we performed. The results show that carbon electrodes are a preferable conductive substrate for RZx electrodeposition and that RZx is derived from both resazurin and L-phenylalanine molecules. We also demonstrate that RZx can be embedded into a polymer matrix for decreased surface roughness and increased simplicity of synthesis. Next, we discuss the microscopic, spectroscopic, diffraction, and behavioral characteristics of RZx. We prove that RZx is organic, crystalline, and chemically distinct from resazurin. We also illustrate that RZx is sensitive to pH changes, with a near-Nernstian response. Bioassays indicate that cellular attachment of mammalian cells to the electrodes explored herein is unfavorable and that surface treatments would be necessary to promote it. On the other hand, the bioassays illustrate that RZx does not induce any toxic effects in the surrounding medium.

Material and Methods for RZx Synthesis/Characterization

Materials and Equipment

Brain-Heart Infusion powder (#53286-500G), resazurin sodium salt (#R7017-5G), calcium chloride (#C4901-100G), magnesium chloride (#M8266-100G), PEDOT:PSS solution (#483095-250G), and Chitosan (#419519) were purchased from Sigma Aldrich. Potassium chloride (#PX1405-1) and sodium chloride (#SX0420-3) were purchased from EMD Millipore. Lithium chloride was gifted from Mauricio Terrones’ Group at Penn State. Tryptone powder was purchased from Fischer Scientific (#BP1421100). Tris-Glycine 10X was obtained from Dot Scientific (#DS101110). Dulbecco’s Phosphate Buffered Saline (DPBS) 1X was purchased from
Corning (#21-030-CV). Single-donor whole-blood samples were bought from Innovative Research Inc (#IWB1K2E10ML). Hydrochloric Acid 1.0 N was acquired from VWR Chemicals BDH (#BDH7202-2). Nafion™ D521 (manufactured by The Chemours Company) was provided as a gift by Dr. Hickner’s Group at Penn State University. Pyrolytic graphite sheets (PGS) were purchased from Mouser Electronics (#EQ-PG-017) and MTI Corporation (#667-EYG-S121803). Thin-film gold electrodes were deposited using electron-beam lithography on glass. Nickel (Ni) electrodes were fabricated as detailed elsewhere (Butler et al., 2019). The platinum wire counter electrode (CE, # MW-4130) and Ag/AgCl reference electrode (RE, # MF-2052) used in electrochemical experiments were purchased from BASi Inc. Polyimide sheets for the creation of laser-induced graphene (LIG) were procured from DuPont de Nemours, Inc. (Kapton® GS, 500 mil). All optical images were captured using Nikon Eclipse LV150N. Optical density data at 600 nm (OD$_{600}$) was measured using the Eppendorf Biophotometer D30.

**Electrodeposition of RZx**

Resazurin 1 mM was prepared in Brain-Heart Infusion (BHI) broth and vortexed thoroughly. For the studies investigating the effect of electrolyte composition on RZx formation, BHI was replaced with the specified medium, i.e. S#1 (100 mM KCl + 1 mM resazurin), S#2 (100 mM KCl + 2 g/L glucose + 1 mM resazurin), or S#3 (100 mM KCl + 10 g/L tryptone + 1 mM resazurin). Electrochemical deposition of RZx was performed in a standard three-electrode setup. The working electrode (WE), Ag/AgCl RE, and platinum wire CE were connected to the PalmSens4 potentiostat (BASi Inc.). Subsequently, 100 μL of resazurin solution was pipetted onto the WE. Then 50 sequences of differential pulsed voltammetry (DPV) were performed at room temperature from -0.2 V to -0.8 V at a scan rate of 25 mV/s, pulse amplitude of 25 mV, and pulse duration of 70 ms. The solution was allowed to settle for 10 minutes before being removed and replaced with a fresh solution, followed by another 50 DPV sequences. Finally, the device was removed, washed in a stream of deionized (DI) water, and dried with clean/dry compressed air. For reference, an image of the electrodeposition setup (using transparent liquid in place of resazurin-dyed BHI) is presented in Figure 2.1.
Fabrication of Nafion/RZx/PGS Electrodes

PGS was first cleaned in isopropanol 99.5% (5 min), ethanol 200 Proof (5 min), and DI water (5 min). PGS was gently blow-dried with clean/dry compressed air and mounted onto a glass slide using a copper tape contact. Separately, a strip of Kapton® tape was placed on a cutting board and a 4 mm diameter circular window was created with a biopsy punch. The Kapton® tape was placed over PGS, excluding the electrode window. Electrochemical deposition of RZx was then performed on the assembled PGS electrode following the process previously described. After cleaning and drying the device, 25 \( \mu L \) of Nafion 2% in ethanol was spin coated on the electrode at a spin rate of 4000 rpm for 30 seconds. A drying time of 30 minutes was sufficient before testing the sensors. Prior to running any electrochemical test, the devices underwent a critical stabilization step in BHI to ensure stable performance. The treatment consisted of cyclic voltammetry between -0.5 and 0.35 V until the oxidation peak voltage was stable (at least 10 cycles).

Figure 2.1. Electrodeposition setup. The WE, RE, and CE are shown. A transparent drop of liquid is used in place of resazurin-dyed BHI for enhanced visibility. The site of deposition on the WE is indicated by the arrow.
Fabrication of Laser-Induced Graphene Electrodes

Laser-Induced Graphene (LIG) is a 3D form of porous graphene that can be created through the laser ablation of a carbonaceous material (Chyan et al., 2018; Kaidarova and Kosel, 2020; Lin et al., 2014; Wan et al., 2020). We have utilized a well-developed process to generate the LIG electrodes (Lin et al., 2014). Specifically, a CO$_2$ laser was utilized to locally ablate the surface of a polyimide sheet in a high throughput and patternable fashion. First, a polyimide sheet (Kapton®, 500 GS) of desired specifications was cut from the stock. Then, the polyimide was washed with IPA and affixed to a glass slide using double-sided tape. The sheet was placed in a CO$_2$ laser writing system, the VSL2.30 Desktop Laser System (Universal Laser Systems Co., 25 W). A photo of the setup is presented in Figure 2.2. Next, the desired electrode design was imported into the Universal Control Panel from a CAD file. Subsequently, the system parameters were set to 26% power, 29% speed, and 1000 PPI (pulse per inch). Finally, the electrode design was patterned onto the polyimide by running the system.

Figure 2.2. Image of the VSL 2.30 Desktop Laser System (Universal Laser Systems Co., 25 W). The bottom unit is an air filtration system.
Creation of Nafion/RZx/LIG Electrodes

An LIG electrode was first contacted with copper tape. The local contact area was covered in epoxy (Loctite, #30671) to inhibit liquid interaction with the interface. After the epoxy was cured, the copper lead was further covered with polyimide tape (Kapton®), except at the very end. Electrodeposition electrolyte was prepared by adding 1 mM resazurin powder to BHI broth. The solution (~ 40 mL) was utilized in a C3 Cell Stand (BASi Inc., EF-1085). Next, the LIG electrode was connected to the PalmSens4 potentiostat (BASi Inc.) as the WE and immersed in the electrolyte. A Pt wire CE and Ag/AgCl RE were also introduced. A total of 300 sequences of differential pulsed voltammetry (DPV) were performed at room temperature from -0.2 V to -0.8 V at a scan rate of 25 mV/s, pulse amplitude of 25 mV, and pulse duration of 70 ms. Afterwards, the device was removed, immersed in Ultrapure MilliQ deionized (DI) water for 5-10 minutes, and gently dried with clean/dry compressed air. Nafion 2% diluted in ethanol was spin coated onto the dry electrode at a spin rate of 4000 rpm for 30 seconds. After at least 2 hours wait time, the finished electrode was stabilized in BHI as described previously.

Electrochemical Measurements

Electrochemical data was measured using PalmSens4 (BASi Inc.) in a standard three-electrode system (RZx-based WE, Ag/AgCl RE, and platinum CE). Unless otherwise indicated, 200 μL sample solution was deposited on the sensor and characterized using cyclic voltammetry (CV) with scan rate of 100 mV/s from -0.5 to 0.35 V. The voltage associated with the peak oxidation current ($E_{ox}$) was extracted from the CV curves. Differential $E_{ox}$ (as compared to some baseline solution) served as the sensor signal, designated as $\Delta E_{ox}$. For a visual interpretation of $\Delta E_{ox}$, see Figure 2.3. Between different testing solutions, the samples were cleaned by pipetting a drop of deionized water, allowing it to sit for at least 10 seconds, then wicking it off with a laboratory wipe.
To investigate the ion selectivity of the device, a buffered solution free of monoatomic ions (besides H\(^+\) and Cl\(^-\)) was created using Tris-Glycine. Firstly, the Tris-Glycine 10X was diluted to 1X in MilliQ Ultrapure DI water. Then, the 1X solution was titrated to the desired pH values (pH 4-8) using HCl. The pH was confirmed with a Thermo Scientific Orion ROSS Ultra Glass Triode pH electrode. 1 mM and 10 mM concentrations of LiCl, NaCl, KCl, CaCl\(_2\), and MgCl\(_2\) were created for each pH value. Finally, each permutation was measured with the Nafion/RZx/PGS sensor. For the temperature sensitivity tests, Nafion/RZx/PGS sensors were placed on a calibrated polyimide thin-film heater (Omega Engineering, #KHLVA-202/2). Electrochemical data for BHI was measured at various temperatures after 10 min equilibration to obtain the temperature response. The data for these experiments can be found in Figure 2.15.
RZx-Embedded Polymers as Working Electrode

In addition to deposition of RZx on an electrode, we also investigated whether RZx crystals can be embedded in polymers (Nafion, Chitosan, PEDOT:PSS). Embedding the crystals in polymer prevents sharp crystals from contacting and possibly puncturing cells. It also increases the uniformity of the surface while decreasing roughness.

First, stock dispersions of the polymers were created. For Nafion, this was 2% Nafion (diluted from 5% with ethanol). For chitosan, the stock was 1% chitosan in 2% acetic acid. For the PEDOT:PSS, the solution was used as made. Then, resazurin powder was added at a concentration of 10 mM. The resazurin/polymer liquid was spin coated onto a clean PGS electrode at 4000 rpm for 30 sec. After at least 30 minutes drying time, the coated PGS WE was hooked up to the PalmSens4 potentionstat with Pt wire CE and Ag/AgCl RE. Then, 200 μL of BHI was dropped onto the WE and 25 sequences of DPV were performed at room temperature from -0.2 V to -0.8 V at a scan rate of 25 mV/s, pulse amplitude of 25 mV, and pulse duration of 70 ms. Fresh BHI was placed on the WE and the CV signal was stabilized as described previously.

Bioassay Experiments on Electrode Materials

The bioassay experiments for assessing biocompatibility and antibacterial properties were generously conducted by Su Yan in Dr. Jian Yang’s group at Penn State using samples provided and prepared by the author. Two types of cells were investigated: the L929 mouse fibroblast cell line (mammalian) and Staphylococcus aureus (S. aureus, Gram-positive bacterial). The assays were performed under two differing conditions: in culture medium (films present) or in leaching medium (no films). For leaching medium, a 1 cm² sheet of material was incubated in 2 mL culture medium for 24 hours at room temperature. Then, the supernatant liquid (leaching medium) was removed and used in place of the base culture medium.

For cell attachment/growth experiments using the L929 mouse line, various film materials (or no film) were punched out such that they fit into the wells of a 96-well culture plate. The films – if present - were then adhered to the bottom of the wells using medical device epoxy (Loctite, #30671). A total of 3 ≤ n ≤ 5 samples was used to collect data for each condition. Initially, L929 cells were seeded onto the bottom surface of the wells at a density of 1 × 10⁴ cells/cm². Then the
samples were incubated in culture medium or leaching medium at 37°C and 5% CO_2 for 24 hours. Subsequently, a Cell Counting Kit 8 (CCK-8) was used to measure the proliferation of attached cells (OD measurement at 450 nm). The signals are measured relative to the blank control (medium only). The measured absorbance is linearly related to cell concentration over a wide range.

For bacterial growth experiments, *S. aureus* was first cultured to an OD_{600} of 0.06 in culture medium. Then the culture was diluted 1:100 in either culture medium (films present) or leaching medium (no films present). For samples with film present, 1 cm² sheets were utilized in 2 mL of culture medium. A total of n = 5 samples was used to collect data for each condition. The samples were all incubated for 24 hours at 37°C. Then, OD measurements at 600 nm were performed relative to the blank control (medium only). The data for these experiments can be found in Figure 2.16.

**RZx Synthesis Mechanism**

Resazurin is an organic dye that is commonly used in fluorescence or colorimetric assays for measuring microbial viability/metabolic activity (Abcam, 2020; Chen et al., 2018; Khazalpour and Nematollahi, 2014; Sandberg et al., 2009). It is composed of a set of planar, tricyclic rings and possesses a deep purplish-blue color in solution. Resazurin is minimally fluorescent, but it is redox-active and can be irreversibly reduced to a pink, highly fluorescent compound called resorufin (Khazalpour and Nematollahi, 2014). Subsequently, resorufin can undergo a further reversible reduction to a colorless and non-fluorescent molecule, dihydroresorufin (Khazalpour and Nematollahi, 2014). The following reactions outline the commonly-observed resazurin reduction pathways:

\[\text{Resazurin} + 2e^- + 2H^+ \rightarrow \text{Resorufin} + H_2O \quad (2)\]

\[\text{Resorufin} + 2e^- + 2H^+ \rightarrow \text{Dihydroresorufin} + H_2O \quad (3)\]

In the present work, we electrochemically reduce resazurin at the working electrode by utilizing differential pulse voltammetry (DPV). The devised process ensures that the product of this reduction process (i.e. RZx) is not water-soluble, unlike resorufin and dihydroresorufin. As such, the synthesis method and the choice of the solution composition both result in an overall
reaction distinct from (2) and (3). Moreover, we believe that RZx belongs to the larger family of charge transfer salts (Batail et al., 1998). These materials are crystalline solids composed of redox-active ions and are often synthesized through electocrystallization (Batail et al., 1998). A well know example is TTF-TCNQ, whose cationic/anionic components are cyclic, like resazurin and certain amino acids (Pauliukaite et al., 2007). Resazurin is known to be electroactive and can have cationic, anionic, or neutral forms depending on the pH (Khazalpour and Nematollahi, 2014). Similarly, amino acids (depending on identity and pH) can similarly be cationic, anionic, or neutral. So, the creation of an ordered redox-active salt at the electrode is thus highly possible (Batail et al., 1998).

Figure 2.4a schematically shows the RZx synthesis process. RZx crystals are electrodeposited in a solution of 1 mM resazurin and amino acids supplied by 10 mg/mL of tryptone - which is a pancreatic digest of casein protein used to enrich culture media (Atlas, 2004; Hankes et al., 1948; Savoie et al., 1988) – by applying a sequential DPV approach. The DPV curves for sequence # 1, 2, 25, and 50 are shown, illustrating a decrease of the resazurin reduction peak until reaching a plateau. This suggests progressive blocking of the electrode surface by RZx.

Figure 2.4a shows exemplary optical and scanning electron microscopy (SEM) images of RZx deposited on a pyrolytic graphite sheet (PGS). Our study of the effect of electrolyte composition on RZx synthesis shows that phenylalanine – which is one of the 21 proteinogenic amino acids – plays a key role in deposition of stable, non-soluble RZx crystals. Moreover, the choice of the conductive substrate (thin film gold, rough nickel, and graphitic layer) directly affects the morphology and density of RZx crystals, with the optimum deposition on PGS compared to metallic layers. After RZx deposition, Nafion is spin-coated onto RZx (Figure 2.4b). Nafion is a biocompatible cation-exchange membrane which we used to mitigate potential damage to bacterial cell walls due to sharp edges of RZx crystals (see SEM images in Figure 2.6). Additionally, Nafion increases the peak height at the sensor’s characteristic oxidation peak, \( E_{ox} \), and hence enhances the device’s sensitivity (see appendix, Figure A.0.1). The final step in
fabrication of RZx-based sensing electrodes is to stabilize it using sequential cyclic voltammetry (CV), Figure 2.4c. The role of sequential stabilization is discussed in the appendix, Figure A.0.2.

Figure 2.4. Synthesis of RZx electrode. a) Electrochemical deposition of RZx using sequential differential pulse voltammetry (DPV). Optical and SEM images of RZx/PGS are shown in the inset. A representative DPV voltammogram is presented on the right. b) Spin-coating of Nafion on the RZx electrode. c) Stabilization of the electrode using cyclic voltammetry in BHI, pH 7.4.

**Studying Parameters Affecting RZx Synthesis**

In this section, we study the effect of conductive substrate and composition of the electrodeposition solution on formation of RZx, including its crystal morphology, surface coverage and adhesion. We identify each of these parameters as being critical in the synthesis of the crystals layer.

**Impact of the Conductive Substrate**

We explored three chemically distinct electrode materials to evaluate their impact on RZx layer formation: gold (Au), nickel (Ni), and PGS (see Figure 2.5 and Figure 2.6). The electrodeposited nickel electrodes yielded a moderate density of large x-shaped RZx crystals that are strongly adsorbed to the surface (e.g. they are resistant to vigorous and repeated washing in deionized water). In contrast, the smooth gold electrodes yielded an initially similar density of much smaller crystal sizes with poor adhesion (Figure 2.5). Even a gentle rinse in DI water was enough to dislodge many of the deposited crystals.
Ni is significantly easier to oxidize than Au, and is likely partially or completely oxidized at the surface (Lambers et al., 1996; Pinnel, 1979), as confirmed by our XPS analysis. Besides the difference in surface chemistry of Ni and Au, there is a crucial difference in terms of their orbital availability: Au atoms have completely filled 5d orbitals, while Ni atoms in Ni and NiO both have a 3d\(^8\) electron configuration. The presence of unfilled d orbitals in Ni increases its reactivity and penchant for orbital hybridization (Mittendorfer and Hafner, 2001; Yamagishi et al., 2001). For Au, there is an additional relativistic phenomenon, whereby its outermost 6s orbital contracts with respect to the 5d orbital, making the single 6s electron less accessible than it would otherwise be and further reducing its reactivity (Bartlett, 1998).

Figure 2.5. Effect of surface roughness on RZx morphology and adhesion. Scale bars are all 100 \(\mu\)m. Images (a)-(c) are taken at the same position. a) Optical image of bare e-beam evaporated Au electrode, which has low RMS surface roughness. b) Optical image of RZx deposition on gold electrode, showing low density of coverage and small crystal sizes. c) Optical image of the RZx/Au electrode after being washed in a stream of DI water. Many crystals have been washed off of the surface of the electrode, confirming poor adhesion. d) Optical image of RZx/Ni electrode. Although possessing a similar density of crystals when compared with (b), the crystal size is greatly enhanced and the adhesion is significantly stronger.

In agreement with the preceding chemical characteristics, computational methods with benzene or CO adsorption on metals suggest that Ni is the more favorable electrode for resazurin adsorption (Jenkins, 2009; Mittendorfer and Hafner, 2001; Yamagishi et al., 2001). This is
consistent with our RZx synthesis results showing higher surface coverage on Ni compared to Au (Figure 2.5d). Figure 2.5a shows a bare Au electrode, while Figure 2.5b shows the Au electrode after electrodeposition, yielding very sparse growth of crystals. Figure 2.5c shows the surface after being rinsed in a stream of DI water; it is evident that crystals have been washed from the surface due to poor adhesion/bonding. Figure 2.5b and c are contrasted with Figure 2.5d which shows more coverage of RZx on the nickel electrode surface.

Additionally, the improved formation of RZx on Ni electrode could also be influenced by differences in the RMS surface roughness. The nickel electrode, which was prepared as detailed elsewhere (Butler et al., 2019), has a high RMS surface roughness of ~58 nm. In contrast, the gold electrode is expected to have low nm-level surface roughness (Mahapatro et al., 2006). The significance of electrode surface roughness on crystal nucleation and growth has been previously shown in formation of magnetite and gold crystals using the electrodeposition method (Huang et al., 2009; Jeon et al., 2016). Increased surface roughness decreases the homogeneity of the electric field across the electrode (Huang et al., 2009; Jeon et al., 2016; Lazić and Persson, 2010), leading to more complex diffusion zone profiles centered on surface peaks and aiding in nucleation at these sites (Huang et al., 2009; Jeon et al., 2016). The grain orientation at the electrode surface could also contribute, since higher energy faces will decrease the nucleation rate (Ghavidel et al., 2012); however, that has not been explored in this work.

In addition to two common metal substrates (Au and Ni), we studied RZx deposition on a pyrolytic graphite sheet (PGS). The optical and SEM images (Figure 2.6) illustrate that PGS provides significantly higher density of RZx crystals compared to Au and Ni. Figure 2.6a presents an optical image of large but sparse RZx crystals on Ni, which is contrasted with the high density of small RZx crystals on PGS in Figure 2.6b. Figure 2.6c and d contain higher magnification SEM images of the RZx on Ni and PGS, respectively. Besides the density disparities, the SEM images reveal two characteristic crystal shapes, namely, parallelepipeds and crosses. The crosses are more prevalent as particle size increases. The very predictable geometry is strong evidence that the material is highly crystalline, which is further justified using x-ray diffraction (XRD) and second harmonic generation (SHG) as discussed in the Materials Characterization section. We believe the excellent deposition of RZx on PGS is due to enhanced \( \pi - \pi \) interactions between the aromatic rings of resazurin (the precursor) and graphene layers in PGS. Such enhanced \( \pi - \pi \) interactions between graphene structures and dopamine (which has an aromatic ring) has been
discussed previously (Fernández and Castellani, 2017; Zhang et al., 2017). Similar conclusions have been made regarding the interaction of graphene materials and organic dyes with aromatic rings (Minitha et al., 2017; Rathinam et al., 2017; Xiao et al., 2016; Yan et al., 2014).

Impact of Composition of the Electrodeposition Solution

Next, we studied the effect of chemical composition of the electrodeposition electrolyte on formation of RZx. Considering PGS results in optimum surface coverage, we have used this substrate for the rest of the studies in this manuscript, unless otherwise noted. In our initial synthesis experiments, we observed that RZx was formed in BHI as the electrolyte. BHI is a common bacterial culture medium which contains the necessary nutrients for bacterial growth,

Figure 2.6. Surface chemistry affects RZx synthesis. a) and b) Optical images of the crystals on a nickel electrode (surface roughness 58 nm) and PGS, respectively. c) and d) SEM images of the crystals on the nickel electrode and PGS, respectively. Compared to nickel surface, the nucleation sites are more prevalent on PGS, resulting in a uniform, dense coverage of RZx crystals with smaller size.
mainly carbon sources (e.g. glucose), tryptone (source of amino acids and peptides), and salt. In order to understand the key components for synthesis of RZx, we performed systematic electrodeposition studies using resazurin solutions (1 mM) with combinations of the key nutritional elements: 100 mM KCl only (S#1), KCl and glucose (S#2), and KCl and tryptone (S#3). Figure 2.7 compares the DPV curves and optical images of the PGS post-DPV among S#1 (Figure 2.7a), S#2 (Figure 2.7b), S#3 (Figure 2.7c), and a BHI + resazurin reference (Figure 2.7d). The solid and dashed lines indicate the DPV sequence # 5 and sequence # 25, respectively. Only in S#3 (with tryptone), resazurin is reduced to RZx as evidenced from the decrease of the current with the sequence number. Moreover, the peak positions and shape of S#3 are similar to BHI solution with a major peak around -0.26 V and a minor peak around -0.39 V. The dual peaks indicate that reduction of RZ to RZx is a two-step process. S#1 and S#2 are significantly different with a major one around -0.57 V and a minor one around -0.43 V. These two reduction peaks are associated with the reduction reactions presented in Equations (2) and (3).

Once tryptone was identified as the key ingredient within BHI, we performed further experiments to narrow down which components of tryptone were most important. Since tryptone is a protein digest, it contains peptides and amino acids. There exist far too many permutations of even short peptide sequences to test, so we investigated select amino acids to assess whether they were responsible for RZx growth. There are twenty-one naturally occurring proteinogenic 𝛼 amino acids, grouped into four categories based on their physicochemical properties: charged side chains, polar side chains, hydrophobic side chains, and special cases. We decided to test two amino acids with charged side chains (histidine, glutamic acid), two amino acids with polar side chains (serine, glutamine), three amino acids with hydrophobic side chains (alanine, phenylalanine, tryptophan), and two special cases (cysteine, glycine). For each of the nine amino acids, at least 2 g/L was added to 100 mM KCl + 1 mM resazurin. PGS working electrodes were prepared and electrodeposition processes were attempted according to the protocol described in the methods section of this chapter; the only change was the total number of DPV sequences run. In all cases, at least 100 sequences of DPV were performed. If any deposition was apparent, more sequences were run to enhance the effect. After DPV, each of the electrodes were stabilized in BHI and then their CV responses were recorded in fresh BHI.
Deposition was apparent under the microscope only for two of the amino acids: histidine (2 g/L) and phenylalanine (10 g/L). The histidine-derived crystals (300 runs) had a slightly different appearance compared to the RZx crystals: they were more needle-like and had less of a metallic sheen to them under the microscope. The CV did show evidence of redox activity with two overlapping oxidation peaks and similar overlapping reduction peaks. The presence of two peaks and significant redox reversibility differentiate the crystals from the normal RZx crystals. The DPV curves, CV curves, and optical image of the histidine-derived crystals can be found in the appendix, Figure A.0.3.

Figure 2.7. **Tryptone is a key component for RZx electrodeposition.** a) DPV voltammogram in 100 mM KCl + 1 mM resazurin (S#1). Inset: optical image of the substrate showing lack of growth. b) DPV voltammogram in 100 mM KCl + 2 g/L glucose + 1 mM resazurin (S#2). Inset: optical image of substrate showing lack of growth. c) DPV voltammogram in 100 mM KCl + 10 mg/mL tryptone + 1 mM resazurin (S#3). Inset: optical image of substrate showing RZx growth. d) DPV voltammogram in BHI + 1 mM resazurin as reference. Inset: optical image of substrate showing excellent RZx growth. Solid lines indicate the sequence #5, dashed line indicate sequence #25. All scale bars are 50 μm.
The phenylalanine-derived crystals (350 runs), although larger in size and less densely packed on the surface than RZx crystals, appear remarkably similar in terms of shape and color. Figure 2.8a shows an optical image of the phenylalanine-derived crystals. The inset compares the molecular structure of phenylalanine and resazurin. In terms of the DPV curves, the differences between the two deposition conditions are easier to spot, as presented in Figure 2.8b. It is evident that, although there are two reduction peaks for each case, the potentials at which those peaks occur are significantly shifted and the ratio between them is altered. This is not entirely unexpected due to the major differences in chemical makeup/complexity of the two precursor solutions. Figure 2.8c compares the CV curves of RZx and phenylalanine-derived crystals. The CV data shows that there is a very pronounced oxidation peak and a less distinct reduction peak for the phenylalanine-derived crystals, which approximates the CV response of RZx/PGS in BHI. From these results, there is strong evidence that phenylalanine is essential for RZx deposition.

**Figure 2.8. Characterization of crystals derived from a solution of phenylalanine and resazurin.** a) Optical image of the crystals obtained during the electrodeposition process. The shape and color are equivalent to RZx. Inset: the molecular structure of resazurin and phenylalanine. b) DPV voltammograms contrasting electrodeposition in BHI + resazurin (BHI) and phenylalanine + resazurin (F). c) CV voltammograms comparing electrochemical response of RZx (BHI) and phenylalanine-derived crystals (F) in BHI.

It should be noted that after attempted electrodeposition for each amino acid case, the CV curves did show limited redox activity even if no deposition was apparent; however, the shapes of the curves did not closely match that of RZx/PGS. We attribute this redox activity to residual resazurin molecules, which is expected to adsorb well to graphitic materials (Minitha et al., 2017; Rathinam et al., 2017; Xiao et al., 2016; Yan et al., 2014).
Impact of Embedding RZx in a Polymeric Matrix

It is clear from scanning electron microscopy (SEM, Figure 2.11 and Figure 2.12) and optical profilometry (Figure 2.13) images presented in the next section that RZx crystals can introduce significant surface roughness and sharp corners to substrates. This is especially evident on PGS, which has minimal texturing on the scale of RZx crystals. While the addition of a spin coated Nafion layer likely decreases the overall roughness due to planarization effects (Bornside, 1990; Larson and Rehg, 1997), there may still be significant roughness and sharp corners across the surface. It is well known that significantly sharp features can damage adjacent cells (Bolotsky et al., 2019), which may not be desirable for certain applications. We hypothesize that synthesizing/embedding the RZx crystals within a polymer, such as Nafion, will decrease the overall surface roughness and reduce the size/number of sharp edges. Additionally, the electrode functionalization process is slightly simpler for embedded RZx than for Nafion-coated RZx.

Figure 2.9. Comparison of CV curves for polymer-embedded RZx. The Nafion + 1 mM resazurin does lead to redox activity, but the peaks are fairly non-descript. In contrast, Nafion + 10 mM resazurin induces very prominent redox peaks with high reversibility. The Chitosan + 10 mM resazurin also leads to very prominent redox peaks with high reversibility, although the current values decreased rapidly with increasing number of scans. The PEDOT:PSS + 10 mM resazurin does not induce much redox activity, which makes it a poor candidate for the present mode of sensing.
We investigated three polymer matrices for embedding RZx: Nafion, Chitosan, and PEDOT:PSS. Figure 2.9 shows CV curves for completed devices (see methods) from 1 mM resazurin in Nafion, 10 mM resazurin in Nafion, 10 mM resazurin in chitosan, and 10 mM resazurin in PEDOT:PSS. Comparing 1 mM resazurin and 10 mM resazurin in Nafion, it is clear that 1 mM was insufficient while 10 mM resazurin was sufficient; thus, 10 mM was set as the standard concentration in each polymer solution. Based on the CV curves, various conclusions can be reached. Firstly, Nafion-embedded-RZx and Chitosan-embedded-RZx are much more redox reactive than the PEDOT:PSS-embedded-RZx, as illustrated by the pronounced oxidation/reduction peaks or lack thereof. Secondly, embedding the RZx yields a much more reversible redox reaction (the reduction peak height is similar to the oxidation peak height). Overall, embedding RZx in a polymer seems like a promising approach to creating redox-active working electrodes.

Material Characterization of RZx-Based Electrodes

Microscopic, Spectroscopic, and Crystallography Characterization

Considering the predominance of organic molecules in the precursor solutions, it is expected that RZx is entirely organic. To confirm this, X-ray Photoelectron Spectroscopy (XPS) was performed on RZx samples grown on electrodeposited Ni electrodes and PGS electrodes. XPS spectra of materials were obtained using Physical Electronics VersaProbe II. The data is presented in Figure 2.10a. The major difference between the analysis of RZx/Ni and RZx/PGS samples is the amount of oxygen. The disparity can be attributed to the fact that the density of RZx on the RZx/Ni sample is much lower, meaning that some of the Ni surface – which can be easily oxidized – is exposed to the beam. This is an additional advantage of deposition on PGS; not only does it provide higher surface coverage compared to traditional metallic surfaces (see Figure 2.5), the substrate is generally biocompatible and chemically stable (Cook et al., 1999; More et al., 2008), making it resilient against surface corrosion and/or oxidation.

Although the geometry of RZx suggests formation of a crystalline phase (see Figure 2.6), XRD data provides a clear confirmation. The X-ray diffraction (XRD) spectra were measured
using Malvern Panalytical Empyrean (3rd gen.) with Co source operated at 40 mA and 40 kV. We compared XRD measurements for three samples in Figure 2.10b: PGS, RZx/PGS, and resazurin powder (RZ). The PGS substrate has two sharp peaks associated with its layered structure. Those same peaks show up for the RZx/PGS sample, with one new prominent crystal peak (belonging to the RZx) appearing at $2\theta = 33^\circ$. The resazurin precursor material was also probed with XRD for comparison, confirming that RZx is structurally distinct from the resazurin powder. The inset of Figure 2.10b presents a magnified view of the curves around $2\theta = 30^\circ$ to highlight the distinct difference between RZx and resazurin.

![Figure 2.10. Surface chemistry and crystallinity of RZx. a) XPS analysis of RZx on Ni or PGS. The data confirms that the crystals are organic. b) XRD spectra for PGS, RZx/PGS, and resazurin powder. The RZx peaks do not correspond to resazurin powder peaks, confirming that RZx is crystallographically distinct from resazurin sodium salt. Inset: magnification of the XRD spectra between $28^\circ < 2\theta < 35^\circ$.](image)

In addition to XPS analysis, Energy Dispersive Spectroscopy (EDS) elemental analysis and mapping were performed on RZx/PGS samples. The high spatial resolution of the technique was utilized to complement the lower resolution XPS data. The data was captured using Quanta 250 at 3-5 keV. Two sensing materials were probed by the technique: BHI-derived RZx/PGS (B-RZx/PGS) and Tryptone-derived RZx/PGS (T-RZx/PGS). Both sheets were affixed to a glass slide and conformally coated with 4 nm of iridium to avoid substrate charging. Since the iridium is not of quantitative interest here, it was excluded from analysis. Because EDS has significant penetration through the crystals and into the PGS, its ability to provide quantitative data is limited; however, its qualitative abilities are useful here. The EDS spectrum for B-RZx/PGS is presented...
in Figure 2.11a and corroborates the XPS data showing that RZx is organic and mainly composed of carbon. Figure 2.11b-e shows the local SEM image used for EDS and the associated elemental maps. The elevated nitrogen and oxygen concentrations at the sites of the crystals proves that both elements are substantial constituents of RZx. The EDS spectrum for T-RZx/PGS is presented in Figure 2.12a: the elemental ratios of C, N, and O are almost identical to the B-RZx/PGS. Similarly, the local SEM and elemental maps of T-RZx/PGS (presented in Figure 2.12b-e) are
nearly equivalent to their BHI counterparts. Taken together, the evidence is strong that T-RZx/PGS and B-RZx/PGS are equivalent materials.

In order to better understand the surface morphology of the RZx/PGS electrodes, we performed optical profilometry. A representative map for PGS and RZx/PGS is shown in Figure 2.13. As expected from optical images, the maps and associated line scans demonstrate that deposition of RZx increases the roughness of the PGS electrodes.
We also characterized RZx crystals using second harmonic generation measurements. SHG is a nonlinear optical process where two photons of fundamental frequency $\omega$ are annihilated and a photon with second harmonic frequency $2\omega$ is simultaneously created. Strong SHG can be generated from non-centrosymmetric crystals, including 2D materials (Janisch et al., 2014; Li et al., 2013; Malard et al., 2013; Shen, 2002; Yin et al., 2014). SHG can be used to evaluate material synthesis quality (e.g., amorphous vs. crystalline) and probe crystalline symmetry via polarization studies. SHG characterization was performed using a Ti:Sapphire mode-locked femtosecond laser (Tsunami, Spectra-Physics, center wavelength: 805 nm). The laser beam was focused through a 0.55 NA, 50X long working distance objective lens (Mitutoyo). The SHG signal was collected in reflection mode, separated from the laser beam by using a dichroic mirror, and focused through a

Figure 2.13. Optical Profilometry of PGS and RZx/PGS. The maps (2D and 3D) and the line scans confirm that the RZx/PGS has an increased surface roughness compared to PGS, stemming from high density of crystals deposited on PGS.
lens into a spectrometer (Princeton Instruments). A 450 nm short-pass filter was used on the spectrometer to suppress the laser pump and ambient light. All signals were collected with a spectrometer integration time of $t_{\text{integration}} = 5$ sec.

Three samples were probed for SHG: PGS, RZx/PGS, and Nafion/RZx/PGS. PGS by itself produced no observable SHG signal. Both RZx/PGS and Nafion/RZx/PGS produced strong SHG signals. The existence of SHG implies that RZx occurs as a non-centrosymmetric crystal.

Figure 2.14. **SHG of RZx/PGS and Nafion/RZx/PGS.** a) Log-log plot of SHG intensity vs. input power for RZx/PGS. Inset image shows SHG spectra in blue and the laser spectra in red. b) Log-log plot of SHG intensity vs. input power for Nafion/RZx/PGS. Inset image shows SHG in blue and the laser spectra in red. c) SHG spectra of RZx/PGS centered at 402.5 nm. d) SHG spectra of Nafion/RZx/PGS centered at 402.5 nm. For (c) and (d): Flat blue spectra in each Figure represents background signal which was subtracted from each measurement.

Three samples were probed for SHG: PGS, RZx/PGS, and Nafion/RZx/PGS. PGS by itself produced no observable SHG signal. Both RZx/PGS and Nafion/RZx/PGS produced strong SHG signals. The existence of SHG implies that RZx occurs as a non-centrosymmetric crystal. Figure 2.14a,b show log-log plots of intensity vs. input power for RZx/PGS and Nafion/RZx/PGS, respectively. SHG has a quadratic relationship with respect to its input power, as can be seen with the linear fit of slope $\approx 2$ in the log-log plots (exact fitted slope shown in the Figures). The inset
images in Figure 2.14a,b show the normalized spectra of the SHG and the laser signals. SHG intensities are reported with arbitrary units (a.u.). Figure 2.14c shows the SHG spectra for RZx/PGS where the input average power ranges from 2 mW-10 mW in increments of 1 mW. Figure 2.14d shows the SHG spectra for Nafion/RZx/PGS, where the input average power ranges from 2 mW-8 mW in increments of 1 mW. The spectra reported in Figure 2.14c,d are collected in the spectrometer with a 1200g/mm grating. The 1200g/mm grating provides a high resolution for the sharp SHG spectrum. The SHG characterization demonstrates a potential avenue to explore in future works, e.g. using RZx as an element for optical sensing.

**Studying the Ionic and Temperature Sensitivity of RZx Electrodes**

When performing CV characterization of Nafion/RZx/PGS in BHI, the most prominent redox characteristic is an oxidation peak centered around -0.2 V vs. Ag/AgCl. This peak, which we define as $E_{ox}$, is shown in Figure 2.4c during the electrode stabilization step. The presence of this peak is indication of the favorable redox activity of the RZx crystals. We next explored ways to utilize the redox activity of RZx for sensing purposes. We studied the effect of pH on the electrochemical response of Nafion/RZx/PGS using Tris-Glycine buffer with pH ranging from 4 to 8. The oxidation peak, $E_{ox}$ indicated by dashed lines in Figure 2.15a, increases to more positive potentials as pH decreases. Such shift is predicted by the Nernst equation:

$$E = E^0 + 2.303 \frac{RT}{nF} \log (Q_r)$$

where $E$ is the reaction potential, $E^0$ is the standard electrode potential, $R$ is the gas constant, $n$ is the number of electrons in the reaction, and $Q_r$ is the reaction quotient. For a reaction involving equal transfer of $\text{H}^+$ and $e^-$ (as expected here), the idealized slope is $dE/d(pH) = -59.2$ mV/pH. Experimental sensitivities can diverge from the Nernstian values based on thermodynamic considerations (Amemiya et al., 2003). For our sensor, Figure 2.15b plots the change of $E_{ox}$ vs. pH, confirming a near-Nernstian response of -53.4 mV/pH. This sensitivity is similar to and competitive with the values obtained using state-of-the-art polymer-based potentiometric pH sensors (Ghoneim et al., 2019; Korostynska et al., 2007), which hover around the Nernstian limit of -59.2 mV/pH. However, the simplicity of RZx deposition, its eco-friendly and readily available precursors, and low-cost scalable production make this material very attractive as a pH sensor.
We also studied the proton selectivity of the electrodes by spiking the Tris-Glycine pH buffers with 10 mM of various cations, including Li⁺, Na⁺, K⁺, Ca²⁺, and Mg²⁺. As can be seen in Figure 2.15c, Δ𝐸_{ox} (compared to baseline buffer) changes insignificantly (≤ 5 mV) in the pH range relevant to the time scale of the antibiotic susceptibility testing (60 min, see Figure 3.1). This implies a high degree of proton selectivity achieved by RZx. To see an alternate presentation of the data that highlights how insignificant the Δ𝐸_{ox} is, see appendix Figure A.0.4.

![Figure 2.15. pH response and temperature sensitivity of Nafion/RZx/PGS. a) Cyclic voltammograms showing the relevant oxidation peak E_{ox} and its relationship with solution pH. b) Extracted E_{ox} values from Figure 4a and the linear regression. The pH sensitivity is found to be -53.4 mV/pH, which is near-Nernstian. Data collected from n = 4 measurements, 1 sample. c) Proton selectivity of the sensor against common cations (monovalent and divalent). The signal shift is less than 10 mV, especially around the pH values of interest relevant to bacterial analysis, demonstrating high selectivity. Data from n = 2 measurements, 1 sample. d) Temperature sensitivity of the sensor from 25 to 45°C. A small temperature dependence of -0.975 mV°C is obtained by linear fitting of the curve. Data from n = 2 measurements, 1 sample.](image-url)

We additionally calculated the temperature sensitivity of Nafion/RZx/PGS electrodes. Solution temperature, whether by ambient conditions or external constraints, can fluctuate from
day to day or measurement to measurement, especially in field applications. Thus, a low
temperature sensitivity is desirable for greater precision. The temperature response of the device
within 25 – 45°C is shown in Figure 2.15d. Applying a linear fit to the data yields a small
temperature dependence of \( \frac{dE_{ox}}{dT} \approx \) -0.975 mV/°C, which is relatively consistent – albeit
smaller than – the value expected by the Nernst equation (4). Explicitly, using pH = 7.3 (expected
medium pH at 25°C):

\[
\frac{dE}{dT} = 2.303 \frac{R}{nF} \log(H^+) = -1.449 \text{ mV/°C} \quad (5)
\]

The obtained temperature sensitivity is similar to or smaller than start-of-the-art potentiometric
pH sensors (Horiba Scientific, 2018; W. D. Huang et al., 2011; Inzelt, 1990; Wen and Wang,
2014).

Bioassay Results

Since many biosensors, including the RZx-based biosensors described later, come into
contact with live cells/tissues, it is important to understand their interaction with those cells over
time. Whether it is beneficial to have a favorable or unfavorable effect on the growth/health of
cells depends on the cell type and application. For example, implantable and wearable sensors
will be expected to come into contact with body tissues for extended periods: thus, unfavorable
interactions with the host body need to be engineered out. Taking the example of a smart wound
dressing, it is further desirable for the surface to have antimicrobial properties (but not anti-tissue-
formation properties) in order to mitigate persistent infection (Simões et al., 2018). As an initial
investigation, we performed bioassays on RZx materials using both mammalian (mouse L929
fibroblast) and bacterial (\( S. \text{ aureus} \)) cells. Two different carbon electrode materials were studied:
PGS and laser-induced graphene (LIG). The PGS is smooth and non-porous, while the LIG is
very rough and porous (see methods for preparation details). Six sample types were prepared:
PGS, RZx/PGS, Nafion/RZx/PGS, LIG, RZx/LIG, Nafion/RZx/LIG. Due to our observation that
RZx materials tend to stain solutions pinkish over the course of many hours, we separated the
bioassays according to whether the RZx material was directly present or just the leached solutes
(leaching medium, see methods for details). This approach meant we could separate physical
effects of the RZx film on cells from chemical effects of RZx films on cells.
For the mammalian cells, attachment/growth was assessed for all six sample types. A low value for the OD\textsubscript{450} measurement indicates that the cells failed to attach to the available surfaces and/or that they failed to propagate. The data is presented in Figure 2.16a. Since the epoxy used to immobilize the films actually promotes attachment/growth, it is not considered a factor in low signal value. Compared to the control, it appears that all of the films dramatically hindered

**Figure 2.16. Bioassays on RZx electrodes and related materials.** a) Mouse L929 fibroblast attachment growth assay on various films: (A) PGS, (B) RZx/PGS, (C) Nafion/RZx/PGS, (D) LIG, (E) RZx/LIG, Nafion/RZx/LIG, and (G) epoxy films. In all cases, except the epoxy control, the films impeded attachment/growth compared to cells only. b) Mouse L929 fibroblast attachment growth assay in the presence of leaching medium. For all three cases, the leaching media promoted attachment/growth. c) Culture growth data for *S. aureus* bacteria in the presence of films/leaching medium. The two films with RZx crystals decreased the growth relative to the control, possibly due to physical damage from the crystals. On the other hand, leaching medium from RZx-containing materials seems to promote the culture propagation.
attachment/growth. The exact reason for this ubiquitous hinderance is not clear at this time, but it is known that surface wettability, surface charge, and surface treatment can all affect both the attachment and growth of mammalian cells on surfaces (Webb et al., 1998). It could also be that leached solutes are cytotoxic to the cells used; to that end, we used leaching medium from RZx/PGS, LIG, and RZx/LIG to test whether that is the case. The results are presented in Figure 2.16b. For all three types, the leaching medium actually aided cell attachment/growth compared to the control. Thus, we conclude that cytotoxic leaching is not responsible for the poor attachment/growth on the films. Further research using surface engineering on these electrode films could help mitigate the deleterious effect on mammalian cells propagation.

For the bacterial cultures, we investigated growth of *S. aureus* in the presence of films/leaching medium for the following sample types: RZx/PGS, LIG, and RZx/LIG. After 24 hours of incubation at 37°C, the cultures were probed with optical density measurements; the cell concentration is approximately linearly related to OD_{600} value. The results, shown in Figure 2.16c, indicate that growth of the colonies in the presence of the RZx-coated films is slightly hindered compared to the control. On the other hand, the LIG film seems to promote the growth of the colony. The fact that the two films coated with RZx crystals were worse for growth suggests that either chemical toxicity or physical damage could play a role. To identify whether chemical toxicity is a factor, we performed leaching experiments with the same sample types. The results show that growth is actually promoted for leaching media from RZx samples; thus, it would appear that physical damage to cells from RZx is the cause of the hindered growth.

**Chapter Summary and Conclusion**

In this chapter, we have introduced the RZx crystals as a sensing material and extensively characterized/optimized them. We first presented the synthesis conditions of RZx from BHI and resazurin on PGS. Then we investigated the effects of electrode chemistry and electrolyte composition on RZx formation and morphology. The results clearly showed that graphitic carbon electrodes are optimal materials for electrodeposition and that RZx synthesis requires both resazurin and L-phenylalanine, two eco-friendly and easily available reagents. We additionally demonstrated that RZx can be imbedded into polymer matrices. Then, we explored the characteristics of RZx. Results indicate that RZx is organic (C, N, and O) and crystalline. It also
increases the surface roughness of smooth electrode surfaces such as PGS. Optically, RZx samples showed SHG, which is further confirmation of their crystallinity and a potential area for further investigation. As a sensing material, RZx was proven to be pH sensitive and selective, with minimal temperature sensitivity. Lastly, bioassays showed that cell attachment and growth on RZx is not favorable without further surface engineering, but the RZx does not have any toxic effects in the surrounding medium.

With these results, there is clear indication that RZx has sensing capabilities that can be leveraged. In the next chapter, we apply these sensing capabilities to bacterial monitoring. We demonstrate that RZx-based sensors can accomplish the vital task of rapid antibacterial susceptibility testing (RAST).
Chapter 3

**Organic Redox-Active Crystalline Layers for Reagent-free Electrochemical Antibiotic Susceptibility Testing (ORACLE-AST)**

The contents of this chapter are reproduced from our paper published in Biosensors and Bioelectronics (Bolotsky et al., 2021). Herein, to enable reagent-free electrochemical sensing of bacterial viability we developed a reagent-free electrochemical RAST method (ORACLE-AST) based on novel, organic redox-active crystal layers, termed as RZx. We demonstrated the performance of ORACLE-AST using *Escherichia coli* (*E. coli*) K-12 exposed to different concentrations of ampicillin ($\rho_{AMP}$) and kanamycin ($\rho_{KAN}$). The method successfully determined ampicillin and kanamycin MICs in 60 minutes without requiring any redox reagent. Furthermore, the method enabled measuring bacterial viability in complex media, including whole blood and milk. The sensors possess a long shelf-life (< 12% degradation in ~ 60 days) in ambient storage condition. Furthermore, RZx can be synthesized on LIG. The Nafion/RZx/LIG sensors similarly show sensitivity towards bacterial metabolic activity. RZx-based electrochemical sensors can thus provide a portable, low-cost, simple-to-use, and rapid phenotypic RAST approach for potential use in both traditional settings and resource-limited areas. Moreover, the developed reagent-free electrochemical sensors might enable *in situ* probing of cellular metabolic activity for applications beyond AST, for example in microbial fuel cells and early detection of microbial infection in wounds or catheters.

**The Importance of Antibiotic Susceptibility Testing Technologies**

According to the Centers for Disease Control and Prevention (CDC) and the World Health Organization (WHO), antimicrobial resistance (AMR) is one of the most serious global health threats of the 21st century (Centers for Disease Control and Prevention, 2019; World Health Organization, 2018). In the United States alone, there are annually more than 2.8 million infections caused by antibiotic resistant pathogens, with more than 35,000 cases leading to death (Centers for Disease Control and Prevention, 2019). Furthermore, it is estimated that with the current rate of spread of AMR, around 10 million people globally could die annually due to its
direct effect by 2050 (O’Neill, 2016). In clinical settings, antimicrobial susceptibility testing (AST) is a standard procedure for identifying the minimum inhibitory concentration (MIC) of a chosen antibiotic (Syal et al., 2017). Despite high accuracy and low cost, gold standard clinical ASTs require at least 16 hours after bacterial isolation to produce reliable results (Khan et al., 2019), forcing the physicians to prescribe high dosages of broad-spectrum antibiotics (Luyt et al., 2014; Ventola, 2015). Hence, there is an urgent need to develop rapid ASTs (RASTs), not only to improve clinical outcomes, but also for AMR stewardship (Syal et al., 2017).

**Literature Review of Antibiotic Susceptibility Testing Methods**

There are numerous RAST methods that are being developed, relying on a wide range of technologies and signal transduction schemes (Bard and Lee, 2018; Khan et al., 2019). In general, RAST methods can be divided into phenotypic or genotypic approaches (Bard and Lee, 2018). Genotypic methods do not directly measure susceptibility, instead relying on detection of known or suspected resistance-related nucleic acid sequences to predict the susceptibility of a pathogen (Khan et al., 2019). Normally, since the available concentration of the target sequence is low, the genotypic methods require an amplification step, such as polymerase chain reaction (PCR) (Athamanolap et al., 2017; Fluit et al., 2001; Rolain et al., 2004), isothermal recombinase polymerase amplification (RPA) (Lillis et al., 2016), or loop-assisted isothermal amplification (LAMP) (Khazaei et al., 2018; Law et al., 2018; Schoepp et al., 2017, 2016). By combining the amplification with fluorescence tagging and detection, susceptibility predictions can be generated in about one hour (Li et al., 2017). However, despite the speed of the genotypic methods, they require expensive/specialized reagents, fluorescence detection equipment, and skilled personnel (Bard and Lee, 2018; Khan et al., 2019; Li et al., 2017) to carry out and interpret the results. Furthermore, the genotypic methods require knowledge of the resistance genes *a priori* and contamination-related false-positives can still occur (Khan et al., 2019). Also, due to the complex interaction of genetic markers in determining resistance, extensive understanding of the relevant microbe’s biology is required. This makes it difficult to apply the genotypic methods broadly across a range of microbial strains (Ellington et al., 2017). Also, due to the ever-evolving nature of microbial genetics, novel resistance mechanisms – which have not been characterized – can cause false negatives in these techniques (Khan et al., 2019).
Compared to genotypic RAST methods, phenotypic techniques have the benefit of providing more clinically-relevant susceptibility results since they directly assess the physical state of bacterial cells under the influence of a given antimicrobial agent. Numerous phenotypic techniques have been developed, ranging from optical (Baltekin et al., 2017), to mechanical (Longo et al., 2013), to electronic (Safavieh et al., 2017), to electrochemical (Besant et al., 2015) methods. Among various RAST methods, to the best of our knowledge, Accelerate Pheno™ (Accelerate Diagnostics, Inc., Tucson, AZ, USA) is the fastest FDA-approved phenotypic technology (Accelerate Diagnostics, 2020). By combining fluorescence in situ hybridization (FISH) and optical/fluorescence microscopy, this method utilizes morphokinetic image data to provide AST results directly from blood cultures in 6-7 hours. However, a drawback of optical techniques (Baltekin et al., 2017; Cermak et al., 2016; Choi et al., 2013; Etayash et al., 2016; Fredborg et al., 2013; Lu et al., 2013; Mohan et al., 2013; Price et al., 2014; Yu et al., 2018) is that they utilize complex and relatively expensive optical equipment (Yu et al., 2018) (such as 100x objectives, lasers, etc.), cannot effectively probe opaque solutions, and often require specialized/short-lived labeling reagents that can interfere with cellular biology and/or antibiotic action. These limit their field application, especially in resource-limited regions.

In comparison to optical ASTs, electrical and electrochemical methods are more compatible for point-of-care application. ASTs based on measuring the change of impedance (non-Faradaic) (Brosel-Oliu et al., 2019; Ebrahimi and Alam, 2017; Kara et al., 2018; Safavieh et al., 2017; Song et al., 2019; Yang et al., 2020) and electrochemical signal (Faradaic) (Besant et al., 2015; Chotinantakul et al., 2014; Ertl et al., 2000; Liu et al., 2014; Mishra et al., 2019; Rao R et al., 2020) have been developed. The impedance-based electrical RASTs, which do not involve any electrochemical reactions, have been demonstrated primarily using microfluidics and/or interdigitated electrodes. The microfluidic components help to concentrate, direct, or immobilize bacteria from a culture (Safavieh et al., 2017; Yang et al., 2020), while the interdigitated electrode arrangement helps increase the sensing area and improve signal-to-noise ratio (Brosel-Oliu et al., 2019; Ebrahimi and Alam, 2017). While these sensors do not require a reference electrode, they suffer from unintended channel clogging in microfluidics-based methods (Hassanpourfard et al., 2016; Mukhopadhyay, 2005), have limited compatibility with bacteriostatic (e.g. non-lytic) antibiotics (Brosel-Oliu et al., 2019; Ebrahimi and Alam, 2017), require specific captures molecules (Safavieh et al., 2017; Song et al., 2019), and/or have
difficulty probing non-motile bacteria (Kara et al., 2018). RAST methods based on field effect transistors (FET) have been also developed (Y. Huang et al., 2011; Ibarlucea et al., 2017; Kumar et al., 2020; Pourciel-Gouzy et al., 2008), including affinity-based FET sensors (Kumar et al., 2020), and pH-sensitive FETs (Y. Huang et al., 2011; Ibarlucea et al., 2017; Pourciel-Gouzy et al., 2008). The pH-sensitive FETs, which fit into the larger field of ion-sensitive bioelectrodes, monitor metabolic acidification/alkalization as a function of antibiotic concentration. Some issues that need to be solved are sensor lifetime in complex media, charge screening issue with FETs, and measurement reproducibility.

Electrochemical ASTs measure change of the redox state of a redox-active molecule as induced by bacterial metabolism (Besant et al., 2015; Chotinantakul et al., 2014; Ertl et al., 2000; Liu et al., 2014; Mishra et al., 2019; Rao R et al., 2020). Viable cells reduce a redox-active reagent, such as resazurin (Besant et al., 2015; Mishra et al., 2019) or ferricyanide (Chotinantakul et al., 2014; Ertl et al., 2000; Rao R et al., 2020) mixed in the culture medium, leading to change of the voltammetric signals. The magnitude of the Faradaic current depends on the bulk concentration of the reactant state and, by extension, the bacterial metabolic activity which changes in response to antibiotic treatment (Besant et al., 2015; Chotinantakul et al., 2014; Mishra et al., 2019). A major problem with current electrochemical ASTs is the need to mix a redox reagent into the culture medium which can interfere with antibiotic action or normal cellular function and hence, make interpretation of the MIC values challenging.

Materials and Methods

Bacterial Culture and Testing with Antibiotics

Ampicillin sodium salt was purchased from Sigma Aldrich (#A9518). *E. coli* K-12 was generously provided by J. Kovac’s lab at Penn State University. The culture was stored as a frozen stock at −80 °C and resuscitated every 2 weeks to maintain a fresh inoculum. The resuscitation was performed by streaking onto a Brain Heart Infusion (BHI) agar and then incubating at 37 °C for 20 ± 2 hr. An isolated colony from the agar was inoculated into BHI broth, shaken at 210 rpm, and maintained at 37 °C for 20 ± 2 hr. Next, the overnight culture (~0.5-1×10⁹ CFU/mL)
(Zhou et al., 2020) was diluted 1:100 in a round-bottom falcon tube with 10 mL of fresh medium (BHI, fat-free milk, or whole blood). Then, the tube was placed in a shaking incubator at 37°C and 210 rpm. To ensure consistent data from test to test, we incubated cells for ~1 hour (lag time), followed by adding antibiotic (ampicillin or kanamycin); this represents time $t_0 = 0$ min. For each sample and time point (multiples of 30 min), the culture was probed using both OD$_{600}$ and ORACLE-AST (following the procedure detailed in Chapter 2). MIC values were validated by measuring OD$_{600}$ after overnight culturing (18-24 hrs) as the gold standard.

Testing Sensors with Milk and Whole Blood: Sample Preparation

We tested the sensor performance in low-fat milk purchased from a local supermarket. After sterilizing the milk using an autoclave, we spiked it with overnight E. coli K-12 culture at a ratio of 1:100. The suspension was then incubated at 37°C for one hour at 210 rpm, the end of which marked $t_0$. Ampicillin was added at $t_0$. Optical density data could not be generated due to the solution opacity.

For testing sensors using whole-blood, 1 mL of overnight E. coli K-12 culture was added to a fresh microcentrifuge tube and spun at 6500 rpm for 8 min (Thermo Fisher Scientific Heraeus Pico 17 Centrifuge). Afterwards, 900 μL of supernatant was removed and discarded, leaving the pelleted bacteria. Next, 900 μL of whole blood was added to the tube containing the bacterial pellet. The resulting bacteria/blood mixture was vortexed to create a homogenous solution and served as the “blood overnight culture.” AST proceeded from this overnight culture, as explained in the previous subsection. Optical density data could not be generated due to the solution opacity.

Statistical Analysis

Probability values (p-values) in Figure 3.1 were calculated using test statistics (t-values) and the degrees of freedom (DoF = $n - 1$). The t-value is defined as $t = \frac{x - \bar{X}}{S_x}$, where $x$ is an estimator value of some statistic, $\bar{X}$ which is the hypothetical value, and $S_x$ is the standard error of $x$. The hypothesis being tested is that $\frac{\delta E_{ox}}{\delta E_{ctrl}}$ for non-MIC values of antibiotic concentration
\( (\rho_{\text{Antb}}) \) is the same as MIC. A p-value of 1 means this hypothesis is true and a p-value < 0.05 means that the hypothesis is most likely not true. The t-values were fed into an open-source statistical calculator (GraphPad, 2020) along with the DoF.

**Performance of the ORACLE-AST Platform**

To demonstrate the application of RZx for reagent-free electrochemical AST, we chose *E. coli* K-12 strain (model strain) treated with two antibiotics (ampicillin and kanamycin); testing procedure is shown in Figure 3.1a. The two model antibiotics inhibit bacterial growth through different mechanisms of action. Ampicillin inhibits cell wall synthesis, which eventually leads to cell lysis at sufficient concentration (Gualerzi et al., 2013). Kanamycin is an aminoglycoside that disrupts protein synthesis (Gualerzi et al., 2013). For each experiment, cultures with various antibiotic concentrations, \( \rho_{\text{Antb}} \), were tested using ORACLE-AST. In parallel, the turbidity of the samples was measured using OD readout at 600 nm (OD\(_{600}\)). MIC values were determined based on OD\(_{600}\) of the overnight culture, which is the required time for reliable AST decision according to FDA and CLSI guidelines (Clinical and Laboratory Standards Institute, 2012).

The sensor signal is defined as \( \Delta E_{\text{ox}} = E_{\text{ox}} - E_{\text{baseline}} \), where \( E_{\text{baseline}} \) is the oxidation peak for the cell-free medium. The sensor results for ampicillin- and kanamycin-treated cultures are shown in Figure 3.1b and 3.1d, while the corresponding OD\(_{600}\) data is presented in the appendix Figure A.0.5. From overnight incubation, the MIC of ampicillin and kanamycin were determined to be 8 \( \mu \text{g/mL} \) and 16 \( \mu \text{g/mL} \), respectively. Per CLSI guideline, these values indicate that the *E. coli* K-12 is classified as susceptible to both antibiotics (Clinical and Laboratory Standards Institute, 2012).

The sensor data with ampicillin (n = 6 measurements, 3 samples) shows that \( \Delta E_{\text{ox}} \) for the control culture (no antibiotic) rises to approximately 100 mV after 120 min, e.g. \( t_{120} \). The rate of increase of \( \Delta E_{\text{ox}} \) decreases with increase of \( \rho_{\text{Antb}} \). At MIC, the signal reaches a ceiling of ~50 mV denoted by a horizontal line. A similar ceiling occurs around ~15 mV for the kanamycin data (4 \( \leq \) n \( \leq \) 6 measurements, 2-3 samples). If the signal raises above this threshold, the antibiotic concentration is sub-MIC. For bactericidal concentrations, the signal is below this threshold (the shaded region).
Figure 3.1. Electrochemical antibiotic susceptibility testing using RZx/PGS sensors. a) Overnight culture of E. coli K-12 is diluted 1:100 in BHI and incubated for 1 hour (OD<sub>600</sub> = 0.125 ± 0.025). Different antibiotic concentrations are then added and the cultures are tested using the RZx/PGS sensor. A representative voltammogram from which the sensor signal, ΔE<sub>ox</sub> (namely the shift of the oxidation peak potential) is extracted. b) & d) Plot of ΔE<sub>ox</sub> vs. time for E. coli K-12 cultures treated with different concentrations of ampicillin and kanamycin, respectively. The red zones indicate the threshold of signal magnitude for bactericidal concentrations (above MIC). The sensor can determine MIC of both antibiotics in 60 min. Error bars for n = 4-6 measurements (2-3 samples) are smaller than the symbols. c) & e) Normalized rate of signal change with respect to control, i.e. δE<sub>ox</sub>/δE<sub>ctrl</sub>, where δE<sub>ox</sub> = E<sub>ox</sub> - E<sub>ox,t0</sub> and δE<sub>ctrl</sub> corresponds to the control (untreated) culture. Presented at different incubation times for the ampicillin- and kanamycin-treated samples in part b) and d), respectively. The p-values (outlined in methods) are shown where * ≤ 0.0275 and ** ≤ 0.0025. Error bars are for n = 4-6 measurements, 2-3 samples.
Figure 3.1c and 3.1e highlight the sensor ability to identify MIC in 60 min more clearly by plotting the normalized rate of signal change with respect to the control sample at different time points, \( \frac{\delta E_{ox}}{\delta E_{ctrl}} \), where \( \delta E_{ox}(t_i) = E_{ox}(t_i) - E_{ox}(t_0) \) for different concentrations of antibiotics and \( \delta E_{ctrl} \) is the same signal for the control sample. The probability \( (p) \) values, which are discussed in the Materials and Methods of this chapter, are \(< 0.05\), confirming a statistically-meaningful identification of MIC at 60 minutes. It should be noted that accurate prediction of MIC using the OD\textsubscript{600} growth curves takes at least 6 hours (vs. 60 minutes using ORACLE-AST), because cells can adapt to the treatment and grow after the overnight incubation despite an initial decline of turbidity at sub-MIC concentrations. Similar trends have been reported elsewhere (Zhou et al., 2020). On the other hand, the RZx-based sensor is more resilient to this disparity because it measures the metabolic activity of cells, which is a clear signature for bacterial viability as compared to cell division measured using OD reading. Note that cells can still metabolize despite being non-dividing, for example in the Lag growth phase (Bertrand, 2019; Butler et al., 2019; Martin, 1932).

To further demonstrate the performance of RZx-based sensors for differentiation between antibiotic-susceptible and –resistant cells, we measured the response of ampicillin-resistant \( E. coli \) to ampicillin. The results are shown in the appendix Figure A.0.6, confirming that while \( \Delta E_{ox} \) is small at the MIC value of \( E. coli \) K-12 (\( \rho_{Antb} = 8 \mu g/mL \)), \( \Delta E_{ox} \) for the resistant strain is large and similar to the non-treated \( E. coli \) K-12 strain.

**Proposed Working Principle**

To understand the mechanism(s) by which RZx responds to bacterial viability, we designed the experimental process outlined in Figure 3.2a. We measured pH of supernatant of \( E. coli \) K-12 control culture at different time points, \( t_i \). Figure 3.2b-left plots the extracted supernatant pH values. Using the \( E_{ox} \) vs. pH curves (Figure 3.2b-middle) for pH buffers, we calculated the expected \( \delta E_{ox}(t_i) = E_{ox}(t_i) - E_{ox}(t_0) \) for the supernatant (SUP) and compared it with bacterial culture data. The extracted and measured \( \delta E_{ox} \) are plotted in Figure 3.2b-right. The blue line denotes the difference between the two signals, indicating that although pH is a major signal component, there are other factors boosting the signal magnitude. It should be noted
that without RZx, there is no Faradaic peak in CV curves using Nafion/PGS electrodes (appendix Figure A.0.7), confirming the signal is due to RZx.

**Figure 3.2. RZx’s sensing mechanisms in response to bacterial metabolism.** a) Procedure to obtain the pH values of the bacterial supernatants. Supernatants were tested with pH strips. b) pH is not the only factor RZx responds to; RZx can directly oxidize respirating cells. The pH values of supernatants of E. coli cultures (starting at OD$_{600}$ = 0.1) were measured using pH strip (left). $E_{ox}$ of pH buffers were measured (middle). The extrapolated curve (based solely on pH) was then compared to the signal obtained for a culture sample (right). The difference in signal magnitude demonstrates that RZx is responsive to both pH of the medium as well as being able to directly oxidize cells.

Considering that the additional signal is observed in the presence of live whole cells in the medium, it could be attributed to oxidation of redox-active compounds/metabolites secreted by cells. For example, Butina et al. developed a PEDOT:PSS-based potentiometric sensor for detection of uropathogenic cells based on measurement of secreted redox-active compounds such as extracellular thiols, NADH, flavins, ascorbic acid, glutathione, and cysteine (Butina et al., 2019). Moreover, bacterial energy metabolism through catabolism results in conversion of non-ionized sugars (such as glucose in the culture medium) to smaller organic acids (such as pyruvic acid) (Trotter et al., 2011; Yang and Bashir, 2008; Yasid et al., 2016). It is important to note that Nafion is permeable to small molecule metabolites, such as amino acids, especially cationic ones (Chum, 1983; Sikdar, 1985). Thus, we believe that changes in the concentration of cationic small
molecules (resulting from metabolism) and excreted redox-active compounds are responsible for the additional $\Delta E_{ox}$ in RZx-based sensors. As a future investigation, further exploration with different bacterial species in minimal medium (such as M9 culture broth) and control studies with spiked redox-active compounds can elucidate the detailed mechanism.

Considering the dominant sensing mechanisms of RZx, the sensors are well suited for monitoring viability of pathogens that induce significant pH changes during growth and/or cellular maintenance. Organisms that fit this description are varied and fit into numerous categories, such as urinary tract infections (UTIs) (Bono and Reygaert, 2019; Lai et al., 2019), blood pathogens (Lone et al., 2015), or respiratory tract infections (Savic and McShan, 2012). Sensing the pH changes induced by cellular proliferation has been demonstrated using other methods (Y. Huang et al., 2011; Ibarlucea et al., 2017; Lee et al., 2012; Owicki and Wallace Parce, 1992; Pourciel-Gouzy et al., 2008; Ratzke and Gore, 2018; Sánchez-Clemente et al., 2018; Tang et al., 2013), but to the best of knowledge, this is the first report of an electrochemical sensor based on pH used for AST.

**Testing Complex Samples, Sensor Stability, and Scalable Manufacturing**

In addition to testing in culture broth (BHI), we demonstrated the sensor ability to detect bacterial viability in complex solutions: milk and whole blood (Figure 3.3a and b). We spiked human blood with *E. coli* K-12 and ampicillin and performed electrochemical testing using Nafion/RZx/PGS sensors. The sensor successfully can differentiate between growing cells ($\rho_{AMP} = 0 \mu g/mL$) and inhibited culture ($\rho_{AMP} = 32 \mu g/mL$) in 60 min, as depicted in Figure 3.3a,b. As expected, gold-standard OD$_{600}$ measurements were not possible due to the opacity of the solution. In contrast, since electrochemical sensing is insensitive to optical transparency, RZx-based sensors had no difficulty in analyzing cell viability in milk solution. Similar to the results in BHI culture media, differentiation between the bactericidal concentration and the control was possible as early as 60 min (Figure 3.3a). Compared to tests in BHI, the magnitude of $E_{ox}$ was significantly reduced, which is likely due to the slightly acidic nature of milk. We also demonstrated the sensor ability to directly analyze infected whole blood. The sensor successfully can differentiate between growing cells ($\rho_{AMP} = 0 \mu g/mL$) and inhibited culture ($\rho_{AMP} =$
32 μg/mL) within 60 min (Figure 3.3b). The data for Figure 3.3b was collected by Ritvik Muralidharan of the Ebrahimi Bioanalytical and Biosensor Engineering Lab at Penn State.

In order to assess the sensor stability, we compared the *E. coli* K-12 data collected on day 1 and day 60. The sensor surface was cleaned with MilliQ Ultrapure DI water after the first day and stored in ambient conditions (Figure 3.3c). The signal in this case was defined as $\delta E_{ox}(t_i) = E_{ox}(t_i) - E_{ox}(t_0)$. There was only a minor difference (less than 12%) in $\delta E_{ox}$ after 2 months demonstrating that the sensor is highly stable.

**Figure 3.3. Testing complex samples and sensor stability.** a) Plot of $\delta E_{ox}$ vs. time for *E. coli* K-12 spiked in milk and treated with ampicillin. b) Plot of $\delta E_{ox}$ vs. time for *E. coli* K-12 cultures spiked in whole blood and treated with ampicillin. c) The sensor response is almost the same as the initial testing even after 2 months of storage in ambient condition. At the 90-minute mark, the response has only decreased 12%. All error bars are for $n = 2$ measurements.

**Testing Other Bacterial Species (Salmonella Typhimurium)**

To evaluate the applicability of the sensing platform for testing other bacterial species and/or other growth media, we changed the growth medium and the bacterial species. Specifically, we cultured *Salmonella enterica* TLI in lysogeny broth (LB) Lennox from Fischer Scientific (BP1427-500). The following data was collected on Nafion/RZx/PGS by Kayla Root of the Ebrahimi Bioanalytical and Biosensor Engineering Lab at Penn State. The progression of sensor signal was monitored over the course of 120 minutes. Figure 3.4a plots the $\Delta E_{ox}$ (no antibiotics added) with error bars ($n = 9, 3$ experiments). The data clearly indicates that the RZx responds to the metabolic activity of *Salmonella*, similar to *E. coli*. The lowered magnitude of the signal at each time point (compared to *E. coli*) is likely a result of slower growth rate, differences
in the released metabolites, and/or disparities in the buffering capacity of the medium. Figure 3.4b shows the associated OD\textsubscript{600} data (n = 3). It is clear that Salmonella is slower growing than the E. coli K-12, which could contribute to the smaller signal magnitudes.

**Figure 3.4. Viability monitoring for Salmonella in LB Lennox.** a) The data (n = 9), shows the $\Delta E_{ox}$ signal obtained over the course of 120 minutes for Salmonella cultured in LB. There is clear response to the culture growth. b) The OD\textsubscript{600} data (n = 3) is presented here. It is evident that growth is occurring, although the rate is slower than for E. coli K-12.

**Testing with RZx-Embedded Polymers**

As mentioned in Chapter 2, embedding RZx into a polymer matrix is a promising alternative sensor design and can prevent potential harmful effects of sharp crystal edges on biological cells (especially important for wound dressing applications). In order to test the feasibility of the sensor and compare it to the non-embedded design, we investigated two devices in parallel. One was a Nafion/RZx/PGS device and the other was an Nafion-Embedded-RZx/PGS device. We monitored the growth of an E. coli K-12 culture (untreated) according to the methodology laid out in the beginning of this Chapter. The results of the experiment are presented in Figure 3.5. The $\Delta E_{ox}$ signal for each sensor is nearly identical, providing evidence that embedding the RZx during synthesis does not degrade performance.
Laser-induced graphene (LIG) is a 3D and porous form of graphene currently being studied as a simple, scalable, and versatile electrode material for use in a range of applications, especially sensing technologies (Chyan et al., 2018; Kaidarova and Kosel, 2020; Wan et al., 2020). We investigated RZx formation on LIG, which was created using direct laser writing on polyimide sheets. The laser induces the ablation of the underlying polyimide using a well-developed process (Lin et al., 2014). In this instance, a CO₂ laser was utilized to locally initiate the ablation, yielding patternable electrodes for utilization in flexible devices. Due to the high conductivity, porosity, and graphitic nature of LIG, it is an excellent sorbent of organic dyes, such as resazurin (Rathinam et al., 2017). Thus, feasible RZx deposition at high surface density was achieved, followed by spin-coating of Nafion to develop Nafion/RZx/LIG sensors. The synthesis of LIG, morphological characterization of LIG and RZx/LIG, and the performance of Nafion/RZx/LIG sensors are illustrated in Figure 3.6. Considering the significantly larger availability of active sites in LIG compared to PGS, process optimization can enable rapid investigation of antibiotic effects on lower initial cell concentrations ($5 \times 10^3$ CFU/mL to comply

![Monitoring with Embedded RZx](image)

**Figure 3.5.** Comparison of device that do and do not use embedded RZx. The $\Delta E_{ox}$ signal for each sensor configuration is presented. Notably, the sensitivity of the device with embedded-RZx is nearly identical to the Nafion/RZx/PGS sensitivity.

**Laser -Induced Graphene as a Substrate for RZx Electrodeposition**

Laser-induced graphene (LIG) is a 3D and porous form of graphene currently being studied as a simple, scalable, and versatile electrode material for use in a range of applications, especially sensing technologies (Chyan et al., 2018; Kaidarova and Kosel, 2020; Wan et al., 2020). We investigated RZx formation on LIG, which was created using direct laser writing on polyimide sheets. The laser induces the ablation of the underlying polyimide using a well-developed process (Lin et al., 2014). In this instance, a CO₂ laser was utilized to locally initiate the ablation, yielding patternable electrodes for utilization in flexible devices. Due to the high conductivity, porosity, and graphitic nature of LIG, it is an excellent sorbent of organic dyes, such as resazurin (Rathinam et al., 2017). Thus, feasible RZx deposition at high surface density was achieved, followed by spin-coating of Nafion to develop Nafion/RZx/LIG sensors. The synthesis of LIG, morphological characterization of LIG and RZx/LIG, and the performance of Nafion/RZx/LIG sensors are illustrated in Figure 3.6. Considering the significantly larger availability of active sites in LIG compared to PGS, process optimization can enable rapid investigation of antibiotic effects on lower initial cell concentrations ($5 \times 10^3$ CFU/mL to comply
with CLSI guidelines for AST methods) (Clinical and Laboratory Standards Institute, 2012). A proof-of-concept experiment is presented in Chapter 4.

A schematic of the LIG synthesis process is shown in Figure 3.6a; the inset shows the flexibility of LIG on polyimide. We deposited RZx onto the LIG in BHI, using the method discussed in Chapter 2. Optical profilometry maps of the RZx/LIG electrode were taken to illustrate and quantify the high roughness of the as-prepared substrate. A representative map can

Figure 3.6. Characterization and antibiotic susceptibility testing with Nafion/RZx/LIG. a) The synthesis of laser-induced graphene (LIG) and fabrication of the device is shown schematically. A CO₂ laser ablates the surface of polyimide, yielding LIG that can then be functionalized with RZx. Working and counter electrodes are patterned by the laser method. The inset shows the flexibility of LIG on polyimide. b) Optical profilometry of an LIG electrode. The image clearly shows the laser-written lines. An associated height profile is shown along the direction of arrow. c) SEM image of an RZx/LIG electrode, showing the highly porous and 3D structure. Scale bar is 5 μm. Inset: Raman spectrum of the LIG. d) Nafion/RZx/LIG sensor used to probe E. coli cultures in whole blood. Differentiation between the control (0 μg/mL ampicillin, black) and inhibited cell (16 μg/mL ampicillin, red) starts in 60 min. This confirms the device’s applicability for use with clinically-relevant samples.
be viewed in Figure 3.6b. Figure 3.6c shows an SEM image of the RZx/LIG, which has large porous networks of thin graphitic sheets; the inset contains the Raman spectrum of pure LIG, with the D, G, and 2D peaks of graphene/graphite visible (Malard et al., 2009).

After RZx deposition, spin-coating of Nafion and stabilization in BHI proceeded as before. The finished Nafion/RZx/LIG devices were utilized for AST testing of *E. coli* K-12 culture spiked in whole blood (prepared as outlined in the methods section and performed by Ritvik Muralidharan of the Ebrahimi Bioanalytical and Biosensor Engineering Lab at Penn State.). Figure 3.6d shows the evolution of the $\Delta E_{ox}$ signal over the course of 120 min. Two different culturing conditions were compared: $\rho_{AMP} = 0 \mu g/mL$ (control) or $\rho_{AMP} = 16 \mu g/mL$ (inhibited). The sensor was able to easily distinguish growth vs. non-growth conditions in 90 minutes. The ability of these sensors to work directly in blood is promising for their potential use in point-of-care applications with patient samples.

**Screen Printed Carbon Electrode on Paper as a Substrate for RZx Electrodeposition**

Besides PGS and LIG, other carbon-based electrodes can be utilized for bacterial monitoring. For example, screen-printed carbon electrodes can be functionalized with RZx. As proof, we utilized a paper-based sensor with on-chip electrodes, including a screen-printed carbon WE. The RZx was deposited onto the surface using the methods outlined in the previous chapter. Once the Nafion/RZx/Paper-Electrode device was completed, we tested its ability to differentiate between viable (control) and non-viable (16 $\mu g/mL$ ampicillin) *E. coli K12* cultures ($1 \times 10^7$ CFU/mL initial). $E_{ox}$ data was collected at 30-minute intervals by Ritvik Muralidharan of the Ebrahimi Bioanalytical and Biosensor Engineering Lab at Penn State. The results are shown in Figure 3.7. The error bars (n = 3) are too small to be seen behind the symbols and the inset shows a photo of the flexible paper electrode. Like the RZx/LIG and RZx/PGS sensor types, this one is able to differentiate between viable and non-viable cultures within 60 minutes. This is further evidence that carbon-based electrodes serve as ideal electrodeposition surfaces for RZx-based devices.
Chapter Summary and Conclusion

In this chapter, we investigated RZx-based sensors as a RAST platform. RAST is an important technology for combatting AMR, and fast results are key. We have showed that using Nafion/RZx/PGS reagent-free electrochemical sensors, we can accurately predict the MIC of a model bacteria (E. coli K12) exposed to antibiotics (both ampicillin and kanamycin) in just 60 minutes. The parallel OD measurements required at least 6 hours to accurately predict MIC. We identified that the sensors work primarily by sensing changes in pH, although there were other contributions, likely by bacterial metabolites. Furthermore, we showed that the Nafion/RZx/PGS devices could probe complex and optically transparent liquid sample, which optical based methods could not. The sensors were also sensitive to the growth of S. enterica, possessed long shelf-lives in ambient, and could be reused with only small degradations in signal (12% after 60 days). Lastly, we showed that Nafion/RZx/LIG sensors, as low cost, scalable, and patternable functional electrodes, could also be used to rapidly probe bacterial viability.

Based on these results, we wanted to show a proof-of-principle platform using a compact microfluidics design in conjunction with RZx/LIG. This is the focus of the next chapter. The microfluidics would decrease the necessary sample volume, increase ease of handling, and

Figure 3.7. Performance of a Nafion/RZx/Paper-Electrode device. The sensor is able to differentiate between viable (control) and non-viable (16 μg/mL ampicillin) E. coli K12 cultures in 60 minutes. Error bars are too small to be seen behind the symbols. This performance rivals the LIG- and PGS-based devices. Inset: photograph of the paper electrode undergoing flexing.
confine bacteria near the sensing surface. The compact on-chip design (Ag/AgCl pseudoRE) would increase portability, decrease size, and facilitate easier measurements. By validating this proof of principle design, we would show that RZx-based sensors could be implemented in practical point-of-care testing.
Chapter 4

Microfluidic-Integrated RZx/LIG Sensors for Real-time Monitoring of Bacterial Viability

In this chapter, we detail a proof-of-principle microfluidics device with self-contained pseudo-reference electrodes for bacterial monitoring using RZx/LIG. The point of creating such a device is two-fold. Firstly, the compact, on-chip design would provide practical benefits to the measurement system. By utilizing microfluidics, the necessary sample volume is decreased, and setup is made easier (simply placing a drop on the inlet). The microfluidics also make the design more compact for increased portability. The addition of an on-chip pseudoRE further reduces the footprint of the device, increases the ease of conducting a measurement, and (synergistically with the microfluidics) allows for the possibility of a miniature/flexible device. The second point of investigating this design is to demonstrate that RZx-based sensors can be easily integrated into a new architecture without a loss of sensing performance. Versatility of the RZx sensing platform is necessary for it to be practical in real settings, such as in a smart catheter for detecting infection, smart culture microplate for monitoring growth, or smart packaging to sense spoilage (such as inside a milk carton).

The first part of the chapter is devoted to outlining the processes for fabricating and testing the microfluidic device. Next, we introduce the design progression of the system. We identify the effects of channel width, plasma treatment, and epoxy coatings on microfluidic flow. Specifically, a sufficiently large channel width (400 μm), air plasma treatment prior to assembly, and epoxy coatings on select areas of the LIG are all necessary for optimized functionality. Afterwards, we validate the final design by using it to differentiate between live/dead *E. coli* K12 cultures of $1 \times 10^5$ CFU/mL initial concentration in 210 minutes. Finally, we present a summary and conclusion of the chapter.
Materials and Methods

Materials and Equipment

Polyimide sheets for the creation of laser-induced graphene (LIG) were procured from DuPont de Nemours, Inc. (Kapton® GS, 500 mil). Two-part silver epoxy was purchased from MG Chemicals (#8329). Double-sided hydrophilic film (#9960) and double-sided medical tape (#9965) were procured from 3M Company. Brain-Heart Infusion powder (#53286-500G) and resazurin sodium salt (#R7017-5G) were purchased from Sigma Aldrich. Concentrated germicidal bleach was purchased from the Clorox Company. Ecoflex® silicone rubber was purchased from SmoothOn, Inc. The CO₂ laser writing system was purchased from Universal Laser Systems (VSL2.30 Desktop, 25 W). The air plasma system was purchased from Harrick Plasma (PDC-001). The platinum wire counter electrode (CE, # MW-4130), Ag/AgCl reference electrode (RE, # MF-2052), and PalmSens4 potentiostat used in electrochemical experiments were purchased from BASi Inc. E. coli K-12 was generously provided by J. Kovac’s lab at Penn State University.

Fabrication of the sensor-embedded microfluidic device

Firstly, a Kapton sheet 1.5” x 2” was affixed to a glass side using double-sided scotch tape. Then the surface of the Kapton was cleaned with a chemical wipe dampened with isopropanol. Next, a 1.5” x 1.5” sheet of double-sided medical tape and a 1.5” x 1.5” sheet of hydrophilic film were each affixed to a glass slide. Patterns were laser-cut into the films according to the parameters in Figure 4.1. The parameters for the laser were as presented in Table 4.1.

After the patterns were cut into the films, silver epoxy was applied to the tip of LIG RE and each of the four contact pads. Additionally, the area between the LIG electrodes and their contact pads was covered with Ecoflex® silicon rubber to mitigate liquid diffusion towards the contact pads. Simultaneously, a PDMS well was attached over the inlet of the hydrophilic film. The electrodes and hydrophilic film (removed from glass slide) were placed into an air oven at 65°C for four hours to cure. After taking the electrodes out of the oven, 1:10 bleach solution was
dropped onto the RE silver epoxy for 2 minutes before being washed away with DI water. The silver epoxy was thus transformed into an Ag/AgCl pseudoreference electrode (PseudoRE). Next, RZx was electrodeposited onto the LIG WE according to the methods presented into Chapter 2. Subsequently, the electrodes, double-sided medical tape, and hydrophilic film were placed into

<table>
<thead>
<tr>
<th>Material</th>
<th>Horizontal Lines</th>
<th>Vertical Lines</th>
<th>Outline</th>
</tr>
</thead>
<tbody>
<tr>
<td>LIG</td>
<td>50% Power</td>
<td>25% Power</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>90% Speed</td>
<td>75% Speed</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000 PPI</td>
<td>1000 PPI</td>
<td></td>
</tr>
<tr>
<td>Medical Tape</td>
<td>N/A</td>
<td>N/A</td>
<td>50% Power</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>75% Speed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000 PPI</td>
</tr>
<tr>
<td>Hydrophilic Film</td>
<td>N/A</td>
<td>N/A</td>
<td>50% Power</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>60% Speed</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1000 PPI</td>
</tr>
</tbody>
</table>

Table 4.1. *Laser parameters for the microfluidics materials*. LIG underwent two passes of the laser (first horizontal lines and then vertical lines) to improve the uniformity and conductivity of the material.

![Figure 4.1. Microfluidics dimensions in millimeters.](image)


Figure 4.1. *Microfluidics dimensions in millimeters*. Purple = LIG electrodes. Blue = double-sided medical tape. Red = hydrophilic film.
the air plasma system. The system was run at low power and 30 units flow for 60 seconds. The samples were removed. Then, the liners on the double-sided medical tape were removed and it was placed over the electrodes such that the sensing areas were left uncovered. Next, the hydrophilic film and PDMS well were adhered to the exposed double-sided medical tape. A 3D mockup of the completed device is shown in Figure 4.2a. Figure 4.2b shows colored BHI (dark violet) spontaneously being transported through a partially completed device. And Figure 4.2c shows a complete device with *E. coli* culture inside.

![Figure 4.2](image)

**Figure 4.2. Microfluidic device design.** a) 3D schematic of the microfluidic device, produced in AutoCAD. b) Colored BHI (violet) spontaneously being transported through a partially completed device. c) Completed device with *E. coli* culture inside.
Monitoring bacterial growth in real time

Two completed microfluidic devices were placed into a static incubator set to 37°C. The connector cables to the PalmSens4 potentiostat were passed through a small hole in the back of the incubator. The alligator clips at the end were connect to the WE, RE, and CE of whichever device was being probed. The devices were then left alone while the cultures were being prepared.

The optical density at 600 nm (OD<sub>600</sub>) of a fresh overnight culture of <i>E. coli</i> K-12 in BHI (~ 10<sup>9</sup> CFU/mL) was measured with the Eppendorf BioPhotometer D30. Using the conversion OD<sub>600</sub> of 1.0 = 8 × 10<sup>8</sup> CFU/mL, the culture was diluted to a value 1 × 10<sup>7</sup> CFU/mL in BHI. In two separate vials, the culture was then diluted 1:100 in BHI to reach a final concentration of 1 × 10<sup>5</sup> CFU/mL. Ampicillin (Sigma Aldrich, #A9518) was added to one culture at a concentration of 32 µg/mL; this marked time = 0 minutes. Of note, there was no incubation period after the dilution of the cultures to 1 × 10<sup>5</sup> CFU/mL. Then 200 µL of control culture was dropped into the well of one device and 200 µL of ampicillin-treated culture was dropped into the well of the other. At 30 minute intervals, the <i>E<sub>ox</sub></i> signal was measured (n = 6). The first time point to be measured was at 30 minutes (<i>t</i><sub>30</sub>). From the data, a \( \delta E_{ox}(t_i) = E_{ox}(t_i) - E_{ox}(t_{30}) \) signal was generated. Separately, OD<sub>600</sub> measurements from a tube control culture were taken as reference for the expected growth curve.

**Design of the sensor-embedded microfluidic device**

The microfluidics device was conceptualized based on a paper by Ko et al. (Ko et al., 2019). In their paper, they created nanostructured artificial enzymes (nanozymes) made of Au/Pt bimetallic nanoparticles, graphene oxide, and agarose beads (Au@PtNP/GO). The Au@PtNP/GO material is able to catalyze 3,3′,5,5′-tetramethylbenzidine (TMB) in the presence of H<sub>2</sub>O<sub>2</sub>. Using a colorimetric sensing approach based on the TMB redox state, the authors could quantify H<sub>2</sub>O<sub>2</sub> concentration. The authors further implemented the Au@PtNP/GO in a microfluidic device for PoC H<sub>2</sub>O<sub>2</sub> sensing. A schematic of the setup is shown in Figure 4.3. We have utilized their design and modified it to fit our bacterial monitoring application.
The first iteration (Iteration 1) of our bacterial monitoring microfluidic system utilized a 4-layer design of tape/hydrophilic-film/tape/hydrophilic-film. Figure 4.4 shows a 3D representation of the device. In this case, the channel width was nominally only 100 μm to minimize the liquid volume contained within. As opposed to the final iteration (Iteration 3), none of the materials were plasma treated and the design lacked any PDMS well attachment, EcoFlex covering, or silver epoxy on the pads. Attempts to spontaneously flow liquid through the device were unsuccessful, with almost no penetration of BHI into the channel. Liquid could be forced through the channel by placing a PDMS well around the droplet, covering it with parafilm, and then applying periodic pressure to the top of the well with a finger. This did leave many air bubbles in the device. Additionally, due to the hydrophilicity and permeability of the LIG, liquid tended to reach the electrode pads before it ever reached the outlet. Cyclic voltammetry signals from this setup were highly erratic, with the current abruptly jumping from zero at some arbitrary voltage; this could be temporarily fixed by pressing the copper tape contacts into the LIG, signifying a contact issue.

Figure 4.3. Schematic representation of the inspiration for our design. The schematic shows a microfluidic H₂O₂ sensor based on Au@PtNP/GO microbeads. Reproduced from (Ko et al., 2019) with permission from Elsevier.
To address the problem of minimal liquid penetration into the device, a simple set of experiments were conducted. In total, two stacks of materials were prepared. The first consisted of a hydrophobic-film/tape/hydrophobic-film structure. The second consisted of the same structure, except all the materials were plasma-treated prior to assembly. In each case, the double-sided tape had 4 channels cut into it (nominal width = 0 μm, 100 μm, 300 μm, 700 μm). Estimating a cut width of 100 μm, this translates to final channel widths of 100 μm, 200 μm, 400 μm, and 800 μm. On one side of the stack, the hydrophilic film had holes for inlet and outlet. The AutoCAD pattern is presented in Figure 4.5a. Dark violet BHI (colored by resazurin) was dropped onto the inlet of each channel. The amount of time it took for the liquid to reach the outlet is presented in Table 4.2. An image of the samples after experiment completion is presented in Figure 4.5b,c. Notice that liquid tends to diffuse between layer when it encounters an edge (especially for the plasma-treated samples); this is likely a result of preferential delamination at those spots. Extra caution should be taken to strongly press layers together at internal and external edges. From the experiments, it is very clear that wider channels and plasma treatment increase the speed of diffusion greatly.
Based on the preceding findings, the second iteration of the design (Iteration 2) included the following corrections: silver epoxy on the pads to improve contact to the copper tape, Ecoflex over the unexposed LIG, a nominally $400 \mu m$ channel, and plasma treatment of the sample. When flowing colored BHI through the device, the results were more promising. The liquid very quickly reached the first step between the two hydrophilic films, then it was stopped briefly before it flowed into the chamber, but never reached past the second step between the two hydrophilic films. An optical image of the issue is presented in Figure 4.6. The difficulties of liquid flow at the steps makes sense because the double-sided tape is rather hydrophobic. Therefore, the liquid encounters an uninterrupted strip of hydrophobic material right at these locations. To avoid this and simplify the design, the number of layers of the device was reduced.

<table>
<thead>
<tr>
<th>Nominal Channel Width [$\mu m$]</th>
<th>No Treatment</th>
<th>Plasma-Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Approx Time</td>
<td>$\infty$</td>
<td>15 min, 2 min, 10 sec</td>
</tr>
</tbody>
</table>

Table 4.2. Time required for liquid to travel from channel inlet to channel outlet. The experiment utilized a hydrophilic-film/tape/hydrophilic-film stack.

Figure 4.5. Flow experiments on channel width and plasma treatment. a) AutoCAD design for the experiments. Units are in millimeters. b) Optical image of an untreated layer stack after flowing colored BHI flowing through. c) Optical image of a plasma-treated layer stack after flowing colored BHI flowing through.
The third and final iteration of the device was presented earlier in Figure 4.2a. Using a simple polyimide/tape/hydrophilic-film stack ensures that the liquid is always in contact with a hydrophilic surface from inlet to outlet. As such, liquid flow is fast and unimpeded.

![Figure 4.6. Second iteration of device design.](image)
The colored BHI is unable to reach the outlet because the steps between the hydrophilic films.

**Results for Real-time Monitoring of Bacterial Viability**

Two different microfluidics devices (made under the same conditions) were used to probe the growth of two *E. coli* cultures from an initial concentration of $1 \times 10^5$ CFU/mL. One culture was treated with 32 μg/mL ampicillin (above the MIC) and the other served as a control (no antibiotic). The CV curves obtained during the experiment are significantly different than their non-microfluidic counterparts (off-chip CE and RE); the existence of similarly sized oxidation and reduction peaks signifies greater reversibility. CV curves for the control culture at 30 min and 270 min are presented in Figure 4.7a as representative voltammograms. In this case, either the oxidation or reduction peak could (in theory) be used to monitor the growth of the cultures. Further analysis shows that the change in the reduction peak voltage is significantly larger than for the oxidation peak; this would seem to suggest that it is the better signal to use. However, the reduction peak shift for the inhibited culture (32 μg/mL ampicillin) is also much greater. Therefore, using it as the signal falsely gives the sense of a large change in the composition of the medium. Thus, we stuck with using the oxidation peak shift as the signal.
Figure 4.7b presents the $\delta E_{ox}(t_i) = E_{ox}(t_i) - E_{ox}(t_{30})$ signals for both culture conditions. Except at the 60 minute time point, the curves are essentially overlapping in both cases up until 210 minutes. Then at 210 minutes, the signal for the control culture rises at increasingly rapid rates; by 270 minutes, the difference between the two cultures (inhibited vs. non-inhibited) is dramatic. Still, differentiation between the inhibited vs. non-inhibited cultures can be determined within 210 minutes even with a starting concentration of $1 \times 10^5$ CFU/mL. Reference OD$_{600}$ measurements from a tube culture at starting density of $1 \times 10^5$ CFU/mL can be seen in appendix Figure A.0.8. These proof-of-principle results show that this compact chip design for monitoring of bacterial cultures is promising for rapid PoC testing.

![Microfluidics CV Curves](image1)

**Figure 4.7. Monitoring inhibited and non-inhibited cultures with the microfluidic device.** a) The raw CV curves for the control culture are presented at 30 min and 270 min. The oxidation peak potential is marked by the dashed lines. b) $\delta E_{ox}$ signal for the inhibited (32 $\mu$g/mL ampicillin) and non-inhibited cultures. Differentiation is possible in as little as 210 minutes.

**Chapter Summary and Conclusion**

In this chapter, we fabricated and tested a microfluidics-integrated RZx/LIG sensor with on chip pseudo-RE. The design provides a compact and easy to utilize device. We detail the process for fabricating the sensor and we additionally outline how the final design came about. We show that a sufficiently wide channel, plasma treatment, and tactical application of epoxy
can optimize the fluid flow through the device and avoid issues with leakage/bubbles. We validated the system by using it to monitor cultures of *E. coli* K12 at initial concentration of $1 \times 10^5$ CFU/mL (at least two order of magnitude lower than in Chapter 3). Two conditions were explored in parallel: untreated (alive) and 32 µg/mL ampicillin (dead). The devices were able to differentiate between the two conditions within 210 minutes. The advantages of the microfluidics design are in its ease of setup, compactness, portability, low sample volume, and its flexibility.

These proof-of-principle results demonstrate that RZx can be utilized in robust and practical point-of-use applications, especially in resource limited settings. We also believe that compact RZx-based sensors may find use in technologies such as smart catheters, smart wound dressings, smart culture microplates, and smart packaging.
Chapter 5

Summary and Conclusion

In this thesis, we developed a simple, one-step process for synthesis of a novel, highly-stable redox-active organic crystalline layer (RZx) and demonstrated its application to construct reagent-free electrochemical sensors for measuring bacterial metabolic activity in situ. We investigated the effect of substrate material and chemical composition of the electrodeposition electrolyte on formation and morphology of RZx crystals. Specifically, compared to metallic layers, carbon-based electrodes, such as pyrolytic graphite sheets (PGS), laser induced graphene (LIG), and screen-printed carbon electrodes, enabled the highest density of crystal formation and uniform surface coverage. Moreover, our studies confirm that the amino acid phenylalanine plays a key role in formation of stable RZx. Building on RZx’s innate ability to sense the pH change caused by cell metabolism and possibly by directly oxidizing bacterial metabolites, we demonstrated electrochemical sensing of bacterial viability for applications in antibiotic susceptibility testing (AST) and monitoring cultures. As proof-of-principle, RZx-based sensors were tested on *E. coli* K-12 treated with ampicillin and kanamycin. The devices enabled identification of the minimum inhibitory concentration of antibiotics in 60 minutes. We also tested the sensors in complex media - milk and human whole blood - to demonstrate their potential for analysis of raw samples. We further investigated synthesis of RZx on laser-induced graphene electrodes to demonstrate its versatility as a low-cost, scalable sensing material for electrochemical analysis of bacterial viability. This platform may advance the technology for microbiology research beyond AST; for example, as portable probes for reagent-free detection of microbial contamination in food and water samples, studying the effect of host immune response on pathogens’ metabolic activity, drug discovery, microbial fuel cells, etc. As an extension and proof-of-concept, we also showed than a compact and portable microfluidics chip design with RZx/LIG was capable of rapidly monitoring bacterial viability/growth of culture with a low initial cell concentration of $1 \times 10^5$ CFU/mL; this is consistent with the recommended cell concentration for comparison with gold standard methods according to CLSI guidelines (Clinical and Laboratory Standards Institute, 2012).
Appendix

Impact of Nafion on the Sensor Peak Height at $E_{ox}$

Although oxidation peak height is not directly used in the sensor data analysis, it should be sufficiently large compared to the non-faradaic current so that $E_{ox}$ can be easily and accurately identified. We found that addition of a Nafion coating leads to increase of the peak height. As shown in Figure A.0.1 for *E. coli* K12 control cultures at t = 60 min, the extracted height (PSTrace 5.5) is almost 2.2 times larger for the Nafion-coated sensor. In addition to easier signal extraction, the enhanced peak height also prolongs the sensor lifetime (i.e. ability to obtain accurate information).

![Figure A.0.1. Impact of Nafion coating. Peak height and shape comparison between Nafion/RZx/PGS and RZx/PGS. The CV plots are taken from *E. coli* K12 control cultures at t = 60 minutes. The increase of peak height in the case of Nafion/RZx/PGS makes extracting $E_{ox}$ easier and enables the sensor to last for longer.](image)

The apparent increase in peak height for the Nafion-coated sensor can be associated to its proton-exchange properties. According to the Randles-Sevcik equation and its counterpart for irreversible systems, $i_p \propto C_j^* D_j^{1/2}$. Here, $i_p$ is the faradaic portion of peak current, $C_j^*$ is the bulk concentration of species j, and $D_j$ is the diffusion coefficient of j (other variables are omitted because they would not change between the experiments in this work) (Bard and Faulkner, 2001; Elgrishi et al., 2017). The bulk diffusion coefficient of protons, $D_p$, in Nafion is expected to be around $0.5 \times 10^{-5} cm^2/s$ (Ressam et al., 2017; Sel et al., 2013). In contrast, $D_p$ in aqueous solution is expected to be around $9.3 \times 10^{-5} cm^2/s$ (Lee and Rasaiah, 2011). Using the extreme example of a fully protonated Nafion membrane with $\lambda = [H_2O]/[sulfate \ unit] = 22$, the bulk
proton concentration $C_p^*$ in the polymer would be 0.54 M (Spry and Fayer, 2009). Comparing to the $C_p^*$ of BHI broth ($4 \times 10^{-8}$ M), it is clear that the bulk proton concentration in the Nafion membrane should be significantly larger. Thus, we infer that it is the increased bulk concentration of proton in the membrane that is responsible for increased peak height for Nafion/RZx/PGS electrodes vs. RZx/PGS electrode.

**Stabilization of Nafion/RZx/PGS electrodes in Amino Acid-Containing Solution**

Stabilization of the Nafion/RZx/PGS electrode in a medium containing amino acids (such as BHI culture medium) is critical to rapid and accurate AST. Additionally, without this step, the RZx material underneath the Nafion can be rapidly stripped from the surface in solutions with low organic content (like PBS) under applied voltages. The material leaching without stabilization can be observed as staining the solution a deep pink, shown in Figure A.0.2. In contrast, we observed that stabilization of the RZx/PGS electrode does not need to be performed specifically in organic solution containing amino acids (such as BHI) to yield stable RZx. This peculiar disparity may be due to a Nafion-RZx interface that promotes dissolution of the crystals. We believe that electro-polymerization of amino acids creates a passivation layer and stops the dissolution of the crystals (Alhedabi et al., 2017).

![Figure A.0.2. Leakage in a non-stabilized Nafion/RZx/PGS electrode. a) Prior to any voltammetric testing, there is not observable leakage of material. b) After 85 seconds of CV measurement, the solution has been stained a deep pink around the electrode. This is from dissolution/leakage of RZx material. However, if the Nafion/RZx/PGS is properly stabilized in an amino acid-containing organic solution (here, BHI), the leakage is not observed.](image-url)
Characterization Of Crystals Derived From Histidine And Resazurin Solution

In order to illustrate the proton selectivity of the Nafion/RZx/PGS devices, it is helpful to view the data in terms of $E_{ox}$ vs pH (in addition to $\Delta E_{ox}$ vs pH). This alternative perspective is presented in Figure A.0.4. On the left, Figure A.0.4a shows the $E_{ox}$ vs pH curves for a particular experiment with buffer control and solutions spiked with 10 mM NaCl, KCl, and MgCl2. Figure A.0.4b shows the $E_{ox}$ vs pH curves for another experiment with buffer control and solution spiked with 10 mM LiCl. And finally, Figure A.0.4b shows the $E_{ox}$ vs pH curves for a third experiment with buffer control and solution spiked with 10 mM CaCl2. In each experiment, the shift of the curves in response to salt spiking is negligible when viewed over the pH range.

Detailed Data on RZx Ion Selectivity

Figure A.0.3. Characterization of crystals derived from a solution of histidine and resazurin. a) Optical image of the crystals obtained during the electrodeposition process. The shape is more needle-like than RZx and the crystals do not have the same metallic luster. Inset: the molecular structure of resazurin and histidine. b) DPV voltammograms comparing electrodeposition in BHI + resazurin (BHI) and histidine + resazurin (F). The histidine curves only appear to have one prominent peak, as opposed to the two observed with the BHI solution. c) CV voltammograms comparing electrochemical response of RZx (BHI) and phenylalanine-derived crystals (F) in BHI. The histidine-derived crystals yield two overlapping oxidation peaks (and two overlapping reduction peaks), which suggests that their redox behavior is different from RZx.
Figure A.0.4. Ion selectivity presented as $E_{ox}$ vs pH. In all cases, spiking the solution with the salts does not induce a significant shift compared to the control buffer curves. a) Experiment 1: $E_{ox}$ vs pH for the buffer control and solutions spiked with 10 mM NaCl, KCl, and MgCl$_2$. b) Experiment 2: $E_{ox}$ vs pH for the buffer control and solution spiked with 10 mM LiCl. c) Experiment 3: $E_{ox}$ vs pH for the buffer control and solution spiked with 10 mM CaCl$_2$. 
Optical Density Data for E. coli K-12 Experiments

Testing Nafion/RZx/PGS Electrodes with a Drug-Resistant E. coli Strain

As a negative control, we monitored the signal obtained from two E. coli strains exposed to ampicillin. One strain was the ampicillin-susceptible E. coli K-12 (denoted K-12) and the other was ampicillin-resistant E. coli (denoted RES). The MIC value for E. coli K-12 was $\rho_{\text{Antb}} = 8 \mu g/mL$. The data is shown in Figure A.0.6 for supernatants of these samples. While the K-12 strain demonstrated a large decrease in signal at its MIC value of 8 $\mu g/mL$, the RES strain...
presented almost no decrease in signal at the same concentration, showing similar $\Delta E_{\text{ox}}$ as its control (no antibiotic) experiment.

**Confirming the Role of RZx for Redox Sensing**

In order to validate that the RZx is solely responsible for the observed redox peaks during AST testing, we tested Nafion/PGS with *E. coli* cultures. As expected, the cyclic voltammogram was completely non-Faradaic, with no differentiability between cultures. Figure A.0.7 plots the CV curves for the first scan of *E. coli* cultures with $\rho_{\text{AMP}} = 0 \mu g/mL$ at time $t = 0, 90, 150$ minutes.

![Figure A.0.7](image)

**Figure A.0.7** Nafion/PGS electrode cannot detect bacterial metabolism. In order to confirm that the RZx is the redox-active component of the system, *E. coli* culture was probed with a Nafion/PGS device. As expected, no redox activity was observed. The plot shows first-scan CV curves for *E. coli* cultures ($\rho_{\text{AMP}} = 0 \mu g/mL$) at time $t = 0, 90, 150$ minutes. No meaningful differentiation is possible, no peaks were observed, and the overall current was negligible. These results confirm the critical redox role played by RZx.

**OD Data for *E. coli* K12 at Starting Concentration of $1 \times 10^5$ CFU/mL**

To complement the microfluidics validation experiments, OD$_{600}$ data was recorded from a culture tube containing BHI spiked with *E. coli* K12 at initial concentration of $1 \times 10^5$ CFU/mL. Data collection started as soon as the target dilution was achieved, meaning that Lag phase needs to be taken into account. The culture tube was incubated at 37°C and 210 rpm, with measurements occurring at 30-minute intervals. The OD$_{600}$ values are shown in Figure A.0.8. A measurable and reliable signal was obtainable only after 180 minutes incubation.
Figure A.0.8. OD600 data collected from an E. coli culture at an initial concentration $1 \times 10^5$ CFU/mL. A reliable and measurable signal was obtained only after 180 under the culture conditions specified.
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