Detection of drug influx and efflux into/from *Pseudomonas* bacteria by electrochemistry

By

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ABSTRACT

Antimicrobial-resistant bacteria are emerging rapidly and causing antibiotics to become ineffective to treat infectious diseases which pose a threat to global public health. Multidrug-resistant bacteria are spreading rapidly and threaten to make it impossible to treat infections caused by these bacteria. Bacteria possess different types of mechanisms of antibiotic resistance. It is reported that modifications of membrane permeability, such as decreasing drug uptake (influx) and overexpression of efflux pumps that expel antibiotics from periplasm to the outside of the cell, are key contributors to the emergence of multidrug-resistant bacteria. Therefore, the development of rapid, sensitive detection techniques for antibiotic resistance is a significant focus in clinical diagnosis, disease control, environmental monitoring, and food safety. Electrochemical methods are advantageous as analytical tools due to their simplicity, high selectivity, sensitivity, rapid analysis and cost effectiveness. This thesis focuses on the development of robust electrochemical methods to quantify influx and efflux from bacteria. It covers the electrochemical characterization of common antibiotics and antibiotic hybrid molecules. This thesis demonstrates potential applications of drug influx and efflux quantification in bacterial cells using cyclic voltammetry and differential pulse voltammetry.

Table of Contents

List of Figures

List of Tables

Table of Acronyms

MDR – Multi Drug Resistance

CV – Cyclic Voltammetry

GCE – Glassy Carbon Electrode

AMX - Amoxicillin

CIP – Ciprofloxacin

TOB – Tobramycin

GCV – Ganciclovir

AZM – Azithromycin

DPV – Differential Pulse Voltammetry

PBS – Phosphate buffer saline

SECM- Scanning Electrochemical Microscope

MWCNT -multi walled carbon nanotube

CPE – Carbon paste electrode

GO – Graphene Oxide

GST – Glutathione

GMB – Gentamycin

BDD – Boron Doped Diamond

BQ - Benzoquinone

HQ - Hydroquinone

MIP – Molecular Imprinted Polymer

NIP- Non-molecularly imprinted polymer

AuNP- Gold nanoparticles

LOD – Limit of Detection

LOQ – Limit of Quantification

SPCE - Screen Printed Carbon Electrode

List of Symbols

M	Molar
E	Potential
I	Current
V	Voltage
mv	Milli volt
ms	Milli second
R	Reduced
O	Oxidised
n	Number of Electrons
A	Area of the electrode
F	Faraday Constant
R	Gas Constant
R^2	Correlation-coefficient
µ	Micron
υ	Scan Rate
T	Temperature

CHAPTER 1: INTRODUCTION

1.1. Theme of the Thesis

Infectious diseases have been a severe issue in public health throughout the ages. They are some of the main causes of morbidity and mortality throughout the world.[1] However, the incident which brings the light of hope onto the medical sector is the discovery of antibiotics by Alexander Fleming.[2] The discovery of antibiotics passed its infancy period in combatting infectious diseases during the 1930s.[3] Then the golden age hit from 1940 to 1960.[4] People used to think infectious diseases were no longer a threat.[1] But the phenomenon of drug resistance resides in nature, which gives a chance to all species to be fit for survival. Bacteria, the causal agents of infectious diseases, have adapted to cope with this great discovery of antibiotics and finally evolve a new arsenal to survive, which is resistance power to antibiotics.[5] Antibiotics, a medical miracle, face different challenges with this new evolutionary mechanism. Bacteria develop immunity against antibiotics, which causes the antibiotics to become ineffective against those bacteria. This phenomenon is called antibiotic resistance and has become a severe worldwide problem that poses a threat to global public health.[6,7] According to the World Health Organization (WHO), each year, multi-drug resistant tuberculosis causes 230,000 people's deaths worldwide.[8] According to the Public Health Agency of Canada, 18,000 hospitalized patients acquire antimicrobial-resistant infections each year. The Public Health Agency of Canada reported that antimicrobial resistance is expected to kill nearly 400,000 Canadians and cost $400 billion in gross domestic product (GDP) by 2050.[9] In 2015, the WHO launched a global action plan on antimicrobial resistance.[10] The present situation requires an urgent development of simple, rapid, and inexpensive methods for the detection of antibiotic resistance. There is currently no sensor developed for the detection of antibiotic resistance. Electrochemical sensors are a promising approach to detect antibiotic resistance, as they have drawn immense attention as powerful analytical tools in different fields, such as environmental monitoring,[11] biotechnology,[12] and industrial process control[13] because of their high sensitivity, simple instrumentation, fast analysis time, and portability.

1.1.2. Detection of Antibiotic Resistance

The standard method to detect antibiotic resistance is the antibiotic susceptibility test (AST).[14–17] AST is a phenotypic test, which depends on the growth of bacteria in the presence of antibiotics which varies from days to weeks in bacteria, such as in *Mycobacterium tuberculosis*.[18] Other diagnostic methods based on nucleic acid technologies for pathogen identification include PCR, immunological techniques such as ELISA, microarray detection, and fluorescence-based assays.[14–18] These methods are time consuming, not sensitive enough to detect bacteria at concentrations commonly found in bloodstream infections, and require sophisticated equipment and skilled expertise. Physicians are forced to treat infectious diseases empirically, because of the delay in the detection of antibiotic resistance, which leads to inappropriate antibiotic prescription as well as the further spread of resistance.[14,18] It is reported that the delay of administering the correct antibiotics in infections by an hour lowers the chance of survival of patients by 10%.[14] Electrochemical studies can overcome these disadvantages by offering high sensitivity and selectivity, rapid detection time, low-cost, simple instruction methods, and portability, making them an appealing choice to address this severe issue.[11]

1.1.3. Epidemiology of Antibiotic Resistance

Antimicrobial-resistant bacteria are emerging rapidly worldwide and causing antibiotics to become ineffective in treating infectious diseases. Soon after the successful use of penicillin to treat bacterial infections, penicillin-resistant bacteria were identified in 1940 (Table 1).[6] In 1962, methicillin resistant *Staphylococcus aureus* (MRSA) was first spotted in the United Kingdom and in 1968 in the United States.[10] According to a report from the Centers for Disease Control and Prevention (CDC), approximately two million illnesses and 23,000 deaths are caused by antibiotic resistance each year in the United States alone.[19] Several bacterial strains have been classified as multidrug-resistant by the CDC because of gaining resistance to at least one antibiotic in ≥ 3 chemically dissimilar antibiotic classes.[20,21] Only a few antibiotics were still effective in treating infections caused by the "ESKAPE" pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter* species) in 2010.[22] 30% of prescribed antibiotics in the United States are not required, meaning they are unnecessary, according to the CDC.[10] In 2019, MRSA was responsible for bloodstream infections found in 25 countries, and in

49 countries, bloodstream infections occurred due to *E. coli*.[10] A report in Britain estimates that antimicrobial resistance is expected to kill 10 million people per year and cost up to $100 trillion to the global economy by 2050.[1] Each year, the United States could encounter an additional 120,000 surgery site infections and post-chemotherapy infections due to a 30% reduction in the efficacy of antibiotic prophylaxis.[23] According to The European Center for Disease Prevention and Control, antibiotic-resistant bacteria cause approximate 25,000 deaths and cost €1.5 billion to the health sector per year in Europe.[1,24]

Table 1.1: Timeline of antibiotic development, resistance, and global response[10]

1928	Alexander Fleming discovers penicillin
1930s	Prontosil, a sulfonamide, becomes first commercially available antibacterial
1940	Penicillin-R *Staphylococcus* identified
1943	Florey and Chain efficiently purify and scale up production of penicillin
1940–1962	Golden age of antibiotic discovery and production
1950	Tetracycline introduced
1953	Erythromycin introduced
1955	Penicillin use is restricted to prescription only due to widespread misuse
1959	Tetracycline-R *Shigella* identified
1960	Methicillin introduced
1962	Methicillin-R *Staphylococcus* identified
1962	Nalidixic acid introduced (predecessor to fluoroquinolones)
1965	Penicillin-R pneumococcus identified
1967	Gentamicin introduced
1968	Erythromycin-R *Streptococcus* identified
1972	Vancomycin introduced
1979	Gentamicin-R *Enterococcus* identified
1985	Imipenem and ceftazidime introduced
1987	Ceftazidime-R *Enterobacteriaceae* identified
1988	Vancomycin-R *Enterococcus* identified
1996	Levofloxacin introduced

1996	Levofloxacin-R pneumococcus identified
1998	Imipenem-R *Enterobacteriaceae* identified
2000	Linezolid introduced
2000	XDR tuberculosis identified
2001	Linezolid-R *Staphylococcus* identified
2001	WHO launches Global Strategy for Containment of Antimicrobial Resistance
2002	Vancomycin-R *Staphylococcus* identified
2003	Daptomycin introduced
2004/5	PDR-*Acinetobacter* and *Pseudomonas* identified
2008	NDM-1, the New Delhi metallo-carbapenemase, observed in *Enterobacteriaceae*
2009	Ceftriaxone-R *Neisseria gonorrhoeae* and PDR-*Enterobacteriaceae* identified
2010	Ceftaroline introduced
2011	Ceftaroline-R *Staphylococcus* identified
2013	CDC Report on Antibiotic Resistance Threats in the United States
2014	WHO releases Antimicrobial Resistance: Global Report on Surveillance
2015	WHO releases Antibiotic Resistance: Multi-Country Public Awareness Survey
2015	WHO launches Global Action Plan on Antimicrobial Resistance

1.1.4. Factors Leading to the Rise of Antibiotic Resistance

Antibiotic resistance is a complex issue, where various factors are involved in its prevalence.

1.1.4.1. Misuse of Antibiotic in Clinical Practice

The use of antibiotics creates a selective pressure environment in the host receiving antibiotics, favouring the survival of resistant bacteria and eliminating susceptible bacteria. This selective pressure gives a competitive advantage to resistant bacteria, which reproduce in the host body to cause severe infections. Excessive clinical use of antibiotics, especially in developing countries, leads to the rise of antibiotic resistance. Unskilled practitioners in rural areas in developing countries are not aware of the consequences of this global issue and frequently prescribe antibiotics without proper diagnosis. In most developing countries, people can purchase

antibiotics without a prescription. For instance, in rural Bangladesh, it is a very common scenario to purchase medicines from a local pharmacy without any prescription.[6,25]

1.1.4.2. Agricultural and Animal Use of Antibiotics

In the animal industry, the use of antibiotics is prevalent not only for disease treatment. For example, in the poultry industry, the use antibiotics is very common for the prevention of disease and growth promotion. These antibiotic residues finally end up in humans through the food chain and contribute to the development of antibiotic resistance. In the agricultural industry, waste runoff from land treated with chemical fertilizer, municipal wastewater, and industrial agricultural plant water containing drugs are also significant contributors to the development of antibiotic resistance (Figure 1.1). In the United States, the use of antibiotics in agriculture and aquaculture contributes to 80% of the total use of antibiotics.[10,6]

1.1.4.3. Poor Quality of Antibiotics

In addition to the misuse and failure of the therapeutic use of antibiotics, the poor quality of antibiotics is responsible for escalating antibiotic resistance. Antibiotics do not meet the proper quality compliances and regulations. For example, in Nigeria, low quality antibiotics such as ampicillin and tetracycline were found, which cause therapeutic failure. During the distribution of drugs in tropical countries, the storage conditions of drugs are not strictly controlled, which may cause antibiotics to degrade. In developing countries, sometimes drugs are sold on the market having lower concentrations of active substances than those stated on the label. These counterfeit drugs also lead to antibiotic resistance.[25]

1.1.4.4. COVID-19

As I am writing this thesis, the whole world is suffering because of a pandemic of coronavirus disease (COVID-19). COVID-19 was first observed in Wuhan city, China in December 2019. Soon after that, it spread all over the world. The best practice to prevent the spread of the virus is to stay at home and use proper sanitization. Thus, people are using hand sanitizer commonly containing 60% alcohol. A recent study reports the widespread use of antibacterial agents will escalate bacterial resistance. Moreover, hospitalized COVID-19 patients are prescribed antibiotics not to treat COVID-19, but to prevent additional infections by pathogens in the

hospital environment. This increases the spread of antibiotic resistance. This pandemic is affecting every industrial sector and devastating the global economy.[26]

1.1.4.5. Crowding and Unhygienic Conditions

The environment in tropical countries favours the growth and survival of more pathogenic bacteria. In developing countries, most people carry asymptomatic antibiotic resistance and spread it to others in crowded environments like public transport. Improper sewage disposal, unhygienic food practices, and crowded environments in urban areas help the spread of antibiotic-resistant bacteria.[25]

1.1.4.6. Inadequate Hospital Infection Control Practices

Hospital infection control practices are extremely important to prevent the spread of resistant organisms at the community level. The lack of proper infection control measures such as hand washing and the isolation of resistant infection patients contributes to the dissemination of resistant bacteria through the community.[34]

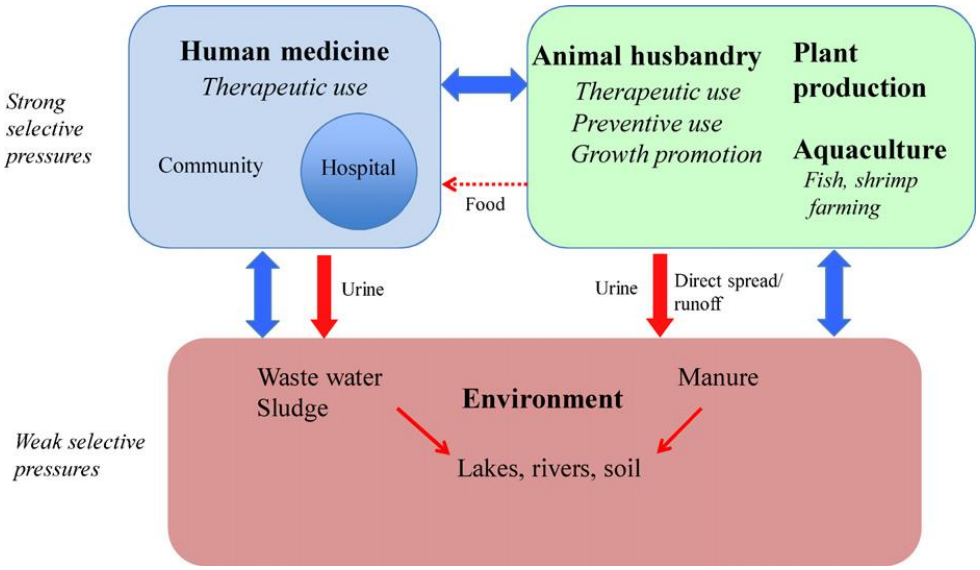

Figure 1.1: Schematic representation of factors affecting antibiotic resistance[29].

1.1.5. Mechanism of Antibiotic Resistance

The complete understanding of antibiotic resistance at the molecular level is crucial to combat this issue and develop a new methodology to detect antibiotic resistance. The mechanisms of antibiotic resistance vary among bacteria. There are different types of mechanisms bacteria gain to attain antibiotic resistance. Antibiotic resistance can be genetically inherent or adaptive. Inherent resistance, also called intrinsic resistance, is gained when bacteria transfer it to the next progeny.[27]

Acquired resistance mechanisms occur due to mutations or the transfer of genetic material from other bacteria, which transfer genetic material encoding resistance mechanisms. The selective pressure environment in the presence of an antibiotic favours bacteria eliciting bacterial adaption to antibiotics and developing an antibiotic resistance mechanism. Plasmids, mobile extrachromosomal circular double stranded DNA, carry the genes responsible for antimicrobial resistance. Transposons (jumping genes) may also contain antibacterial resistance genes and can move to different positions of the genome. Genetic transfer occurs via conjugation, transformation and transduction (Figure 1.2).[6,27,24]

Conjugation: Conjugation is one of the most common mechanisms of gaining resistance. This process takes place with the help of a plasmid transfer when bacterial cells are in direct contact. Plasmids are units of self-replicable, extrachromosomal circular double stranded (ds) DNA. This mechanism transfers resistance capability from one bacterium to others.[6,24,27]

Transformation: Transformation is another process that transfers resistant genetic material. This happens with the transfer of free DNA (naked DNA) found in the environment from dead bacteria close to the receiving bacteria.[6,24,27]

Transduction: The transduction process transfers bacterial DNA via bacteriophages, bacterial viruses which can replicate inside bacterial cells. The bacteriophage infects the bacterial cell and replicates more copies of viral particles. During viral assembly, bacterial DNA can incorporate into phage DNA and the bacterial cell dies and produces new bacteriophages containing bacterial DNA. These new bacteriophages infect new bacterial cells and transfer bacterial DNA.[6,24,27]

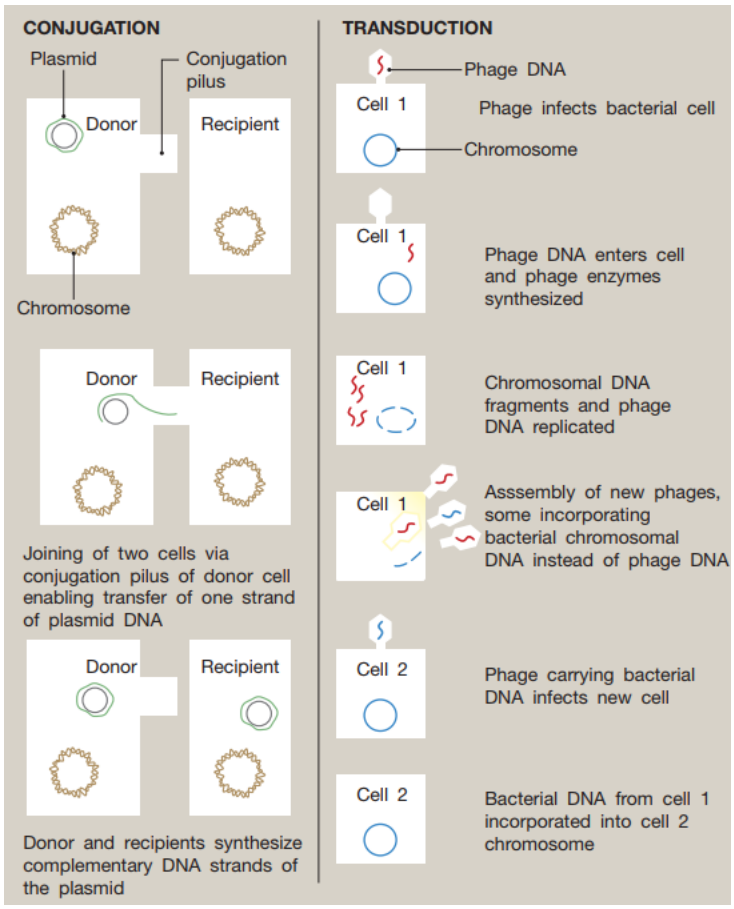

Figure 1.2: Bacterial conjugation and transduction[27]

There are different types of resistance mechanisms through which bacteria demonstrate their resistance capability to antibiotics.[28] They can be broadly categorized into the following four mechanisms (Figure 1.3).

1.1.5.1. Beta-Lactamases

β-Lactamases are enzymes that are capable of hydrolyzing antibiotics containing a β-lactam ring, which renders β-lactam antibiotics inactive. Resistant bacteria produce these enzymes in the periplasmic space and degrade antibiotics. The gene for β-lactamases can be present in plasmids or chromosomes. *Enterobacteriaceae* such as *E. coli*, *Klebsiella spp.*, and *Enterobacter spp.* produce a broad spectrum of β-lactamases, which make these bacteria resistant against penicillin and cephalosporin.[6,22,24,27,29,30]

1.1.5.2. Target Modification

Bacteria can also modify or alter the intracellular target of the drug by changing their binding protein conformation, causing a lack of binding for the drug and a lower antibacterial effect. For example, some penicillin-resistant bacteria change the conformation of penicillin-binding protein (PBP). Macrolide-resistant mycobacterium alters the ribosomal RNA through methylation, which hinders the binding of macrolides. One of the common mechanisms found in fluoroquinolone-resistant bacteria is the mutation of the bacterial DNA gyrase enzyme, which is the target of fluoroquinolone antibiotics.[6,30]

1.1.5.3. Porin Loss/Decreased permeability

The outer membrane of Gram-negative bacteria plays a crucial role in the cellular uptake of small molecules, including nutrients and other chemicals.[31,32,33] Most drugs to date have an intracellular target and must pass the outer membrane to get into the cell and render their effects. This process is mediated by outer membrane protein channels, also called porins, which help small molecules to penetrate the outer membrane and regulate drug permeability. Loss or mutation of these porins restricts the penetration of antibiotics and leads to antibiotic resistance.[22,30] Loss of porin channels in *Enterobacteriaceae* impairs the penetration of β-lactam antibiotics into the periplasmic space where they can access penicillin-binding protein, which causes resistance to β-lactams.[22,27] Many of the outer membrane permeability alterations provide resistance to antibiotics with the synergistic action of efflux pumps.[31]

The outer membrane also acts as a permeability barrier for intrinsic antibiotic resistance.

1.1.5.4. Efflux pumps

Efflux pumps are transport proteins present in both Gram positive and Gram- negative bacteria.[34,35] They serve the crucial role of expelling toxic substances from the cell to the external environment.[36] In Gram-negative bacteria, all efflux pump proteins are located in the inner membrane.[36] The active efflux pump is involved in the extrusion of antibiotics from their site of action to the external environment, which causes decreased susceptibility to the drugs. Overexpression of these efflux pumps is one the most common mechanisms found in the global emergence of multidrug-resistant Gram- negative bacteria.[22,37] Efflux pump-mediated resistance has been found, for example, in *E. coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus.*[37]

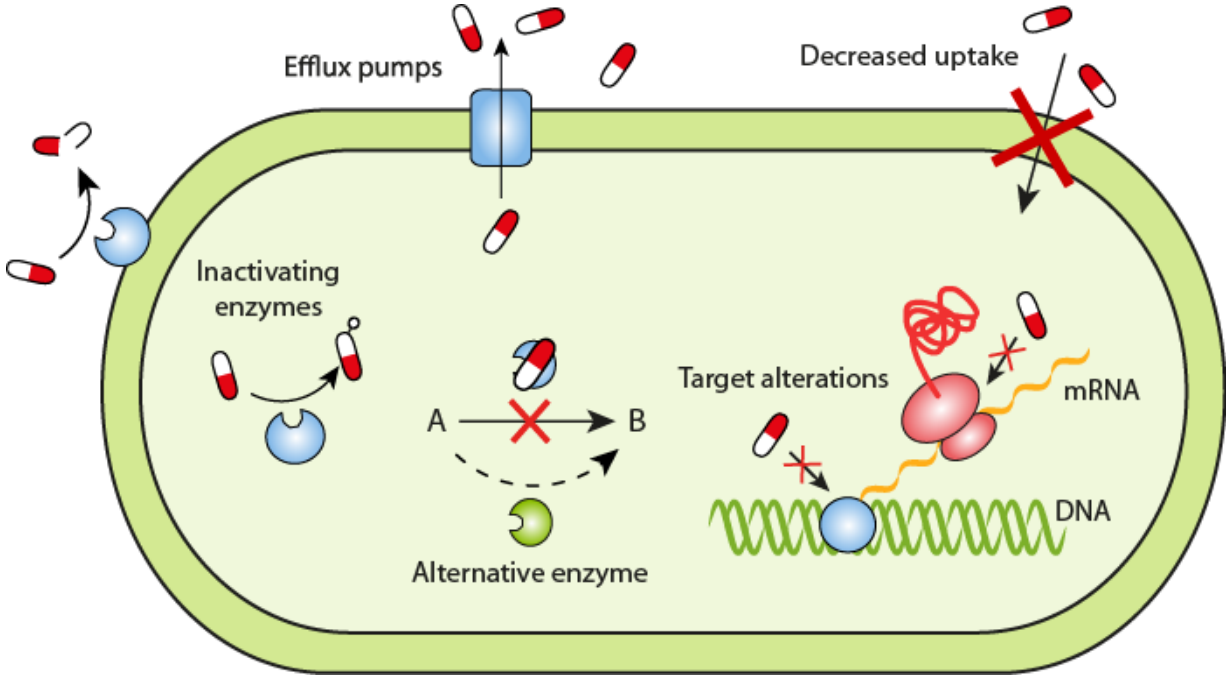

Figure 1.3: Various cellular mechanisms through which bacteria show resistance to antibiotics[28]

1.2. Fundamentals of Electrochemistry

Electrochemistry is the study of chemical processes where electron transfers are involved. In the past decade, electrochemistry has drawn immense attention as a new analytical approach to investigating the electrochemical properties of an analyte solution via a redox reaction.[38–42] Electrochemical redox reactions can be homogenous, involving electron transfer reactions between species in the same phase, such as a solution, or heterogenous, involving electron transfer reactions in different phases, such as an electrode and a solution. An electrode, typically platinum, gold, mercury, or glassy carbon, acts as an electrical conductor where voltage is applied through an external power source, such as a potentiostat. An electron transfer reaction is driven by the energy difference between the electrode and electroanalytical species (Figure 1.5).[38] The following are examples of electron transfer reactions where ferrocenium $[Fe (Cp)_2]^+$ (Cp=cyclopentadienyl), denoted as Fc^+ and ferrocene $[Fe (Cp)_2]$, denoted as Fc:

$$Ox + ne^- \leftrightarrow Red \quad (1.1)$$

Example for homogenous electron transfer: $Fc^+ + [Co (Cp^*)_2] = Fc + [Co (Cp^*)_2]^+$

Example for Heterogenous electron transfer: At an electrode: $Fc^+ + e^- = Fc$

Cyclic voltammetry (CV), chronoamperometry, differential pulse voltammetry, etc. are common electroanalytical techniques for investigating electron transfer processes.

1.2.1. Electrochemical Cell

In CV, an electrochemical cell consists of a working electrode, reference electrode, counter electrode and analyte solution. This is also called a three-electrode setup (Figure 1.4).

Working electrode (WE): The working electrode, composed of redox inert material, carries out the electrochemical reaction. The potential is applied to the working electrode through an external source (potentiostat) against a reference electrode to perform an electrochemical event. Common working electrodes are platinum, gold, glassy carbon, and mercury. Working electrode materials may vary to accomodate different potential windows for different experiments.[38]

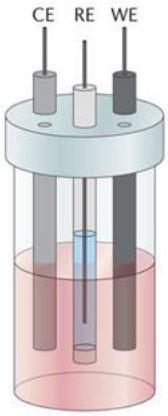

Figure 1.4: An electrochemical cell for a CV experiment with three electrode system, Counter Electrode (CE), Reference Electrode (RE), Working Electrode (WE).[43]

Reference electrode (RE): The reference electrode is the one against which the applied potential of the working electrode is determined in an electrochemical cell. The potential of a reference electrode is defined, stable, and independent of the electrolyte solution. Common reference electrodes used are the saturated calomel electrode (SCE), standard hydrogen electrode (SHE), and silver-silver chloride electrode (Ag/AgCl/) electrode. Reference electrodes have a porous frit that separates them from the solution to minimize the junction potential developed at the interface between two ionic solutions.[38]

Counter electrode (CE): The counter electrode is used in an electrochemical cell to close the electric circuit. As electrochemical reactions begin at the working electrode upon the application of a potential, a current occurs due to the flow of electrons between the working and counter electrodes. It is an inert material, usually a platinum wire or disc, and has a larger surface area than the working electrode so that it does not perturb the kinetics at the working electrode.[38]

1.2.2. Cyclic voltammetry

CV has become a very popular electroanalytical technique to investigate electron transfer processes in solution. In CV, the potential is applied in a cyclic manner (forward and reverse sweeps). A graph obtained from a CV experiment is called a cyclic voltammogram. Figure 1.6 is an ideal cyclic voltammogram. The x axis represents the applied potential and y axis is the obtained current. The arrow in the graph indicates the direction in which the applied potential was scanned. At first, zero current is observed at the initial potential (E_i). As the scanning

progresses towards higher potentials, the current starts to increase, when the potential is sufficiently positive to start oxidizing the analytes. The current signal continues to increase until it reaches the peak, then starts diminishing until it reaches the final potential (E_f). Then, the scan direction is reversed to promote a reduction process. In the reverse scan, the cathodic current starts to develop until it becomes negative enough to start reducing the analyte, and then reaches a peak current. Once the cathodic current reaches a peak, depletion starts and forms a duck-shaped structure. To understand the duck shape of the cyclic voltammogram, we will discuss the Nernst equation.

The relationship between the potential of the electrochemical cell, the standard potential of species, and the activities of chemical species undergoing oxidation and reduction in the system at equilibrium is explained by the Nernst equation.[38]

$$E = E^{\circ} + \frac{RT}{nF} \ln \frac{(Ox)}{(red)} \tag{1.2}$$

$$E = E^{\circ} + 2.3026 \frac{RT}{nF} \log 10 \frac{(Ox)}{(red)} \tag{1.3}$$

E is electrochemical cell potential

E^0 is Standard Potential

F is the Faraday constant

R is the universal gas constant

T is temperature in Kelvin

n is the number of electrons transferred in the cell reaction

Ox is the activities of oxidized species

Red is the activities of reduced species

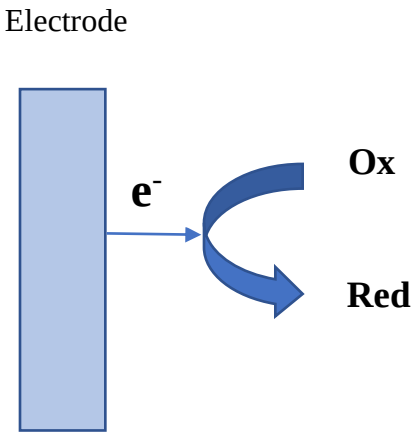

Figure 1.5: Electrochemical reaction at the electrode surface

Activities are replaced with the concentrations of species which are known from experimental conditions. The Nernst equation helps to predict how the system responds to changes in concentration or electrode potential. According to the Nernst equation, as the redox reaction proceeds under the applied potential ($E = E^{\circ\prime} \approx E1/2$), reactants are converted into products until the reaction reaches equilibrium.

There are two types of current flows in a cyclic voltammogram. The Faradaic current is due to the electrochemical reaction, and the non-faradaic current is associated with the movement of electrolyte ions at the electrode-electrolyte interface.

According to the Nernst equation, in a cyclic voltammogram, the concentration of a species changes over time near the electrode surface in response to the applied potential during scanning. As an example, for the ferrocenemethanol/ferroceniummethanol redox couple, in the beginning of the cyclic voltammogram at point A in **Figure 1.6,** no current response is observed at the initial applied potential. As the applied potential at the electrode continues to increase, the electrochemical double layer potential is adjusted by electrolyte ions moving to the surface of the electrode, starting to produce an increase in current. This is called a capacitor current or non-faradaic current. At this time, the applied potential is not high enough to oxidize the electroactive

species. As the scanning proceeds, an increase in faradaic current is observed (Point B) due to the oxidation of,

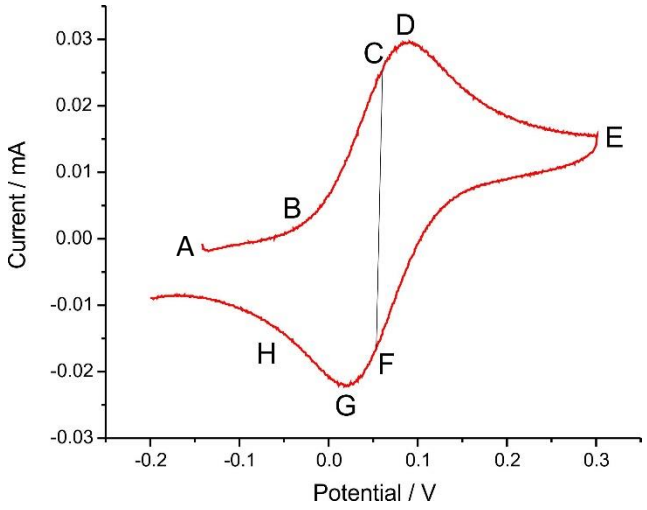

Figure 1.2: Cyclic Voltammograms of Ferrocenemethanol

for example, ferrocenemethanol ($FcCH_2OH$) to ferroceniummethanol ($[FcCH_2OH]^+$) where the applied potential starts to oxidize the species. As the potential continues to increase, more $FcCH_2OH$ is converted into $[FcCH_2OH]^+$ near the surface of the electrode. Depletion of $FcCH_2OH$ occurs at the electrode until the current reaches the peak at point D where mostly all of the $FcCH_2OH$ is oxidized to $[FcCH_2OH]^+$ near the electrode surface. This peak is also called the anodic or oxidation peak. At point D, the concentration of $[FcCH_2OH]^+$ is higher and continues to grow, and the current is limited by the transport of Fc from the bulk solution due to the formation of a diffusional layer. Then, the faradaic current starts decreasing upon scanning towards higher potentials. When the scanning direction is reversed as shown at point E, the concentration of $[FcCH_2OH]^+$ is depleted as it is reduced to $FcCH_2OH$, until the current reaches a peak where all $[FcCH_2OH]^+$ is reduced to $FcCH_2OH$ near the surface of the electrode. This is known as a reduction or cathodic peak. At point C and F, concentrations of Fc and Fc^+ are equal, satisfying the Nernst equation where ($E = E^{\circ\prime} \approx E1/2$). The two observed peaks occur in reversible electron transfer system reactions and are separated as a result of the diffusion of the chemical species to and from the electrode. In an electrochemically reversible redox reaction, the difference between the anodic and cathodic peak potentials is 57 mV at 25˚C. This is also called "peak to peak separation".

1.2.3. Scan Rate

The scan rate of the experiment is an important parameter which controls the rate of the scanning potential. The scan rate indicates how the potential is varied linearly as a function of time. A concentration gradient due to the different concentrations of electroactive species causes them to diffuse to the electrode surface. This movement of analytical species is explained by Fick's first law.

$$\boldsymbol{J_i = -D_i \left(\frac{\partial C_i}{\partial x}\right)} \tag{1.4}$$

Species i will diffuse due to the concentration gradient from a high concentration area to a low concentration area. J_i (mol m^{-2} s^{-1}) is the flux of the species i to the surface of the electrode. D_i (m^2s^{-1}) is the diffusion coefficient of species i. $\partial C_i / \partial x$ is the concentration gradient, where x is the distance from the electrode surface. According to this equation, the greater the concentration gradient, the greater the flux J is. When scanning starts, electroactive species will flow towards the electrode from the bulk solution due to the concentration gradient and the product generated at the electrode surface will diffuse from the electrode toward the bulk solution. This region where the concentration changes occur is called the diffusion layer. The diffusion layer continues to progress with time. Faster scan rates allow less time to form a diffusional layer, which results in increased current.

The Randles-Sevcik equation provides a way to determine the effect of the scan rate on the peak current. The relationship between peak current and the square root of scan rate indicates how mass transport occurs, and whether or not analytes freely diffuse towards the electrode. The Randles-Sevcik equation helps to establish an electrochemically reversible electron transfer system based on the linear relationship between peak current and the square root of scan rate. Using this equation, diffusion coefficients of analytes can be determined.[58]

Randles-Sevcik Equation

$$i_p = 0.446nFAC^0(nFvD_0/RT)^{1/2} \tag{1.5}$$

And the solution at 25˚C

$$i_p = 2.69 \times 10^5 \, n^{3/2} \, A \, D^{1/2} \, C \, \upsilon^{1/2} \quad (1.6)$$

- i_p = current maximum in amps
- n = number of electrons transferred in the redox event
- A = electrode area in cm^2
- F = Faraday Constant in $C \, mol^{-1}$
- D = diffusion coefficient in cm^2/s
- C = concentration in mol/cm^3
- v = scan rate in V/s
- R = Gas constant in $J \, K^{-1} \, mol^{-1}$
- T = temperature in K

1.2.4. Electrolyte solution

An electrolyte solution consists of supporting electrolytes, mainly salts, dissolved in solvents. It allows the ions to migrate in the solution to balance the charge and complete the electric circuit. It also helps increase the solution conductivity and decrease the solution resistance. Higher concentrations of electrolytes are required to limit the migration of electroactive species. An ideal electrolyte solution should dissolve the electroanalytical species and should be stable over the anodic and cathodic potential ranges of the experiment. The non-faradaic current observed in a cyclic voltammogram is due to the movements of these ions.[38]

1.2.5. Mass Transport

The working electrode is immersed in a solution containing analytes and excess supporting electrolytes and kept static. Three modes of mass transport are involved with the movement of the electroactive species to the electrode surface. These are convection, migration and

diffusion.[44] These are explained by the Nernst-Planck equation. The Nernst-Planck equation combines contributions from all three modes of mass transport.

$$J_{(x,t)} = -\left[D\left(\frac{\partial C_{(x.t)}}{\partial x}\right)\right] - \left[\left(\frac{zF}{RT}\right) DC_{(x,t)}\right]\left(\frac{\partial \emptyset_{(x,t)}}{\partial x}\right) + C_{(x,t)}v_{x(x,t)} \qquad (1.7)$$

where D (cm^2s^{-1}) is the diffusion coefficient of the electroactive species, J (mol cm^{-2} s^{-1}) is the flux, C (mol/cm^3) is the concentration of the species, φ is the electrostatic potential, and v_x is the hydrodynamic velocity.

This equation shows that the movement of electroanalytical species towards the electrode surface is proportional to either of three gradients including gradients of concentration, electrostatic potential (migration), and convection (hydrodynamic velocity). All the slopes (gradients) are functions of distance from the electrode surface. By limiting mass transport only to the contribution from diffusion-controlled processes, an electrochemical experiment is performed in a way that eliminates the contributions from migration and convection.

Convection occurs with the help of mechanical force (stirring or vibration). In our electrochemical experiments, working electrodes are kept static and immersed in electroanalytical solutions to eliminate contributions from convection. However, natural convection can also happen due to temperature variations. Thermostated electrochemical cells are used to eliminate this.[44,45] In migration, movements of ions are involved in an electric field. Ions are attracted to the opposite charge. Thus, the use of higher concentrations of electrolytes than the electroanalytical species minimizes the migration of analytes. The concentrated electrolytes are more likely attracted and migrate to the opposite charge. The contributions to the electrochemical signal, therefore, are from diffusion-controlled processes due to the concentration gradient between two points in the electrochemical cell. Analytes move from a point of higher concentration to a point of lower concentration. These conditions allow analytes to freely diffuse to the surface of the working electrode.

1.2.6. Polishing

As the main electrochemical reaction occurs at the surface of the working electrode, it is crucial to carefully clean the working electrode surface and free it from any particles. Cleaning is performed by polishing the electrodes. A water alumina slurry is prepared on a polishing pad and

the electrode is polished in figure 8 motions (Figure 1.7) to make the surface even. After polishing, the alumina slurry residue present at the surface of the electrodes is removed by ultrasonication in a proper solvent. Before taking each measurement, the electrode must be polished and cleaned. Any dirt or impurity present at the surface of the working electrodes can modify the cyclic voltammograms.[38]

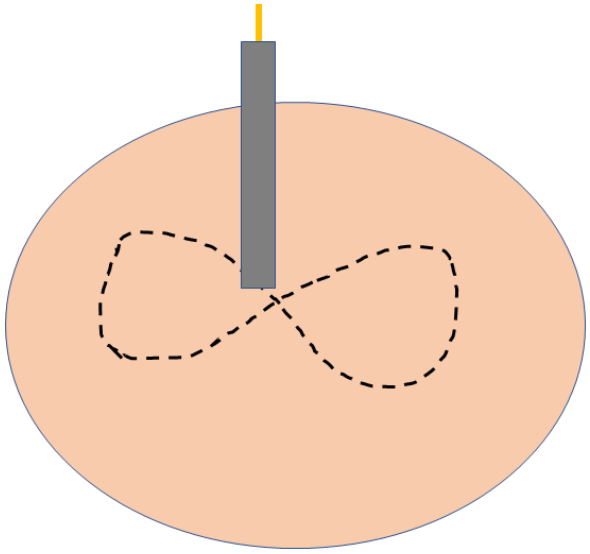

Figure 1.3: Schematic representation of polishing the working electrode

The overall goal of the research is to quantitatively detect the influx and efflux of antibiotics and antibiotic hybrid drugs in *Pseudomonas* bacteria by electrochemistry. This thesis was structured into the following six chapters to reach this goal.

Chapter 2: Chapter 2 presents a review paper published in the *Journal of the Electrochemical Society* (JES) with the title "Electrochemical Approaches and Advances towards the Detection of Drug Resistance". This paper presents a comprehensive literature overview, focusing on the electrochemical characterization of antibiotics and chemotherapeutic agents in the last decade. It also discusses the electrochemical detection of the interaction of drugs with biological cells and the recent progress towards the electrochemical detection of drug resistance.

Islam M.R., Luu H.T.L., Kuss S.: Electrochemical Approaches and Advances towards the Detection of Drug Resistance. Journal of the Electrochemical Society 2020, 167, 045501.

DOI: 10.1149/1945-7111/ab6ff3

Chapter 3: This chapter presents the electrochemical characterization of common antibiotics, namely amoxicillin, ciprofloxacin and tobramycin.

Chapter 4: This chapter presents the electrochemical characterization of a recently synthesized ciprofloxacin-tobramycin hybrid compound. The electrochemical properties of this hybrid compound are investigated by cyclic voltammetry and differential pulse voltammetry using bare glassy carbon electrodes (GCEs). This work was published in the journal *Electrochemistry Communications* with the title "Electrochemical characterization of the antibiotic hybrid ciprofloxacin-tobramycin".

Islam M.R., Singh V., Ammeter D., Schweizer F., Kuss S.: Electrochemical Characterization of the Antibiotic Hybrid Ciprofloxacin-Tobramycin. Electrochemistry Communications 2020, 119C, 106825.

DOI: 10.1016/j.elecom.2020.106825

Chapter 5: This chapter presents the electrochemical quantification of drug influx into *Pseudomonas aeruginosa*.

Chapter 6: This chapter presents the quantification of antibiotic efflux in *Pseudomonas aeruginosa* by electrochemical methods.

Chapter 7: In this chapter, a discussion and future prospects are presented.

CHAPTER 2: Review Literature on Electrochemical Advancement in Detection of Drug Resistance

2.1. Literature on Electroanalytical Studies of Pharmaceuticals

Electrochemical techniques have shown to be promising analytical techniques, which provide advantages over other techniques such as simple instrumentation setup, portability, cost effective and rapid analysis. In the last decade, researchers have focused on developing various electroanalytical techniques to analyze different types of samples, from environmental samples to medical samples. We have published a review paper titled "Electrochemical Approaches and Advances towards the Detection of Drug Resistance" in the Journal of the Electrochemical Society (JES) in 2020 volume 167 issue 4, 045501. The paper presents a comprehensive literature review of the electrochemical characterization of common antibiotics in the past decade. Finding suitable target analytes and their interactions with biological cells needs to be understood in order to detect drug resistance by electrochemistry. This review discusses research articles investigating interactions between drugs and cellular systems. This review is directly related to my research, because we characterized common antibiotics by electrochemistry using cyclic voltammetry, which forms the fundamental basis of electrochemical studies on antibiotic resistance.

Contribution

I did the literature search and wrote Introduction, Electrochemical Characterization of antibiotics, Drug interaction studies, Investigation of bacterial resistance, discussion, and bibliography.

Electrochemical Approaches and Advances towards the Detection of Drug Resistance

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2.2. Abstract

Drug resistance in bacteria and cancer is a growing problem that decreases drug treatment effectiveness and increases the severity of bacterial infections as well as cancer mortality. Due to their high sensitivity, low cost, and rapid analysis time, electrochemical methods have been increasingly employed to tackle this challenge throughout the last decade. This review covers literature on the electrochemical characterization of antibiotics and chemotherapeutic drugs, as well as advances in analyzing interactions between drug compounds and biological cells. Recent developments towards the quantitative detection of drug resistance in bacteria and cancer by electrochemistry are discussed, and the use of specialized electrochemical instrumentation, such as scanning electrochemical microscopy, is highlighted.

2.3. Introduction

The rapid spread of drug resistance in bacteria as well as cancer has developed into a significant threat to the global public health.[46] According to the World Health Organization (WHO), antibiotic resistance is present in every country,[47] and various national and international health organizations, including the United Nations, the Infectious Diseases Society of America, as well as the Public Health Agency of Canada, have called for the urgent development of new treatment and diagnostic strategies.[48,49] The Centers for Disease Control and Prevention reports approximately 10 million deaths worldwide each year in connection with antibiotic resistance.[50] Similarly, drug resistance in cancer is believed to be responsible for treatment failure in up to 90% of metastatic cancer patients.[51] Cellular resistance mechanisms in both bacteria and cancer include cell membrane protein modifications, intracellular drug target alterations, and the over expression of efflux pumps.[52–54] The latter are the result of an over-expression of efflux pump proteins, which enable cells to expel drugs rapidly from the cell interior, before these compounds can take effective action.[53] Drug compounds and efflux pump proteins have recently caught the attention by the electrochemical community to develop new methodologies to understand and detect drug resistance in both bacteria and cancer by electrochemistry.

In recent years, the innovation of electrochemical sensors has attracted immense attention, due to their high sensitivity, rapid analysis and ability to analyze complex samples, such as urine and blood. Although no sensor for the point-of-care detection of antibiotic resistance has been proposed so far, electrochemical sensors have become a powerful tool in various fields, such as environmental monitoring[11], biotechnology[12], and industrial process control[13,55,]. The advantages are clear: Electrochemical sensors are sensitive, inexpensive, and offer the detection of an analyte within seconds. These properties make electrochemistry attractive for its use in medical applications[56] and a number of research articles emerged over the last decade that represent useful first approaches towards the analysis of drug resistance by electrochemistry.

In order to detect drug resistance by electrochemistry, suitable target analytes have to be identified and their interaction with biological cells needs to be understood. This review presents a comprehensive overview of the characterization of drug compounds by electrochemistry during the last decade. A literature review is provided on characterization studies on antibiotics as well as anti-cancer drugs. This forms the fundamental basis for ongoing electrochemical studies on

drug resistance. Research articles are discussed investigating interactions between drugs and biological cells. These drug interactions studies have been a significant focus of interest not only for understanding the mechanism of action of drugs, but also for the design and development of novel pharmaceutical drug candidates.[57] We further present electrochemical approaches, which aim to detect drug resistance in living cells and to further our understanding of drug resistance in bacteria and cancer. A discussion is presented on the most promising electrochemical approaches and techniques, and in an outlook are highlighting potential target points for future research strategies.

2.4. Literature Review

2.4.1. Electrochemical Characterization of Drug Compounds

This section provides an overview of recent publications that are electrochemically characterizing individual drug compounds, which are widely prescribed to treat bacterial infections or cancer. We first discuss electrochemical studies on antibiotics before presenting useful characterization studies on anti-cancer drugs, highlighting successful techniques and electrode materials.

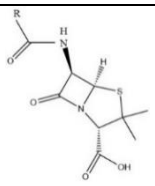
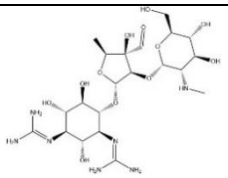
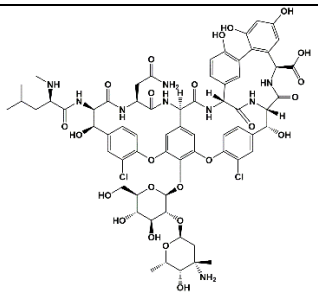
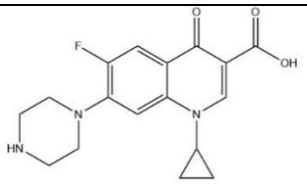
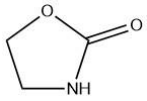
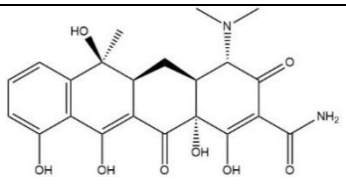
2.4.1.1. Electrochemical Studies on Antibiotics

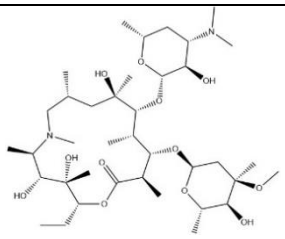
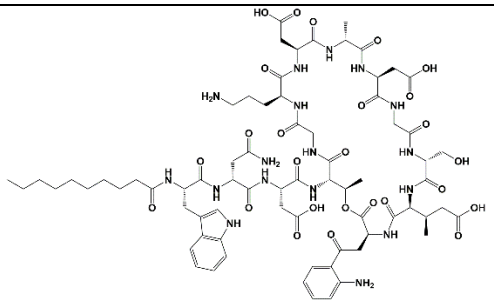
In order to study drug resistance electrochemically, the redox properties of drug compounds must be well understood. Based on their chemical structure, FDA approved antibiotics are commonly categorized into eight different classes, as summarized in **Table 2.1**.[58–61] Since 2009, electrochemical investigations focused mostly on β-lactams[62–70], aminoglycoside[71,72], quinolones[11–13,55,73,74,83–85,75–82] and macrolides[86–92] due to their effectiveness against a large number of bacterial diseases. In the following, we discuss some of the most commonly prescribed antibiotics and their electrochemical properties to evaluate their usefulness for electrochemical live cell studies.

Amoxicillin. The antibiotic class of β-lactams is widely used to prevent and treat various bacterial infections in humans and the field of agriculture.[62] Amoxicillin is a member of this class[66,68] and is the p-hydroxy analogue of ampicillin. It contains variable side chains, which are responsible for its rapid absorption by the human body. The electrochemistry of amoxicillin was investigated by the author *Ağin* in 2016[64] using glassy carbon electrodes (GCEs), which were modified by

electropolymerization of acridine orange. The electrochemical behavior of amoxicillin was characterized by cyclic voltammetry (CV), revealing an irreversible oxidation peak at 0.92 V *vs*

Table 2.1: Classes of antibiotics reported in literature.[58-61]

Class Name	Structure	Mechanism of Action
β-Lactam		Inhibit bacterial cell wall biosynthesis
Aminoglycosides		Inhibit bacterial preotein synthesis leading to cell death
Glycopeptides		Inhibit bacterial cell wall biosynthesis
Quinolones		Inhibit bacterial DNA replication
Oxazolidinones		Inhibit bacterial protein synthesis
Tetracyclines		Inhibit bacterial protein synthesis

Macrolides		Inhibit bacterial protein synthesis
Lipopeptides		Disrupt bacterial cell membrane

Ag/AgCl at a scan rate of 100 mV s^{-1}. This study suggests that the pH of the analyte solution has a profound effect on the peak current and potential. An optimal pH of 5.0 was reported for the determination of amoxicillin and a linear relationship between the anodic peak current as a function of the square root of the scan rate indicated a diffusion controlled process. In this study, differential pulse voltammetry (DPV) and square wave voltammetry (SWV) revealed a limit of detection (LOD) of 1.87 x 10^{-9} M and 1.55 x 10^{-8} M, respectively. No interferences were reported in this publication.

Ampicillin. An interesting strategy to detect ampicillin has been proposed by Wang *et al* using aptamer based differential pulse voltammetry.[62] In this approach, a GCE was modified with double stranded DNA (dsDNA) containing an ampicillin aptamer sequence (Figure 2.1.A). Differential pulse voltammograms showed a pronounced reduction peak. This method resulted in an impressive detection limit of 3.2 x 10^{-11} M and ampicillin was detected in real samples of milk and water.

Ciprofloxacin (CIP). Belonging to the second generation of fluoroquinolones, CIP is used worldwide as an antimicrobial agent to treat infections in humans and livestock.[81,82] Some antibiotics, such as CIP, can react with metal ions to form stable complexes. Several studies have been conducted to electrochemically characterize CIP.[11–13,53,79–82] Its detection through the complexation of CIP with Cd^{2+} was reported by Jun *et al*.[82] A well-defined electrochemical signal was recorded at graphene modified GCEs as a result of the complexation of Cd^{2+} by CIP.

The concentration of CIP was found inversely proportional to the electrochemical signal and an irreversible oxidation peak of CIP in various electrolyte solutions, including acetate buffer solution (ABS), phosphate buffer saline (PBS) and sulphuric acid, was observed. GCE modifications using graphene resulted in a significantly enhanced anodic peak in the presence of Cd^{2+} ions. Overall, this method showed good reproducibility and high selectivity with a detection limit of 5.9 x 10^{-8} M. The main advantage of this approach is that the electrode did not suffer from electrode fouling which is commonly observed during the direct determination of CIP at bare GCEs. This method successfully detected CIP rapidly and sensitively in pharmaceuticals and urine samples.

Norfloxacin (NFX). This fluoroquinole is commonly used to treat gastrointestinal, urinary and respiratory tract infections.[78] As shown by Ke-Jing *et al*, NFX was detected at multi-wall carbon nanotube (MWCNT)/nafion film-coated GCEs during voltammetry.[78] NFX was determined by linear sweep voltammetry at a potential range of 0.3 to 1.4 V *vs* Ag/AgCl, which resulted in a well-defined irreversible oxidation peak current at a peak potential of 1.11 V *vs* Ag/AgCl. The relationship between the peak current and the scan rate at a range of 10–250 mVs^{-1} demonstrated that mass transfer occurs through an adsorption-controlled process and that the pH of the supporting electrolyte solution has an important influence on the electrochemical reaction. The highest oxidation peak was recorded in HAc–NH_4Ac buffer solution at a pH value of 4.4. This method has been used in pharmaceuticals and urine samples with an excellent detection limit of 5×10^{-8} M. Only minimal fouling effects were observed during this procedure.

Tobramycin. Tobramycin (TOB) is an aminoglycoside, which is effective against both Gram-positive and Gram-negative bacterial infections.[71] A modified GCE was prepared by electropolymerization of pyrrole (Py) in the presence of tobramycin to form a molecularly imprinted polymer film on the GCE surface (PPy/GCE). The general approach of fabricating cavities in a polymer matrix based on a specific template molecule is known as Molecularly Imprinted Polymer (MIP) technique and has found useful application in a variety of research fields including electrochemistry, analytical chemistry and biology.[93–95] In the work of Gupta *et al*, mass transfer occurs through a diffusion controlled process where TOB interacts with pyrrole and results in an oxidation peak at 0.72 V *vs* Ag/AgCl. Modified TOB imprinted PPy/GCEs were electrochemically characterized by cyclic voltammetry in the presence of potassium ferricyanide

in KCl and compared to a bare GCE. The reversible redox peak of potassium ferricyanide, which is observed on the bare GCE, is not shown on the modified GCE due to the presence of the polymer. Square wave voltammetry was performed revealing a favorable detection limit of 1.4×10^{-10} M.[71]

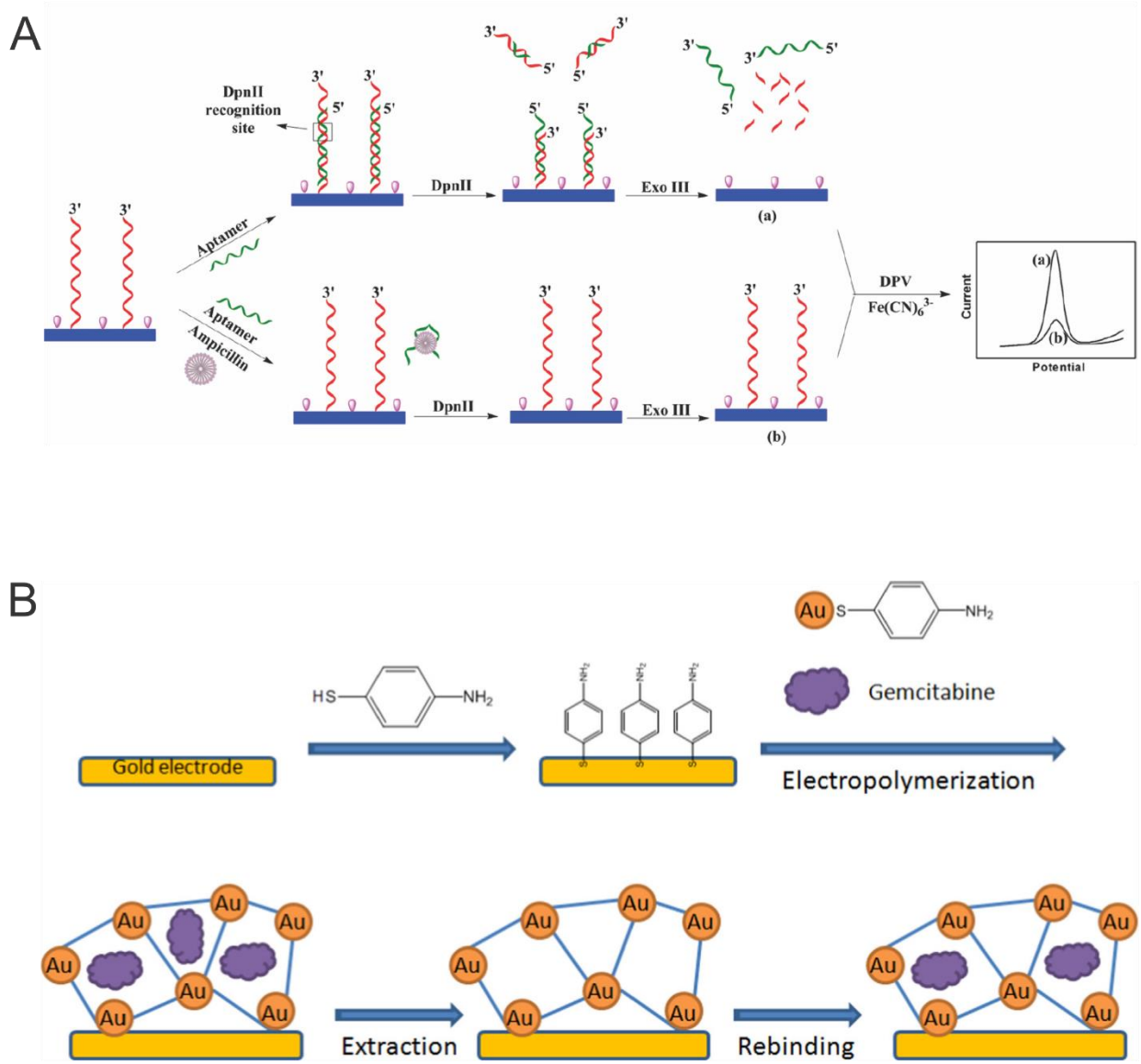


Figure 2.1: Representative examples for successful electrode modification for bioelectroanalytical analyses. A) Electrode modifications with DNA as proposed by Wang et al[62] B) Molecular imprinted polymer modified electrodes as reprinted by Florea et al[96]

Neomycin. Another example for the electrochemical characterization of aminoglycosides is neomycin, which has gained considerable attention due to its effectiveness against Gram-negative and Gram-positive bacteria to treat gastrointestinal infections and mastitis in livestock animals.[72]

Neomycin exhibits a well-defined reduction peak at a potential of -0.2 V *vs* Ag/AgCl as shown by Hamnca *et al.* Square wave voltammetry was carried out at the surface of a polyamic acid/graphene oxide (GO)/screen-printed carbon electrode (SPCE) for the electrochemical detection of neomycin in aqueous solutions. The reduction peak current was shown to increase with increasing concentration of neomycin until the catalytic current enhancement effect at the electrode surface reached a saturation level. The limit of detection was found to be 1.07 x 10^{-6} M. This approach was applied to detect neomycin in urine samples, which presents a reliable and rapid alternative to common chromatography techniques.[72]

Azithromycin. An interesting molecule of the macrolides class is azithromycin (AZM), which is effective against Gram-negative and Gram-positive bacterial infections.[87,91] AZM is highly effective against upper and lower respiratory acute tract infections such as bronchitis, pneumonia, sinusitis, pharyngitis, tonsillitis and otitis, sexually transmitted diseases, skin and soft tissue infections.[87,90,91] The electrochemical behavior of AZM has been investigated by cyclic voltammetry on MIP modified carbon paste (CP) electrodes (MIP/CPs) and non-molecularly imprinted polymer (NIP) modified CP electrode (NIP/CPs). Voltammograms show an irreversible oxidation peak at 0.8 V *vs* Ag/AgCl due to the oxidation of one dimethyl-amino group on the desosamine sugar part of the molecule. This work gives the lowest detection limit of 2.3 x 10^{-11} M, which compares favorable over any other voltammetry methods.[86]

Other existing electrochemical studies on antibiotics not discussed in detail, are summarized in Table 2.2.

Table 2.2: Summary of the recent literature on antibiotics, characterized by electrochemistry. Articles denoted with a star symbol are discussed in detail in this review. RGO = reduced graphine oxide; POT (SDS) = poly(*o*-toluidine) (sodium dodecyl sulphate); CB = carno black; DHP = dihexadecylphosphate; CTAB = cetyltrimethylammonium bromide; BDD = boron doped diamond; GCP = glassy carbon paste; PoAP = poly(*o*- aminophenol); PEDOT:PSS = poly(3,4-ethylenedioxythiophene)-poly(styrenesulfonate); G = graphene; ATP = 4-aminothiophenol; ABA = 4-aminobenzoic acid (4-ABA); IL-G = ionic liquid- graphene; ZSM = mesoporous zeolitic material.

Classes	Antibiotics	Method of Analysis	LOD [M]	Electrode modification	References
B-Lactams	Ampicillin	DPV	$3.2x10^{-11}$	dsDNA/AMP aptamer	62*
	penicillin	CV	$8.0x10^{-16}$	RGO/AuNP	97
		CV	$1.1x10^{-5}$	multisegment nanoparticles	98
	Amoxicillin	CV	$6.0\ x10^{-7}$	POT(SDS)	66
		CV	5.0×10^{-6}	Ni/CR	65
		SWV	$9.0x10^{-6}$	AuNP-PdNP-RGO	69
		SWV	$1.2x10^{-7}$	CB DHP	67
		CV	$1.9x10^{-9}$	poly acridine orange	64*
Aminoglycosides	Neomycin	SWV	$1.1x10^{-6}$	Polyamic acid/GO	72
	Tobramycin	CV	$1.4x10^{-10}$	Polypyrrole	71*
Quinolones	Ciprofloxacin	CV, ASV	$5.9x10^{-8}$	Graphene	82*
		CV, DPV	$5.0x10^{-8}$	CTAB	13
		CV	$1.2x10^{-8}$	$MgFe_2O_4$-MWCNT	12
		CV, LSV	$9.0x10^{-7}$	MWCNT	81
		CV		GO	11
		CV	$3.3x10^{-6}$	BDD	80
		SWV	$3.3x10^{-8}$	GCP	79
		CV	$6.0x10^{-6}$	MWCNT	55

	Levofloxacin	DPV	$1.0x10^{-6}$	PoAP/MWCNT	85
		CV, SWV	$1.4x10^{-8}$	AgNPs-CB-PEDOT:PSS	84
		CV, DPV	$5.3x10^{-7}$	MIP/G-AuNPs	83
		CV, SWV	$2.9x10^{-6}$	BDD	76
		CV	$1.0x10^{-8}$	AgNP	75
	Norfloxacin	SWV	$3.4x10^{-8}$	Polyamic acid/GO	72
		LSV	$5.0x10^{-8}$	MWCNT	78
	Enrofloxacin	LSV	$5.0x10^{-7}$	MWCNT	81
	Ofloxacin	CV, SW-AdAsV	$1.8X10^{-10}$, $2.4X10^{-10}$	MWCNT	74
		CV, DPV	$1.0x10^{-9}$	AuNPs/ATP/ABA	99
Oxazolidonones	Linezolid	DPV	$1.5x10^{-7}$	bare GCE	100
Tetracyclines	Tetracycline	CV	$1.5x10^{-5}$	multisegment nanoparticles	98
Macrolides	Azithromycin	CV		bare AuE	92
		DPV	$7.0x10^{-8}$	$MgCr_2O_4$/MWCNT	91
		CV		MWCNT-DHP	88
		CV		bare GCE	89
		CV	$1.3x10^{-9}$	Ru(bpy)3/ ZSM-5/Nafion	90
		CV, DPV	$1.9x10^{-7}$	IL-G	87
		CV	$2.3x10^{-11}$	MIP	86*

2.4.1.2. Electrochemical Studies on Cancer Drugs

Anti-cancer drugs that were electrochemically characterized in the last decade are categorized into four groups: alkylating agents, antimetabolites, cytotoxic antibiotics, and inhibitors. While alkylating agents, such as cisplatin lead to cell death in cancer by binding to DNA, antimetabolites, such as 6-mercaptopurine and 5-fluorouracil, mimic metabolites to interfere with

processes required for cell division.[101,102] Cytotoxic antibiotics, such as doxorubicin are drugs, which are used to treat cancer in addition to their antimicrobial properties.[101,102] The presented group of inhibitors consists of chemotherapeutic drugs able to inhibit various enzymes. For example, etoposide can interfere with the enzyme topoisomerase II, which is involved in relieving stress on DNA, induced by supercoiling during cell division.[103] The following section discusses recent studies on these four anti-cancer drug categories.

6-mercaptopurine (6-MP). Antimetabolite drugs have been studied extensively using electrochemical methods in the past decade, due to their common use in treating various types of cancers.[96,104,113–120,105–112] 6-MP is an anti-cancer drug used to treat leukemia.[109] There have been several attempts to electrochemically detect 6-MP[109,112,118], such as the recent publication of Kumar *et al* [112], who developed a method that reaches a detection limit of 7.2×10^{-10} M in blood plasma. In this work, the authors synthesize nitrogen-doped hollow carbon nanospheres, coated with Palladium (N-HCNS-Pd) and MIPs. These modifications help to improve the effective area and roughness factor of the electrode, which increases its sensitivity. The modified hollow carbon nanospheres are held on an ionic liquid (IL) modified pencil graphite electrode (PGF) via electrostatic interaction. The authors utilize differential pulse anodic stripping voltammetry (DPASV) to detect 6-MP. A diffusion-controlled and irreversible process for the 6-MP oxidation is presented, which involves two protons and two electrons. Notably, these electrode modifications are stable for 3 weeks if stored at room temperature and in dark conditions.[112]

Other electrode modifications, such as using biomolecules are less stable, but equally valuable due to their effectiveness, as shown by Pattar *et al* in 2015. In this manuscript the authors detect *5-fluorouracil (5-FU),* an antimetabolite drug used to treat various cancer kinds, such as colon cancer[113], stomach cancer[113], breast cancer[113], and cervical cancer.[113] Pattar *et al* employ chitosan, a chemically processed form of chitin[107,110,114,105] in combination with reduced graphene oxide to modify GCEs. This combination creates a rougher electrode surface and results in an electrocatalytic effect to achieve low detection limits. By applying staircase voltammetry (SCV), an impressive LOD of $1.24\text{x}10^{-9}$ M was achieved.[107]

Other successful electrode modification strategies involve the use of nanoparticles[111,106], ionic liquids[104], graphene oxide and carbon-nanotubes[119], methylene blue (MTB)[114], and the use of copper electrodes[117]. Important to point out is the work by Hadi *et al* in 2017, utilizing a

combination of graphene oxide and carbon nanotubes to modify GCEs and SPCEs. This method shows the widest analytical dynamic linear range from 0.05 to $1200x10^{-6}$ M, which is shown useful for the determination of 5-FU in human plasma.[119]

Gemcitabine (GMB) is an anti-cancer drug which is classified as an antimetabolite. Electrochemical detection of GMB can be achieved through the use of bare gold electrodes (AuE), as shown by the authors Naik *et al* in 2013.[115] Detection of drugs at bare electrodes is commonly favorable, because it saves time and costs, but often times results in lower detection limits. Complex electrode modifications to detect GMB using a microporous metal organic framework (MMOF) (MMOF/AuNP/Au electrode) has been proposed by Florea *et al* in 2015.[96] These authors construct a molecularly imprinted MMOF by electropolymerizing p-aminothophenol (PATP) equipped with gold nanoparticles (AuNP) on a gold electrode surface in the presence of GMB (Figure 2.1.B). GMB molecules act directly as templates to create complementary pores in the framework. After the electropolymerization, GMB is extracted, leaving empty binding sites. To analyze samples, the modified electrode is immersed in a solution with GMB, which will bind to complementary sites in the network. This binding will enhance the reduction peak of an electrochemical probe (ferrocyanide/ferrocyanide) and thus, GMB is detected indirectly. This method results in a LOD of 3.4×10^{-15} M. This method provides not only high specificity but also high sensitivity compared to the detection at bare electrodes.[96]

Cytotoxic antibiotics are antibiotics that are found to have anti-tumor activities. These antibiotics have many mechanisms of action. The subgroup of anthracyclines has been studied in the literature and contains antibiotics, doxorubicin[121,122] and daunorubicin[123], extracted from *Streptomyces* bacteria. *Doxorubicin (DOX)* was detected using voltammetry[124] as well as electrochemical impedance spectroscopy (EIS)[121,122]. Rezaei *et al* developed two different methods to utilize immunosensor and EIS to detect doxorubicin at very low concentrations. While one method uses gold electrodes modified with thiol base sol-gel (TBSol-Gel), gold nanoparticles (AuNP), and monoclonal antibody (MAb) (MAb/AuNP/TBSol-Gel/AuE)[122], the other method modifies a stainless steel electrode (SSE) with 3-aminopropyltriethoxysilane (APTES), AuNP, and Mab (MAb/AuNP/APTES/SSE)[121]. Both strategies follow a similar detection principle. Firstly, an electrode is coated with an insulating layer (TBSol-Gel or APTES) at the electrode surface. Secondly, AuNPs are deposited on the electrode by immersing

the electrode in $HAuCl_4$ solution for 180 seconds at -200 mV (with Ag/AgCl as reference electrode). After that, the newly modified electrode is incubated in a doxorubicin MAb. Finally, the electrode is immersed in a 1% bovine serum albumin (BSA) to block non-specific active site. The modified electrode is immersed in a standard solution of DOX prior to sample measurements. The charge transfer resistance, which is obtained from Nyquist diagrams, is used as a signal. The MAb/AuNP/TBSol-Gel/Au and the MAb/AuNP/APTES/SS electrode result in an LOD of 1.7×10^{-13} M and 3.1×10^{-12} M respectively.

Other cytotoxic antibiotics that were studied in the past decade include epirubicin[125], daunorubicin[123], and valrubicin[126]. A common theme in these three studies is the combination of carbon nanotubes with nanoparticles and other components to detect drugs. Shams *et al*[125] introduce a GCE modified with silver decorated MWCNT composite (Ag-MWCNT/GCE). By using square wave voltammetry, this work detects epirubicin with a LOD of 1.0×10^{-9} M. Kong *et al* 2019[123] prepared a nanocomposite from nitrogen decorated reduced graphene oxide and single-walled carbon nanotubes. This nanocomposite is loaded with platinum nanoparticles (PtNP) and is then used to modify a GCE (N-rGO-SWCNTs-Pt/GCE). At optimized conditions, this method achieved an LOD of 5.7×10^{-9} M for daunorubicin. In 2015, Hajian *et al*[126] fabricated an electrochemical sensor based on gold electrode modified with gold nanoparticles/ethylenediamine (EDA)/multi-walled carbon nanotubes (AuNPs/EDA/MWCNTs/AuE). This sensor can detect valrubicin with an LOD of $1.8x10^{-8}$ M in citrate buffer (pH 4.0) and 0.1 M KCl.

Alkylating agents include temodal[127], ifosfamide[128], and cisplatin.[129,130] Temodal was recently detected by the authors Jahandari *et al* in 2019, who propose the use of gold nanoparticles (AuNP) and dsDNA to modify a pencil graphite electrode.[127] One unique feature of this work is the use of computational chemistry to study the intercalations between temodal and guanine in dsDNA structure, which is used to detect the drug. In particular, this interaction between drug and dsDNA decreases the current of the oxidation peak of the guanine base. This method achieved an LOD of $1.0x10^{-9}$ M.

Other chemotherapeutic drugs that have been electrochemical studied in the past, but are not reviewed in detail here are etoposide[128] and the tyrosine kinase inhibitor erlotinib[108]. A summary of anti-cancer drugs investigated by electrochemistry is presented in **Table 2.3.**

Table 2.3: Recent electrochemical work on anti-cancer drugs detection by electrochemistry. Articles denoted with a star symbol are discussed in detail in this review. ($[Co(phen)_3]^{3+}$ = cobalt (III) trisphenanthroline complex; BMPA = biopolymer from babassu mesocarp modified with phthalic anhydride; PFR = porphyran; PANINT = polyaniline nanotube; CuSAE = Copper solid amalgam electrode; AdSLSV = adsorptive stripping linear sweep voltammetry; Pd@PtNP = mesoporous Palladium and Platinum Core shell nanoparticles; AdSSWV = adsorptive stripping square wave voltammetry; PTP = polythiophene; N-rGO = nitrogen-doped reduced graphene oxide; GST = Glutathione-s-transferase; Au-Pd@rGO = gold, palladium and reduced graphene oxide nanocomposite; PUFIX = polyurethane; PPHF = polypropylene hollow fiber).

Classes	Drug	Electrode modification	Method of analysis	LOD [M]	Reference
Antimetabolite	6-Mercaptopurine	MWCNT Paste electrode	LSV	1.0×10^{-7}	[109]
		$[Co(phen)_3]^{3+}$-GO-dsDNA/GCE	DPV	1.5×10^{-8}	[118]
		N-HCNS-Pd-MIP/IL-PGE	DPASV	7.2×10^{-10}	
	5-Fluorouracil	Glucose/CPE	CV, DPV	5.2×10^{-9}	[113]
		BMPA/Flexible AuE	CV, SWV	3.4×10^{-7}	[110]
		Reduced GO-CS/GCE	CV, SCV, SWV	1.2×10^{-9}	
		AuNP-MWCNT-CS/GCE	CV, DPV	2.0×10^{-8}	[105]
		AuNP-PFR/CPE	CV, DPV	6.7×10^{-7}	[106]
		PANINT-AgNP/PGE	DPV	6.0×10^{-8}	[111]
		IL/CPE	CV, DPV	1.3×10^{-8}	[104]
		GO-MWCNT/GCE and SPCE	CV, SWV	1.6×10^{-8}	
		CuSAE	CV, AdSLSV	1.2×10^{-9}	[117]
		MTB/CPE	CV, DPV	2.0×10^{-9}	[114]
	Gemcitabine	AuE	DPV	6.0×10^{-8}	[115]
		MMOF-AuNP/AuE	LSV	3.0×10^{-15}	

Cytotoxic antibiotic	Doxorubicin	MAb-AuNP-TBSol-Gel/AuE	EIS	1.7×10^{-13}	
		Mab-AuNP-APTES/SSE	EIS	3.1×10^{-12}	
		Pd@PtNP-MWCNT/ GCE	AdSSWV	8.6×10^{-10}	[124]
	Mitoxantrone	dsDNA-MWCNT-AgNP-PTP/GCE	DPV	1.3×10^{-8}	[131]
	Epirubicin	Ag-MWCNT/GCE	SWV, CV	1.0×10^{-9}	
	Daunorubicin	N-rGO-SWCNT-PtNP/GCE	DPV	5.7×10^{-9}	
	Valrubicin	AuNP-EDA-MWCNT/AuE	CV	1.8×10^{-8}	
Alkylating agents	Cisplatin	GST/CPE	CV, SWV	8.8×10^{-6}	[129]
		MWCNT/SPCE	CV, DPV	4.6×10^{-6}	[130]*
	Temodal	dsDNA-AuNP/PGE	DPV	1.0×10^{-9}	
Inhibitors	Etoposide	Au-Pd@rGO-L-Cysteine/PGE	DPV	7.2×10^{-10}	
	Erlotinib	MWCNT-PUFIX-PPHF/PGE	DPV	2.0×10^{-8}	[108]*
	Irinotecan	GCE	CV	1.1×10^{-10}	[132]
	Roscovitine	PGE or SPCE	SWV	PGE: 2.0×10^{-7} SPCE: 1.5×10^{-7}	[133]

2.4.1.3. Drug Interactions Studies

Drug interactions studies have been of significant interest to understand the mechanism of drug-cell interactions, which is crucial for the design of novel and effective pharmaceutical drugs.[57] Most of the proposed drug interactions studies in literature focus on the interaction of a drug with DNA molecules.[57,134–137] Triggered by an effective drug, DNA often times is susceptible to various intracellular redox reactions, which lead to DNA damage and cause pathological changes

in cells.[57] Drug molecules bind with DNA either covalently or non-covalently.[135] Non-covalent interactions with DNA occurs primarily in three modes which include groove binding interactions, electrostatic interaction from the exterior sugar phosphate backbone and intercalations between the stacked base pairs of the ds DNA.[57,134,135,] Non-electrochemical techniques that have been used to study drug-DNA interactions, include fluorescence spectroscopy[138], UV-spectrophotometry[139], circular dichroism[138], and mass spectrometry[140]. The electrochemical investigation of interactions between DNA and drug compounds represents a rapid, and reliable alternative, involving mostly DNA-modified electrodes. Thereby, monitoring the oxidation of DNA base pairs enables the direct detection of drug-DNA interactions on the electrode surface.[141]

Due to its well-defined electrochemical properties[82], CIP is an ideal candidate for the investigation of CIP-cell interactions. CIP is known to interact with enzyme bound DNA complexes and triggers conformational changes, which block the DNA replication process resulting in rapid bacterial cell death.[142] Garbellini *et al* studied the interaction of CIP with ds-DNA in solution using boron-doped diamond (CPT BDD) electrodes and square-wave voltammetry.[85] Employing calf thymus DNA, which consist of 41.9% Guanine-cytosine and 58.1% adenine-thymine base pairs, a decrease in the CIP oxidation peak current and an anodic shift of peak potential is observed in the presence of ds-DNA, which indicates the interaction of CIP with dsDNA molecules. In the presence of ds-DNA, the decrease in peak current reveals the reduced amount of free drug concentrations due to the formation of CIP-DNA complexes. Monitoring the interaction time, the authors conclude a possible CIP interaction with DNA occurs by intercalation.[135]

Gemcitabine (GMB) is an antimetabolite, and structurally analog to the cytosine nucleoside, and is commonly used to treat a variety of tumors, such as pancreatic cancer, lung cancer, breast cancer, bladder cancer, and brain cancer.[141] During its cell metabolic pathway, GMB is converted to inactivated 2', 2'-difluorodeoxyuridine (dfdU) by the action of plasma and liver cytidine deaminase and ultimately causes apoptosis by incorporating into DNA. In the work of Tig *et al*, a poly(2,6-pyridinedicarboxylicacid) modified GCE (GCE/P(PDCA)) is used to elucidate the interaction between GMB and DNA molecules by using differential pulse voltammetry whereby the guanine oxidation current signal is used as an indicator. A decrease in

the oxidation peak current for the guanine base pair with increasing concentrations of GMB was observed. The authors propose that this decrease in the guanine oxidation peak current could be explained as a binding of the GMB with ds-DNA at the GCE/P(PDCA) surface, which leads to damage in the oxidizable groups of the DNA electroactive nitrogenous bases. By observing the shifting of peak potentials, conclusions can be drawn about the mode of interaction. A shift towards anodic and cathodic potentials of the oxidation peak indicates intercalation or electrostatic interactions, respectively. However, no measurable shift in the peak potential of guanine oxidation was observed, which suggests that binding of GMB with ds-DNA is neither intercalative nor due to electrostatic interactions.[141]

Curcumin is a plant derived phytochemical compound which exhibits anti-cancer, antioxidant, anti-inflammatory, anti-proliferation effects. Because of its strong antioxidant effects, it is considered as a promising anti-cancer compound.[143] Suhito *et al*[143] proposed electrochemical strategies to measure the effects of curcumin on glioblastoma cells U87MG, an aggressive tumor (Figure 2.2). The authors used Indium Tin Oxide (ITO) as working electrode which was modified by gold nanoparticles and silver nanoparticles (AgNP) to enhance the electrocatalytic surface, which is further treated with cysteine-containing branched arginyl-glycyl-aspartic acid (RGD-MAP-C) peptides to increase cell adhesion property and growth. Differential pulse voltammetry and cell viability assays were performed after 2 days of curcumin treatment at various concentrations. The result showed a clear decrease in cell viability with increasing curcumin concentration. Voltammograms of DPV experiments revealed a decrease of the oxidation peak indicating an anti-cancer effect of curcumin in U87MG cells. The authors concluded that the proposed electrochemical method is more sensitive, accurate, and free from optical interferences compared to traditional colorimetric methods.

Ganciclovir (GCV) is used as an antiviral drug against herpes viruses, varicella-zoster virus, cytomegalovirus and Epstein-Barr virus.[57] In the literature it is reported that GCV is intracellularly converted to its triphosphate form, which inhibits DNA polymerase to elongate the viral DNA during replication by inhibiting the incorporation of deoxyguanosine triphosphate into growing viral DNA.[57] Once the pyrophosphate is released, GCV monophosphate causes a slower replication process of viral DNA by incorporating into the end of a growing chain. Paimard *et al* investigated the drug-DNA interaction of GCV on the surface of GCEs, modified

with Fe_3O_4/cMWCNTs/GCE using cyclic voltammetry. Voltammograms show an oxidation peak of GCV at 0.93 V *vs* Ag/AgCl at the Fe_3O_4/cMWCNTs/GCE. The authors observed a decrease in the GCV anodic peak current and a positive shift in the peak potential at the surface of the modified electrode in the presence of DNA. This decrease in the oxidation peak current can be attributed to a drug-DNA interaction, which is thought to cause a decrease of equilibrium concentration of the free GCV due to GCV-DNA interaction. The positive shift in the GCV oxidation peak potential was explained as an intercalative binding of GCV with DNA molecules.[57]

2.5. Investigation of Drug Resistance by Electrochemistry

Over the last decade, electrochemical research in the area of drug resistance has gone far beyond the characterization of drug compounds, which is nonetheless a crucial first step in recognizing

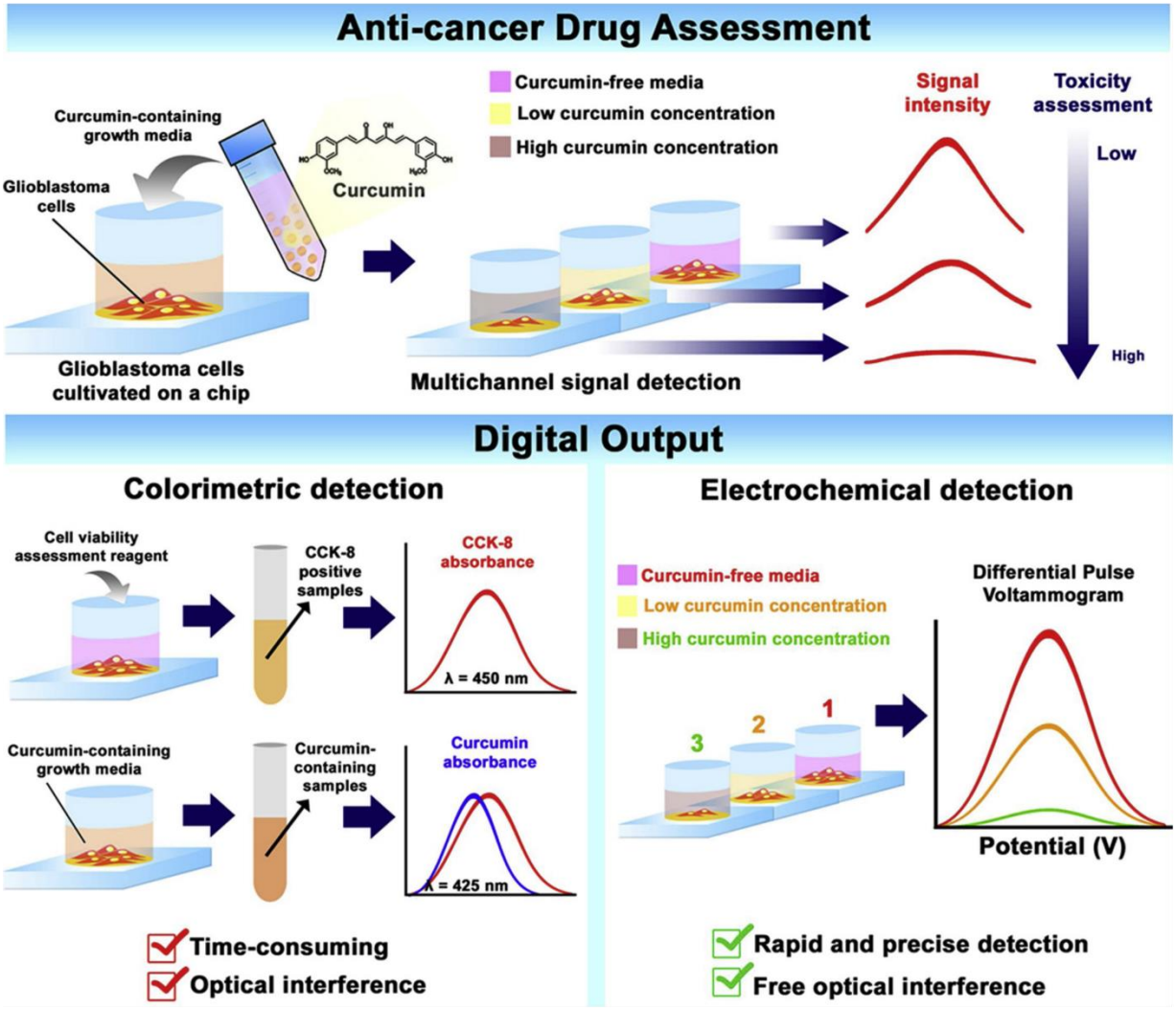


Figure 2.2: Schematic illustration of electrochemical detection of anti-cancer effects of curcumin with human glioblastoma cells as proposed by Suhito et al[143]

drug resistance electrochemically. The following section discusses approaches based on redox mediators as drug resistance indicators, employed during standard electrochemical methods, such as voltammetry, and specialized instrumentation, such as Scanning Electrochemical Microscopy (SECM).

One of the most recent strategies to detect drug resistance in bacteria was reported by Sun *et al* in 2019, who employed p-Benzoquinone (BQ) as a redox mediator and indicator for drug resistance in *Escherichia coli* (Figure 2.3).[50] BQ acts as an electron mediator during bacterial respiration events. Thereby, BQ is enzymatically reduced to hydroquinone (HQ), which diffuses from the cell towards an electrode, where BQ is regenerated at the electrode surface. Monitored by cyclic voltammetry, this mechanism was revealed as a reversible electron transfer reaction. For the quantitative detection of antibiotic resistance, the authors employed bacteria, resistant to trimethoprim, a folic acid synthesis inhibitor, which is known to inhibit the reduction of dihydrofolic acid to tetrahydrofolic acid through binding with dihydrofolate reductase, which leads to the blockage bacterial DNA biosynthesis.[50] During cyclic voltammetry, an increase in peak current was observed with increased resistance phenotype in *E. coli*. The authors concluded that this method is potentially capable of differentiating between wild type *E. coli* and antibiotic resistant *E. coli* with the same concentration, but more studies are needed for the detection in real and complex samples.[50]

The detection of drug resistance in cancer cells has been approached by studying proteins, which are responsible for an increased number of drug efflux pumps when over expressed. Approaches in recent years include impedance spectroscopy and voltammetry, mostly through antigen/antibody modifications of electrodes[144,145] and specialized instrumentation, such as SECM to monitor drug resistance activity in living cells[146–150]. The most recent quantitative electrochemical approaches to measure drug resistance in living cells are discussed in the following section.

In 2015, Chen *et al* assessed the expression of Bcl-2 proteins using an electrochemical immunoassay as outlined in Figure 2.3.[144] In this approach, GCEs were modified with L-Lysine through electropolymerization (PLL/GCE). Functionalized electrodes were then immersed in a glutaraldehyde aqueous solution for 1 hour. Finally, these electrodes were further modified with a

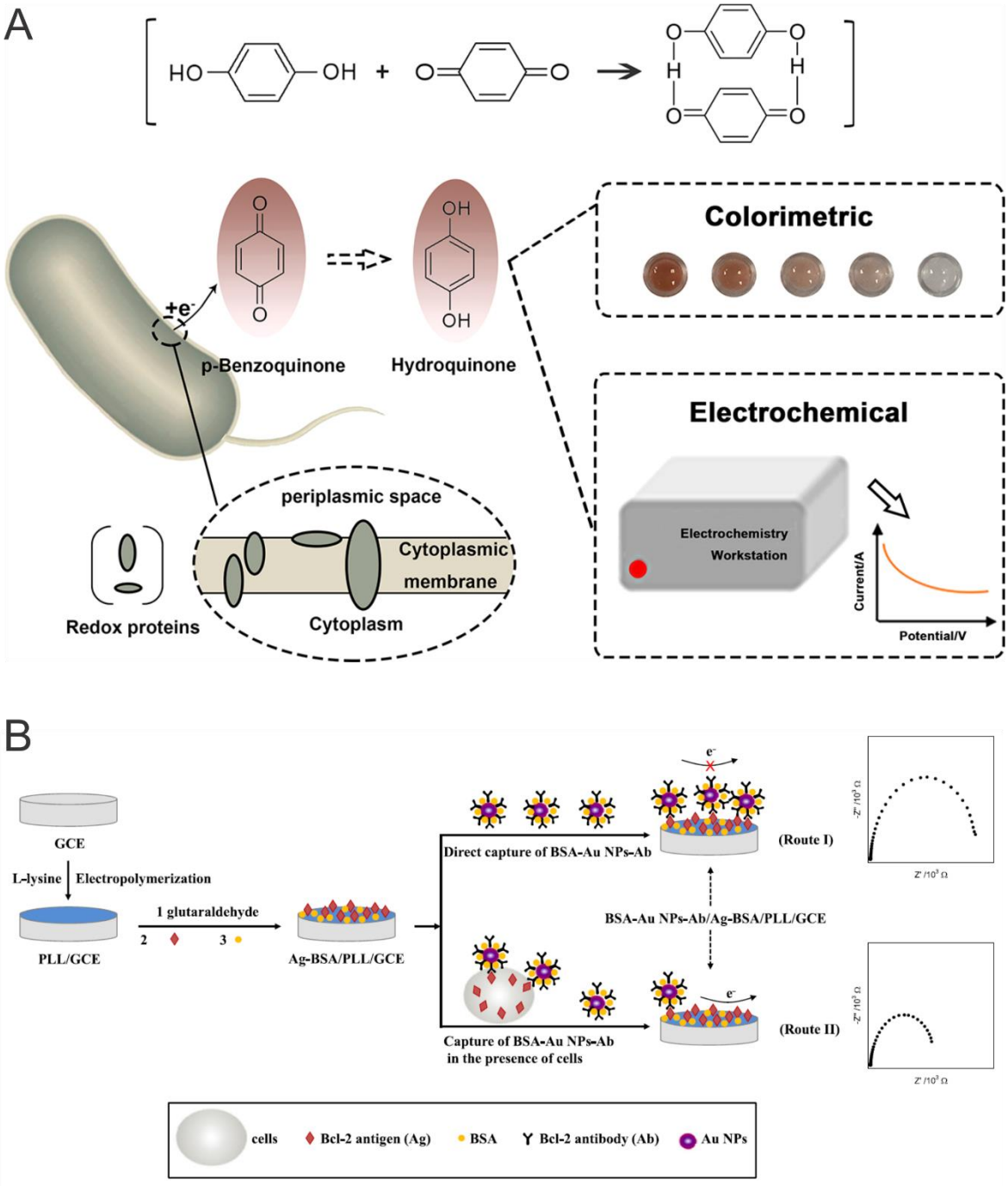


Figure 2.3: Schematic representation of electrochemical approaches towards the detection of drug resistance in A) bacteria (taken from Sun et al[50]) and B) tumor cells, as presented by Chen et al[144]

Bcl-2 antigen (Bcl-2Ag) and BSA to block nonspecific binding sites. To detect the expression of Bcl-2 proteins, this modified electrode (Bcl-2Ag/BSA/PLL/GCE) is exposed to a solution containing gold nanoparticles (AuNP) coated with BSA and Bcl-2 antibody (Bcl-2Ab) in the absence of cells (blank), or in the present of drug resistance or drug sensitive cells for 3 hours. The use of an antibody ensures the specificity of the proposed method. Electrochemical impedance spectroscopy (EIS) revealed changes in charge-transfer resistance between samples. In the absence of cells, the Bcl-2Ab/BSA/AuNP is able to bind to the Bcl-2Ag/BSA/PLL/GCE as it is modified with the antibody. Hence, the charge transfer resistance is lower than in samples containing cells, because of a competition between the electrode surface and the cells as both have Bcl-2Ag. The more the Bcl-2 proteins are expressed, the more Bcl-2Ab/BSA/AuNP bind to cells and hence, the fewer Bcl-2Ab/BSA/AuNP bind to the electrode. Impedance measurements revealed a decrease of charge transfer resistance as the expression of Bcl-2 proteins increases. Since drug resistant cells express more Bcl-2 proteins than drug sensitive cells, the charge transfer resistance measured in the former is smaller than that of the latter.

To investigate drug resistance activity rather than protein expression, cellular drug uptake was monitored in leukemia cells K562 and its multidrug resistant variant K562/A02.[150] This approach by Zhang *et al* in 2011 used carbon nanotube-modified GCEs (CNTs/GCE) to detect daunorubicin resistance caused by an over expression of P-glycoprotein (MDR). This modified electrode is immersed in daunorubicin solutions containing cells. The authors hypothesize that sensitive cells have a higher drug uptake than resistant cells, which leads to a lower concentration of daunorubicin in suspensions of drug sensitive cells compared to daunorubicin suspensions of drug resistant cells. The results show a decrease in peak current with increasing concentrations of sensitive cells.

To study cells under physiological conditions and as close to their natural environment as possible, specialized electrochemical instrumentation, such as SECM, has been advanced and customized specifically for the analysis of living cells.[151–154] In 2011, Kuss *et al* reported a differential electrochemical response during SECM measurements between wild type cells and cells overexpressing the multidrug resistance associated protein 1 (MRP1) in the presence of the redox mediator ferrocenemethanol ($FcCH_2OH$) (Figure 2.4, A-B). MRP1 efflux pumps have the ability to expel drugs, and drugs in conjugation with the reduced form of glutathione (GSH),

from the cell interior. In the literature MRP1 is known as an independent prognostic indicator and its expression is strongly predictive of the survival rate of cancer patients, which makes it an interesting target for drug resistance detection.[155] Using flow cytometry, the authors showed that $FcCH_2OH$ increases the intracellular concentration of GSH and propose a mechanism (Figure 2.4.A) of action in which GSH is expelled from the cells at a higher concentration and reacts with ferrocenium methanol, the oxidized form of $FcCH_2OH$ ($[FcCH_2OH]^+$), regenerating $FcCH_2OH$ in solution. As a result, during SECM studies, an increase in electrochemical current was recorded when the microelectrode passes cells due to the enhanced oxidation of $FcCH_2OH$ at the microelectrode (Figure 2.4.B). Adenocarcinoma cervical cancer (HeLa) cells over expressing MRP1 showed a lower electrochemical current signal. The importance of glutathione as an antioxidant[129] suggests that it is possible to monitor the redox state of cells through this $FcCH_2OH$-glutathione relationship.

Building on their previous work, Kuss *et al* successfully extracted an apparent heterogeneous rate constant for drug resistant and non-resistant HeLa cells from experimental SECM data using cell permeable and impermeable redox mediators.[148] This work determined the samples' apparent heterogeneous rate constant, independent from their topography, which until this point remained a challenge to the SECM community, and presents the first step towards a quantification of multidrug resistance on the single cell level in human cancer cells by SECM. Further publications by the authors in 2015[146,147] demonstrated the application of SECM to cancer cells exposed to green tea catechins (GTC), which are known for their antioxidant properties, and the sensitive monitoring of the cells' electrochemical response over time. In this work, the authors report a simulation model based on the electrode scan velocity during SECM studies for high scan rates and slow scan rates, which enables the determination of the apparent heterogeneous rate constant for cells within minutes. This presents a significant advantage over previous works as results will be more reliable, because cells can be imaged faster and before environmental changes potentially affect the cells metabolism during the experiment. Employing the established relationship between $FcCH_2OH$ and GSH, the authors reported an increase in current when HeLa cells were exposed to GTC. The authors suggested this behavior is due to an enhanced efflux of GTC and GSH as a potential defense mechanism to prevent cell death. The electrochemical current signal re-adjusted to a control value after the removal of GTC from the solution. Although no drug resistant cells were monitored during this study, this work represents

an interesting approach to potentially monitor the initiation of drug resistance in cancer in the future by SECM.

The correlation between over expression of proteins related to efflux pumps, such as MRP1, and functional activity of cells was investigated in 2017 by Polcari *et al*. By exposing wild type and drug resistant HeLa cells to a doxorubicin drug challenge (Figure 2.4.C), the authors monitored the apparent heterogeneous rate constant by SECM and obtained information about the expression of MRP1 by flow cytometry.[156] Comparison of data from both instrumental approaches showed that functional activity and expression do not directly correlate.

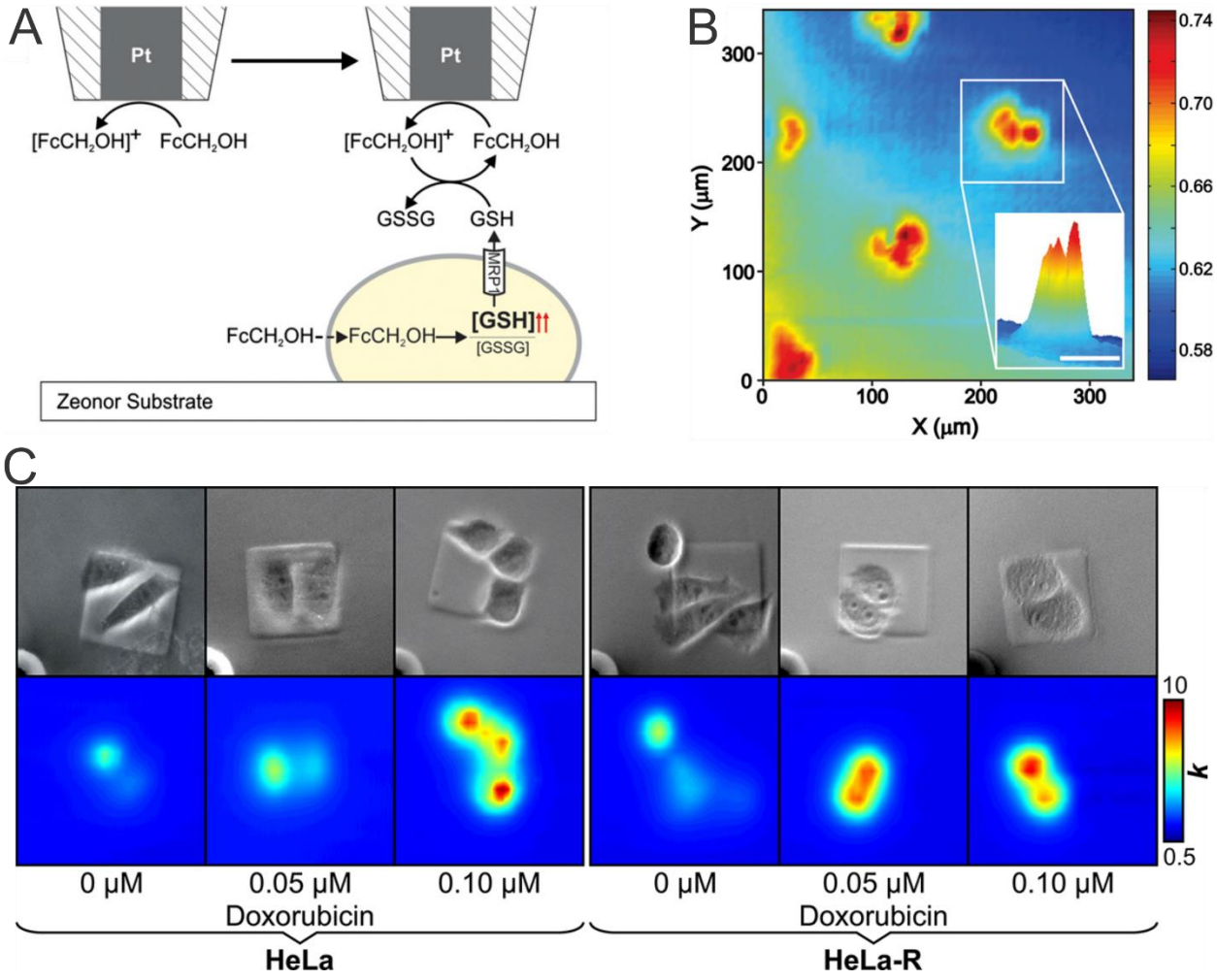


Figure 2.4: Electrochemical quantification of drug resistance using SECM imaging. (a) The relationship of FcCH2OH and GSH was used as drug resistant indicator as reported by Kuss et

al.[148] to electrochemically image HeLa cells by SECM (b) (scale bar: 50 μm.). C) Detection of multidrug resistance protein expression by Polcari et al.[156]

2.6. Discussion, Conclusions and Future Prospects

The development of antibiotics and chemotherapeutics is one of the greatest scientific achievements of the 20th century, enabling the treatment of various infectious diseases and saving many lives of cancer patients.[20] Unfortunately, the emerge of drug resistance due to an overuse of antibiotics or ineffective treatment strategies has evolved to one of the greatest medical obstacles.[10,20,22] To date no electrochemical sensor for the accurate determination of antibiotics in the environment, or the food industry has reached the commercial market and no chemical sensor for the routine detection of antibiotic resistance in patients has been proposed.

As outlined in this review, many common antibiotics, such as amoxicillin, ciprofloxacin, azithromycin, ampicillin have been successfully characterized using electrochemical techniques, such as cyclic voltammetry[12,13,55,66,63,68,64,65,73,87,89,90] in various buffer solutions. **Figure 2.6** presents an illustrative summary of in detail discussed electrode modifications and instrumental approaches towards the electrochemical detection of analytes, such as cell metabolites and drug compounds. These techniques reach incredibly low detection limits, down to the sub-nanomolar range.[62,71,74,97] However, analyzes in buffer solutions do not accurately represent conditions in environmental samples or biological fluids. Inorganic and organic species present in pharmaceutical and biological samples can interfere with the detection of target drug compounds. A few studies [73,78] considered such interferences with antibiotics, such as CIP.[79] Interferences by various species such as Sr^{2+}, Cd^{2+}, Ba^{2+}, Cr, Ni, Ca, Cu, acetate, lysine, L-glutamic acid, L-serine, L-histidine were studied by Lida *et al* and results show influences on the electrochemical signal of CIP of about 5%, which demonstrates the importance of interference studies in quantitative analytical electrochemistry. The analysis of real samples, such as urine and blood is important to exclude interferences by common biomolecules present in samples of such highly complex composition. Interference studies become unavoidable especially when detecting multiple drugs simultaneously. Furthermore, although excellent detection limits have been achieved by electrochemistry during *in vitro* studies, no relationship to expected concentrations *in vivo* has been made in the literature. Potential application of electrochemical techniques for *in vivo* studies of drug detection should address the applicability of the proposed

methods to therapeutic concentrations, which usually lie at the order of 1-7 mg kg^{-1} for individual dosages.[157]

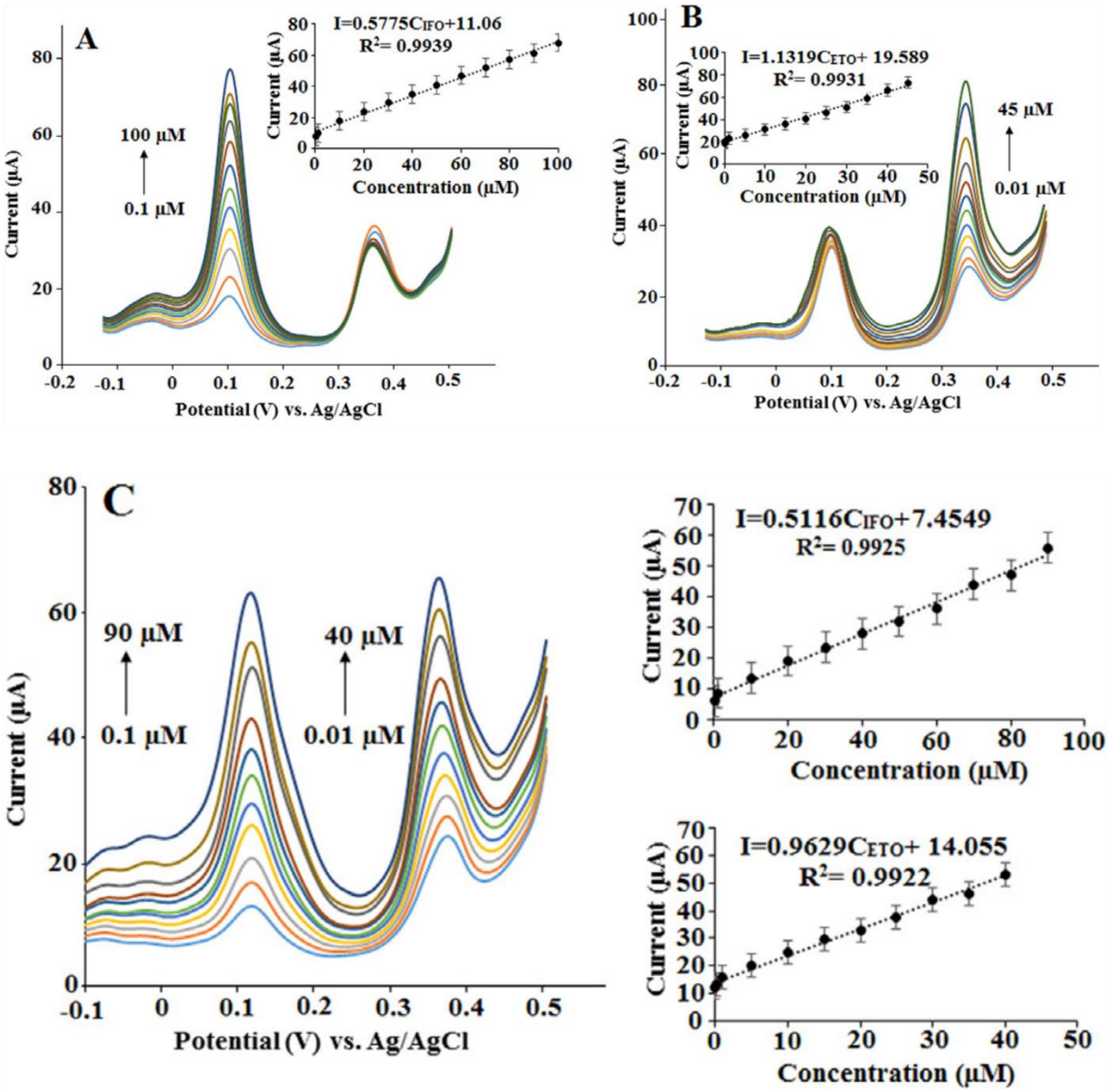


Figure 2.5. Simultaneous electrochemical detection of both anti-cancer drugs IFO and ETO as developed by B. Hatamluyi et al[128]

Combined administration of several chemotherapeutics is considered to increase the effectiveness of the treatment, to minimize side effects, and prevent drug resistance to a specific drug. However, this strategy can lead to multidrug resistance in tumor cells with different drug-unspecific resistance mechanisms, such as MRP1 efflux pumps.[124,128] Hence, it is helpful to develop methods

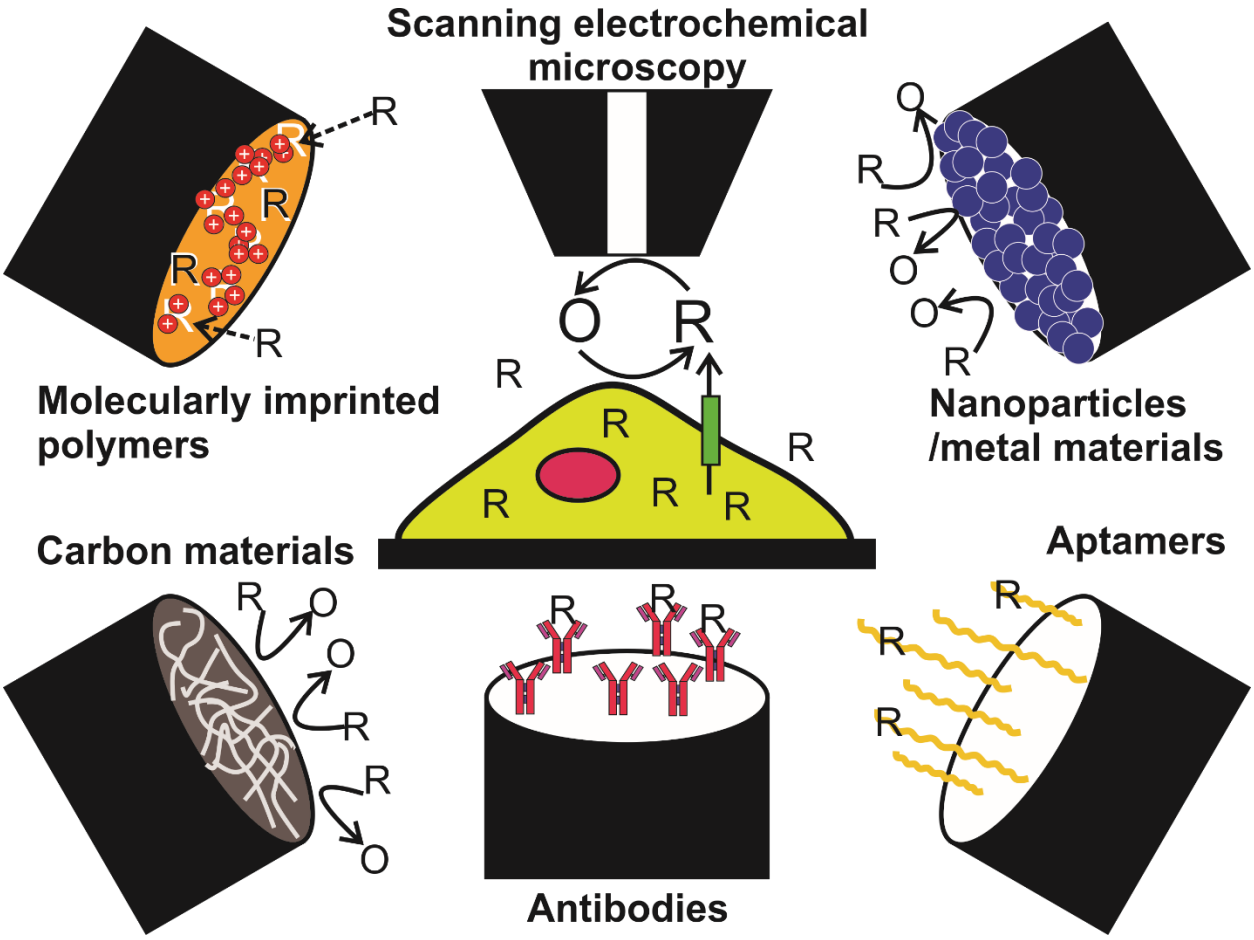


Figure 2.6. Schematic summary of commonly used electrode modifications and techniques to detect analytes, such as cell metabolites or drug compounds. R=reduced species; O=oxidized species.

for the simultaneous detection of common drug combinations. Out of the literature reviewed in this work, only three studies have focused on the simultaneous detection of drug compounds. Hatamluyi *et al* in 2018[128] detected ifosfamide, an alkylating agent, and etoposide, a

topoisomerase II inhibitor, simultaneously. The achieved detection limit of ifosfamide and etoposide are 9.210×10^{-9} M and 0.718×10^{-9} M, respectively. Figure 2.5 demonstrates the simultaneous detection of these two drugs. Furthermore, Es'haghi *et al* presented a method to monitor concentrations of capecitabine, an antimetabolite, and erlotinib, a tyrosine kinase inhibitor, in 2019. Kalambate et al detected doxorubicin, an anthracycline antibiotic. and dasatinib, a tyrosine kinase inhibitor, simultaneously[124]. These studies are great examples for the analysis of mixtures of drugs by relatively simple electrode modifications and many similar studies on new investigational drug compounds, such as antibiotic hybrids, are expected to follow.

The increase of drug resistance in Gram-negative bacteria in particular is a major cause for concern[158,159], as many Gram-negatives cause serious infections, such as pneumonia, and few antibiotics effective against Gram-negatives have been developed due to their innate defense mechanisms including low outer membrane permeability and high number of efflux pumps. Thus, with the rise of drug resistance, many infections caused by Gram-negative bacteria have become untreatable.[160] The development of antibiotic hybrids has gained significant attention, because reports show that some antibiotic hybrids have the ability to increase the efficacy of other antibiotics, which are unable to cross the cell membrane on their own in Gram-negative bacteria.[21] These hybrid molecules represent interesting future targets for the quantitative detection of drug compounds by electrochemistry.

Although the presented review focuses on the direct detection of drug compounds at electrodes, it should be noted that other strategies have emerged to test the efficiency and antimicrobial properties of alternative treatment strategies by electrochemistry. The use of nanomaterials, such as silver nanoparticles, is one example. Unfortunately, the low selectivity of silver was found to affect the growth of human cells surrounding the implants and ineffective antibacterial effects leads to the formation of a bacterial biofilm, which was reported immune to antibacterial drugs. Arrassi *et al* in 2017[161] proposed a holistic approach to release a desired amount of silver ions into human cells. Thereby, linear sweep voltammetry was performed to determine the precise amount of silver ions on conductive surfaces.

Drug resistance is a complex phenomenon associated with different cellular mechanisms, such as intracellular drug inactivation, drug target alteration, drug efflux pumps, cell death inhibition,

and DNA damage repair.[54] Targeting single mechanism to combat this issue is therefore often unsuccessful. Efflux pumps mediated resistance is one of the most electrochemically studied mechanisms, because electrochemistry can easily quantify the flux of electrochemically active species.[54] However, literature shows that efflux pump protein expression is not directly correlated to a profound drug resistance phenotype.[162] Hence, more studies are expected to focus on other drug resistance mechanism rather than drug efflux. The detection of metabolic molecules released from cells by electrochemistry therefore presents an excellent approach to tackle the phenomenon of drug resistance. Combinatorial approaches that incorporate electrochemical methods into the biochemistry toolbox are needed to address both multi-mechanism drug resistance and the analysis of complex samples.

In summary, electrochemistry with its high sensitivity, rapid data output and cost-efficient procedures represents an immense potential to aid in both the detection of resistance and to further the understanding of underlying cellular mechanisms that ultimately lead to a drug resistant phenotype in cells. The development of devices able to detect drug compounds and drug resistance rapidly, sensitively and inexpensively would be of major benefit for clinical diagnoses, disease control, environmental monitoring and food safety. Although no immediate device for the detection of drug resistance has been developed yet, the last decade has provided fundamental steps towards the understanding of electroactive drug compounds and the recognition of drug resistance activity by electrochemistry.

CHAPTER 3: Electrochemical Characterization of Common Antibiotics

3.1. Introduction

Electrochemistry is the study of chemical reactions involving electron transfer processes. Many pharmaceutical compounds are electroactive, which can readily be oxidized or reduced as discussed in detail in Chapter 2.[79] To date, the detection of antibiotics, such as ciprofloxacin, has been conducted using different analytical techniques, such as spectrophotometry,[163] fluorometry,[14] high-performance liquid chromatography,[15] capillary electrophoresis,[164] immunoassay[165]. Most of these approaches have been proposed for the determination of antibiotics in pharmaceutical preparations.[55] Electrochemical methods, such as cyclic voltammetry and chronoamperometry, are inexpensive and are able to detect analytes at very low concentrations, such as at the femto mole range.[80]

This chapter will present the characterization of three widely used antibiotics, amoxicillin, ciprofloxacin, and tobramycin (Figure 3.1).

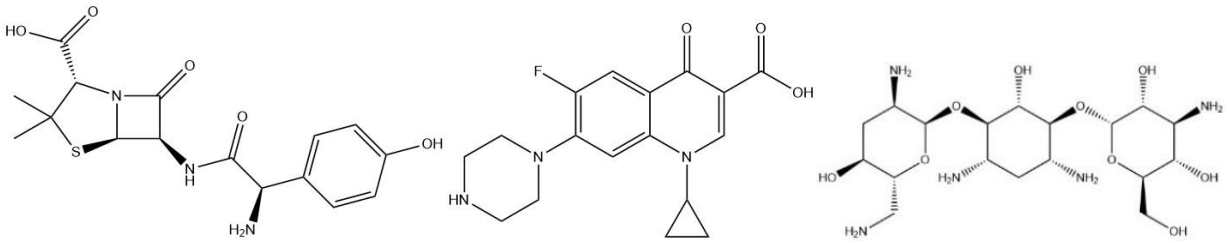


Figure 3.1: Chemical Structure of Antibiotics discussed in this chapter.

Contribution:

I performed all experiments and analysed data. Sabine Kuss designed the research, analysed data, and obtained research funding.

3.2. Experiments and Results

3.2.1. Amoxicillin

Amoxicillin (AMX) is a widely used member of the class of β-lactam antibiotics.[64] Amoxicillin is the *p*-hydroxy analogue of ampicillin and contains variable side chains, which are responsible for its rapid absorption by the human body.[62] It exerts its effect by inhibiting bacterial cell wall synthesis.[60] As it is commonly used to treat bacterial infections, its quantification in drugs and biological fluids is crucial. Various analytical methods have been reported to determine amoxicillin such as high-performance liquid chromatography, spectrophotometry, electrophoresis, and electrochemical methods. Electrochemical methods have shown high sensitivity for the determination of analytes, making these to be excellent procedures. Electrochemical methods are advantageous in comparison with other methods due to their simplicity, low cost, and rapid analysis. Amoxicillin, containing phenol groups on the side chain, can be used for voltammetric determination. Cyclic voltammetry was used to determine amoxicillin using bare platinum (Pt) electrodes. A three-electrode electrochemical cell was setup to perform the experiment: Pt electrode as the working electrode, a platinum wire as a counter electrode and Ag/AgCl as the reference electrode. All voltammetric measurements were performed using a modular SP-200 potentiostat/galvanostat controlled with EC-lab software (BioLogic, France). Temperature (25ºC) was maintained for all voltammetric measurements.

An amoxicillin solution (2mM) was prepared by dissolving the dry into 0.1 M KCl electrolyte solution at pH 7.5. Pt working electrodes are polished on a polishing pad using different grade alumina powders (1 µm, 0.3 µm, and 0.05 µm) before each measurement. After polishing, the electrodes are sonicated in nano pure water for 2 minutes to remove any alumina on the electrode surfaces. Then the working electrodes were immersed into an electrochemical cell containing 2 mM of amoxicillin (AMX)solution. Pt is the choice the of electrode after performing CV on glassy carbon electrodes (GCE) (Figure 3.7) and gold electrodes. Gold (Au) electrodes affect the anodic peak current by oxidizing Au and forming AuO (Figure 3.6).

In Figure 3.2, cyclic voltammetry was performed using a potential range from 0.2 V to 1.2 V at 50 mV s^{-1}. The cyclic voltammogram showed an oxidation peak attributed to oxidation of amoxicillin at 0.9 V at the surface of GCEs. In reverse sweep, no peak was observed indicating

that the oxidation of AMX is chemically irreversible. To study the effect of scan rate, different scan rates were used: 10 mV s^{-1}, 30 mV s^{-1}, 50 mV s^{-1}, 80 mV s^{-1} and 100 mV s^{-1}.

To evaluate the electrochemical behavior, a cyclic voltammogram of AMX was recorded in 0.1 M KCl solution at bare Pt electrodes **(Figure 3.2**). It can be seen from the figure that in absence of AMX, no redox peak was observed during cyclic voltammetry within the potential window of 0.2 V to 1.2 V. CV of AMX produced a broad irreversible oxidation peak at 0.9 V vs Ag/AgCl at a scan rate of 50 mV s^{-1}. To study the effect of the scan rate on the peak current and potential, different scan rates, from 10 to 100 mV s^{-1}, were applied to record the cyclic voltammogram of AMX at bare Pt electrodes (**Figure 3.3**). Figure 3.3 shows that higher scan rates increase the current signal. The plot between the peak current as a function of square root of scan rate revealed a linear relationship (**Figure 3.4**), which indicates the transfer of AMX molecules to the Pt electrode surface is a diffusion-controlled process. From the literature, the oxidation of amoxicillin can be attributed to the phenol group present on the side chain of AMX (see Figure 3.8).[68]

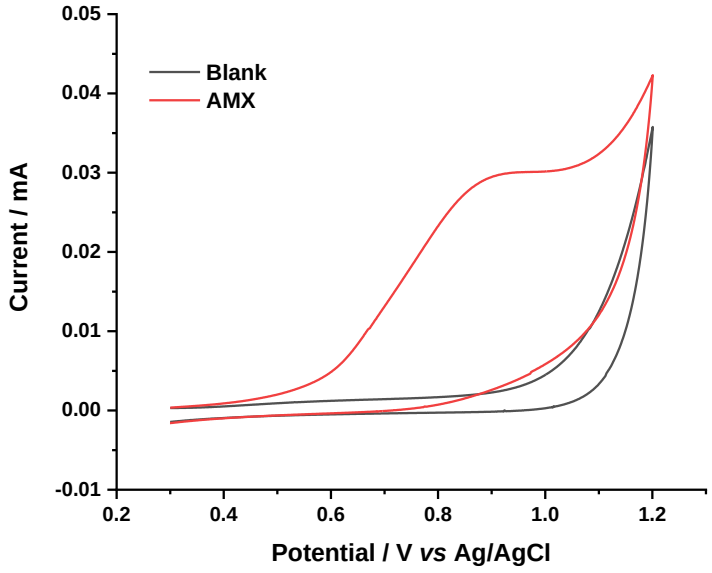


Figure 3.2: Electrochemistry of AMX. CV of 2 mM AMX in KCl at 50 mV s^{-1} at bare Pt electrode (red) and a blank presents a 0.1 M KCl solution.

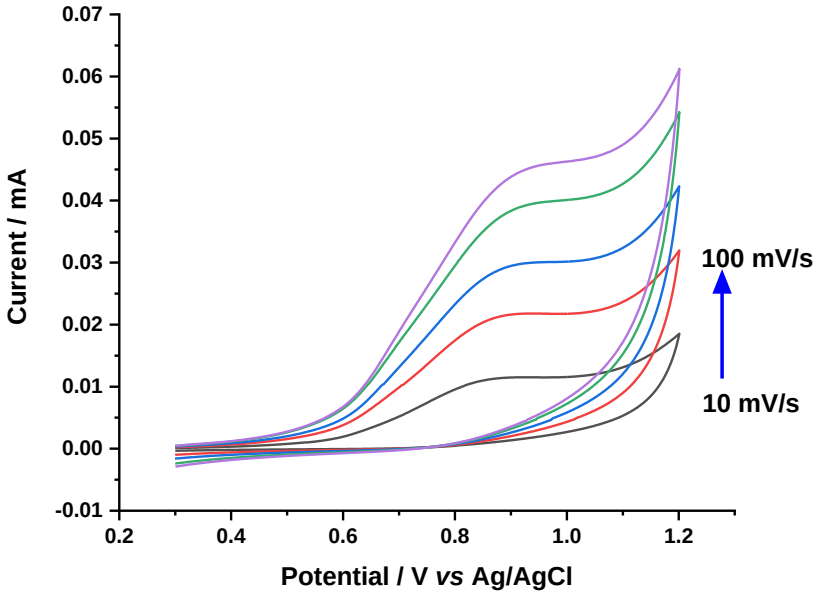


Figure 3.3: Scan rate study of AMX at bare Pt electrode. Five different scan rates ranging from 10 mV s^{-1} to 100 mV s^{-1} were applied to record CV of AMX in 0.1 M KCl.

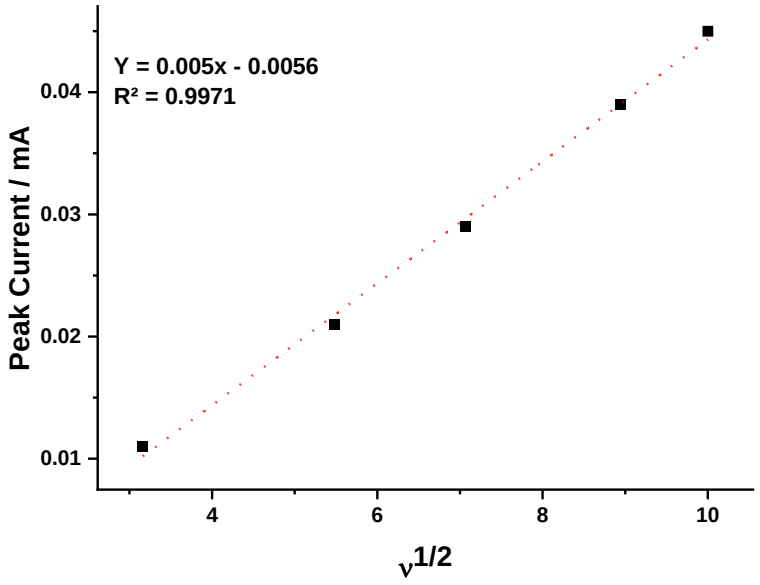


Figure 3.4: Peak current as a function of square root of scan rate of 2 mM AMX in 0.1 M KCl at bare Pt electrodes.

The limit of detection (LOD) is the lowest analyte concentration to be reliably distinguished from the background. In our experiment, LOD is defined the as the lowest concentration with a greater peak height than three times the standard deviation of the blank average. The limit of quantification (LOQ) is the lowest concentration of the analyte to be quantified at defined levels for imprecision and accuracy.[166] In our experiment, LOQ has been defined as the lowest concentration which has a greater peak height than ten times the standard deviation of the blank average. The LOD was studied by a serial dilution of AMX. A CV for each concentration was recorded (Figure 3.5). The detection limit at unmodified electrodes was found to be 3 µM, which is of the same magnitude as values reported in the literature.[65] The LOQ was determined to be 7 µM.

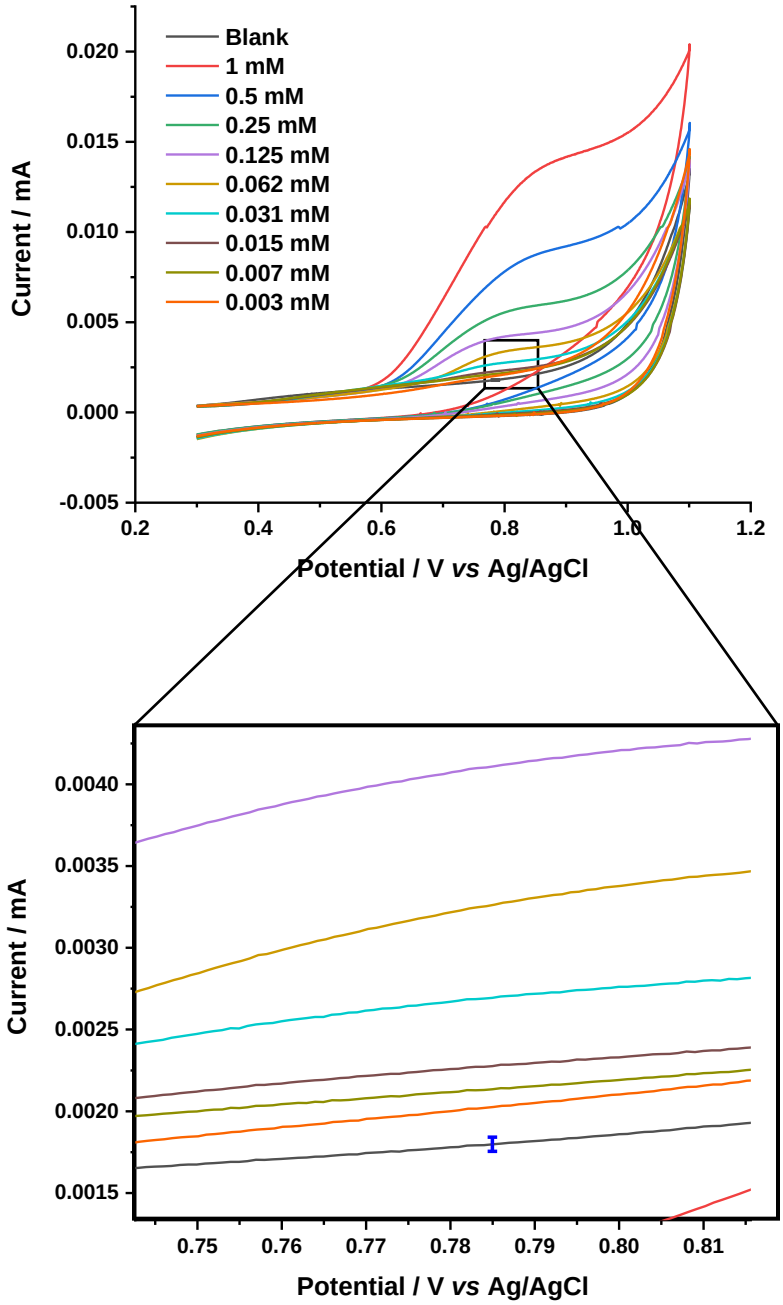


Figure 3.5: Determining the limit of detection of AMX at bare Pt electrode by CV in 0.1 M KCl at a scan rate of 50 mV s^{-1}.

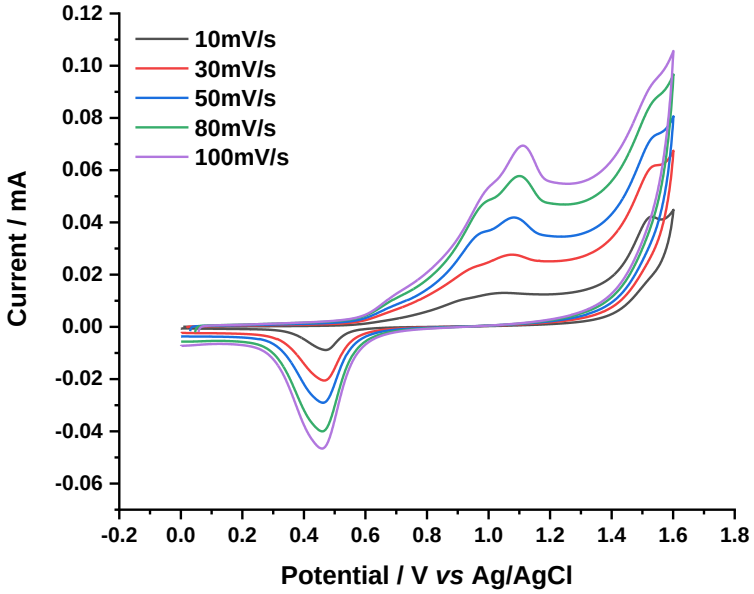


Figure 3.6: CV of Amoxicillin at Au electrode in 0.1 M KCl at different scan rate from 10 mV s^{-1} to 100 mV s^{-1}.

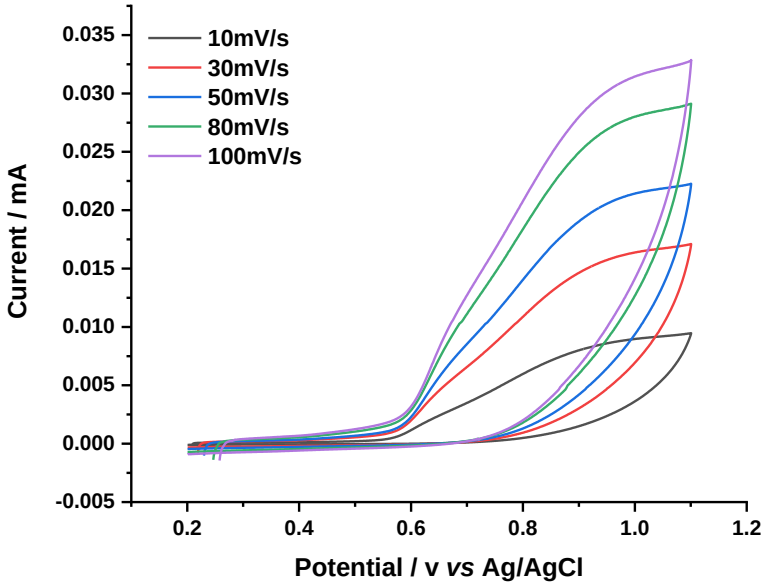


Figure 3.7: CV of 2 mM Amoxicillin at bare GCE in 0.1 M KCl solution at various scan rate ranges 10 mV s^{-1} to 100 mV s^{-1}

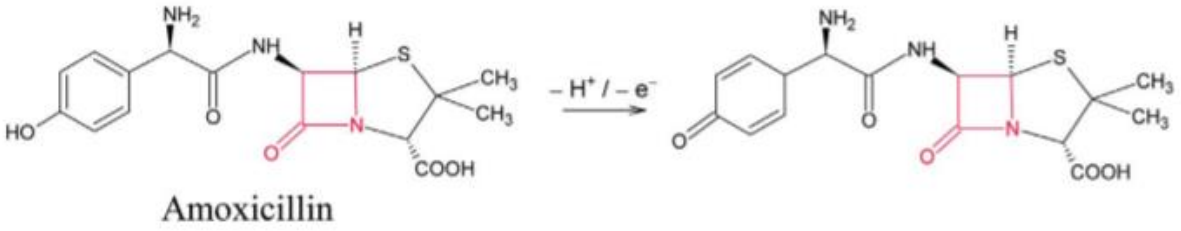


Figure 3.8: Schematic presentation of electron transfer of amoxicillin

3.2.2. Ciprofloxacin

Ciprofloxacin (CIP) is a second-generation fluoroquinolone antibiotic with an expanded spectrum of activity against gram-positive and gram-negative bacteria. Ciprofloxacin causes inhibition of DNA gyrase enzyme required for bacterial DNA replication.[55,79,82] Due to its high activity and comparatively low toxicity, CIP is used worldwide to treat bacterial infections in humans and animals.[83] The electrochemical behavior of ciprofloxacin was investigated by cyclic voltammetry using glassy carbon macro electrodes (GCEs).

Cyclic voltammogram of CIP was performed in various electrolytes solution such as $NaNO_3$, KCl, PBS, HCl. The choice of electrolytes for the further investigation of electrochemical properties of CIP is $NaNO_3$, as it produces the well-defined oxidation peak at lower potential range at bare GCEs compare to KCl, HCl, and PBS. With KCl and HCl, there is a chance of contribution to current from chloride oxidation to the electrochemical signal at higher potentials. For the electrochemical characterization of CIP, GCEs are the choice of electrodes, as they give better results compare with Pt and Au electrodes.

Ciprofloxacin (2mM) was prepared in 0.1 M $NaNO_3$ electrolyte solution at pH 3.0. The result obtained from cyclic voltammetry revealed a profound anodic peak of CIP at bare GCEs at a scan rate of 50 mV s^{-1} within the potential range 0.1 V to 1.4 V (Fig 3.9). In absence of CIP, 0.1 M $NaNO_3$ produced no easily distinguishable redox peak. The reverse sweep of CIP produced no peak current, indicating an irreversible electron transfer process. The irreversible oxidation peak was observed at 1.3 V vs Ag/AgCl at bare GCEs.

Different scan rates were applied to study the effect of scan rate on CIP oxidation at the electrode surface. **Figure 3.10** shows the enhanced electrooxidation current of CIP with increased scan rates. The study of scan rates demonstrated that the linear plot of oxidation peak current versus square root of scan rate indicates that the diffusion of electroactive species (CIP) to the electrode surface occurs (**Figure 3.11**). However, a repetitive cyclic voltammetry study showed a diminished current after the first cycle (**Figure 3.12**), which indicates that the oxidized product of CIP can be adsorbed on the surface of GCEs. Therefore, it can be concluded that transfer of CIP molecules is a diffusional-adsorption controlled process. The pH effect on the oxidation of CIP is crucial, as CIP molecules do not get dissolved completely at higher pH (≥4).

The limit of detection (LOD) was studied by cyclic voltammetry and differential pulse voltammetry. Using both techniques, the detection limit was found to be 7 µM[55] (**Figure 3.13**) and limit of quantification (LOQ) was found to be 31.25 µM (**Figure 3.14**). The oxidation of CIP can be attributed to the amine group of ciprofloxacin structure which is supported by literature (Figure 3.15).[55]

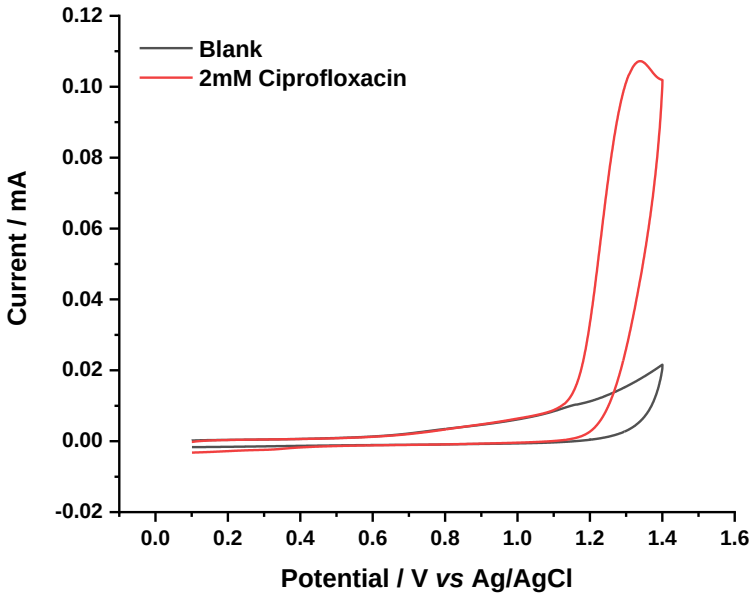


Figure 3.9: CV of ciprofloxacin in 0.1 M $NaNO_3$ at bare GCE at a scan rate of 50 mV s^{-1}. 2 mM CIP in 0.1 M $NaNO_3$ corresponds to red CV and black one is 0.1 M $NaNO_3$ without CIP molecule.

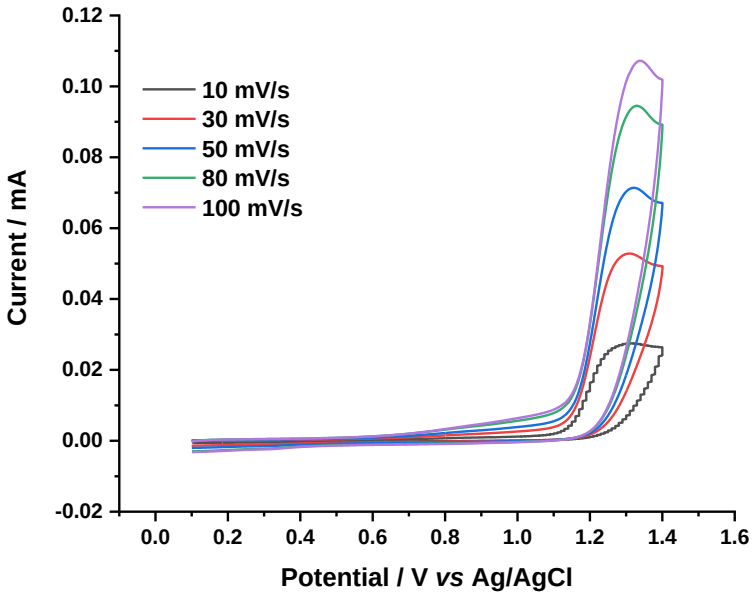


Figure 3.10: CV of CIP in 0.1 M $NaNO_3$ at bare GCE at various scan rate from 10 mV s^{-1} to 100 mV s^{-1}.

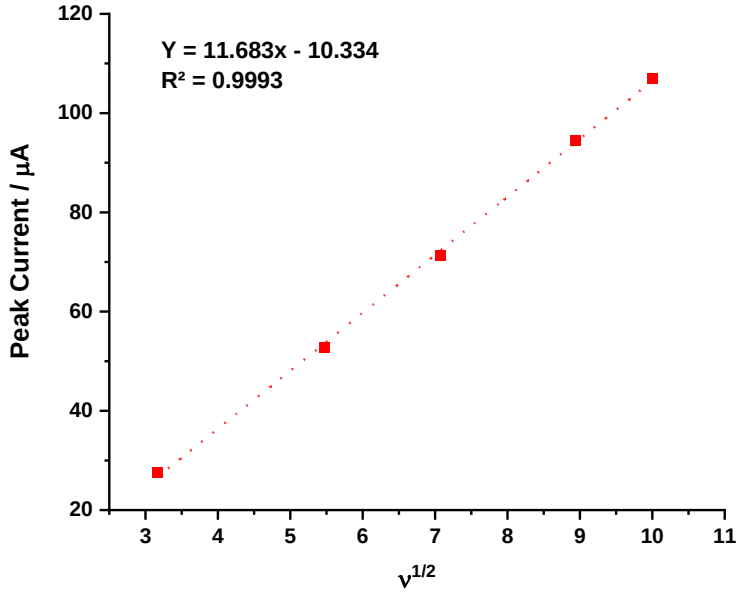


Figure 3.11: Peak current as a function of square root of scan rate of 2 mM CIP in 0.1 M $NaNO_3$ at bare GCE electrodes.

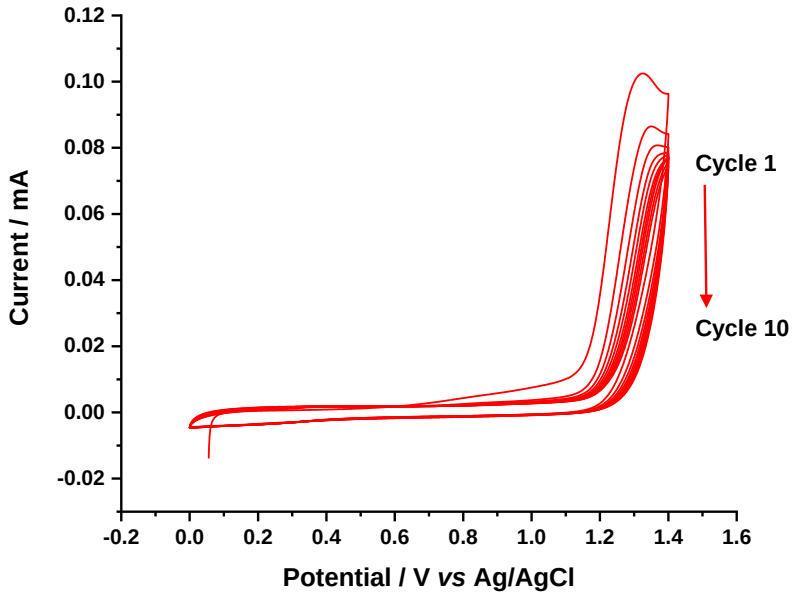


Figure 3.12: Repetitive CV of CIP in $NaNO_3$ at bare GCE at 50 mV s^{-1}

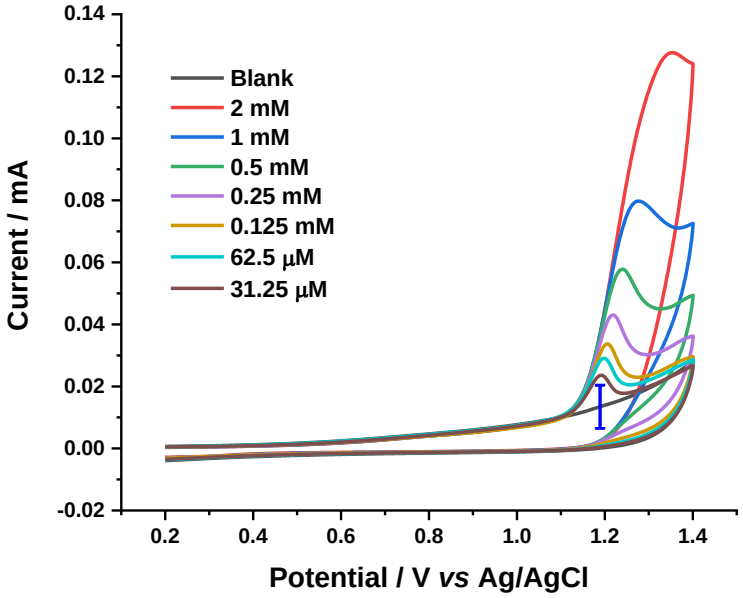


Figure 3.13: CV of CIP in various concentration at bare GCE at a scan rate of 50 mV s^{-1}

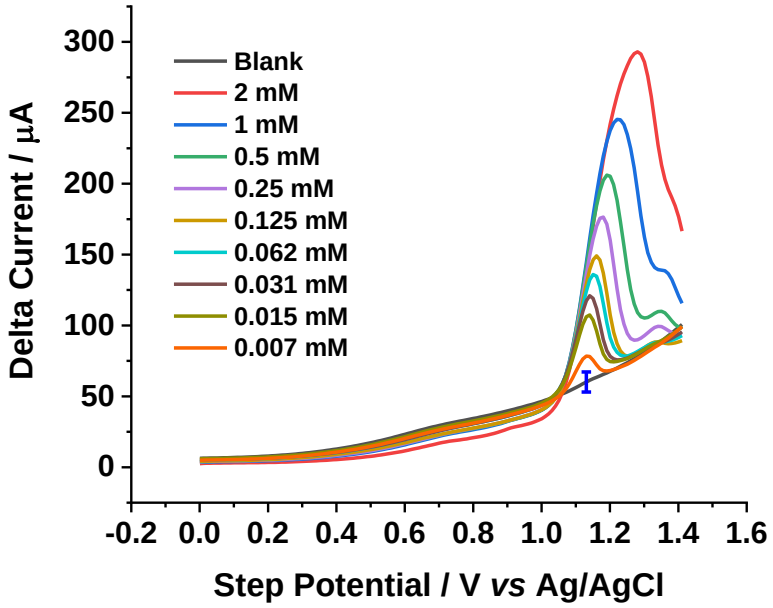


Figure 3.14: DPV of CIP in various concentration at bare GCE in $NaNO_3$ at a scan rate of 50 mV s^{-1}

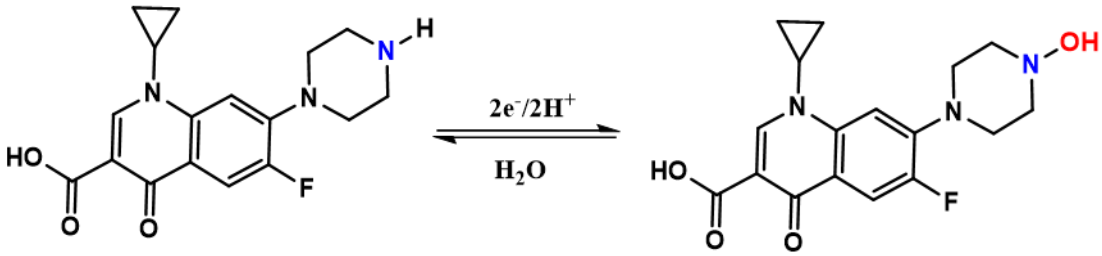


Figure 3.15: Electron transfer scheme of ciprofloxacin

3.2.3. Tobramycin

Tobramycin (TOB) is an aminoglycoside antibiotic, which is effective against both Gram-positive and Gram-negative bacterial infections. It inhibits bacterial protein synthesis which leads to cell death. Tobramycin is a water soluble aminoglycoside composed of an amino sugar linked by glycosidic bond to an aminocyclitol named 2-desoxystreptamine.[167]

For the electrochemical characterization of tobramycin, various electrolytes were analysed and the electrode material used was glassy carbon electrodes (GCEs). In Figure 3.16, the cyclic voltammogram showed electrochemical response for 2 mM TOB in 0.1 M KCl, PBS, $NaNO_3$ solution. It can be observed from Figure 3.16 that the highest electrochemical signal was observed in 0.1 M KCl solution, which makes KCl the best choice of electrolyte for further investigations of TOB by electrochemical techniques.

The result from cyclic voltammetry revealed a broad oxidation peak for 2 mM TOB at 1.54 V vs Ag/AgCl at bare GCEs surface within the potential range of 0.0 to 1.8 V (Figure 3.17). The oxidation of tobramycin is a chemically irreversible electron transfer system, as there is no peak observed in the reverse sweep.

The scan rate study was performed to analyze the effect of scan rate on the oxidation of tobramycin at the GCEs electrode surface. It is seen from Figure 3.18 that the increased scan rate results in enhanced electrooxidation current as well as a shift of the peak current in anodic direction. The linear relationship between peak current versus scan rate demonstrated that the mass transfer of tobramycin to the electrode surface is an adsorption-controlled process with a correlation coefficient $R^2 = 0.9954$ (Figure 3.19).

After performing the first cycle, without polishing the working electrode (GCEs) the second cycle was performed (Figure 3.20). The diminished current in the second cycle indicates that the oxidized product of tobramycin is getting adsorbed at the surface of the working electrode, which blocks oxidation of further tobramycin molecules.

The effect of pH on electrochemical signal of TOB was studied in the range of 3 to 9.7 in phosphate buffer solution (PBS). DPV was performed to study the pH effect. Figure 3.21 showed there is no shift in peak potential with pH, which makes the analysis pH independent. This makes our method promising for the analysis of environmental samples.

Determining the limit of detection for tobramycin was a challenge. As it can be seen (Figure 3.18) the oxidation peak of tobramycin merged with the solvent window, so lowering the concentration of tobramycin made the current in solvent window higher, impairing the oxidizing current from tobramycin.

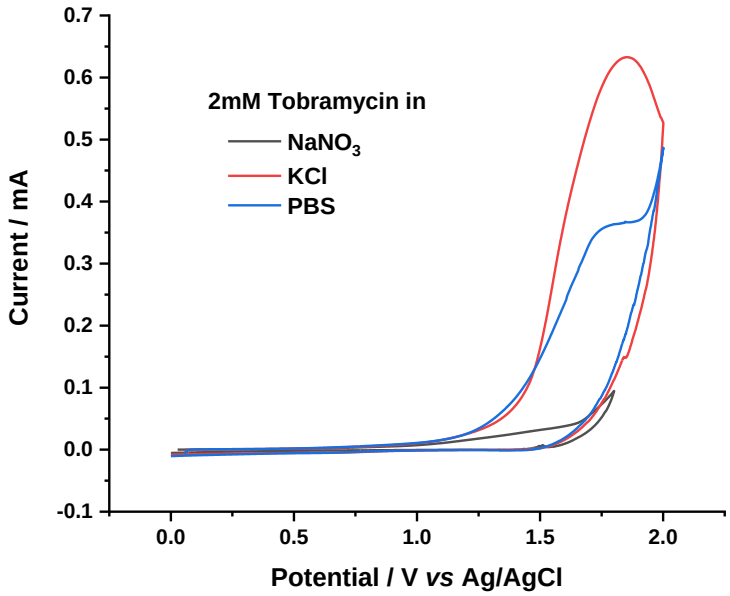


Figure 3.16: CV of 2 mM TOB in 0.1 M of $NaNO_3$, KCl, and PBS solution at bare GCE at a scan rate of 50 mV s^{-1}

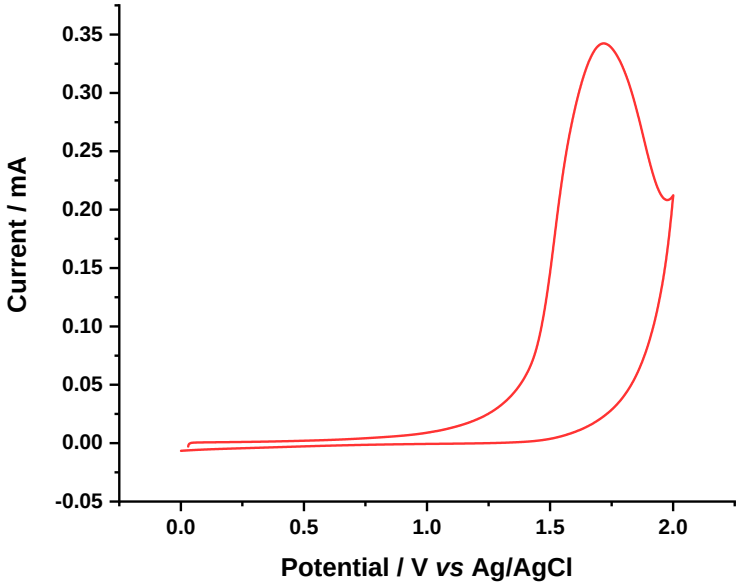


Figure 3.17: CV of 2 mM tobramycin in 0.1 M KCl solution at bare GCE at a scan rate of 50 mV s^{-1}

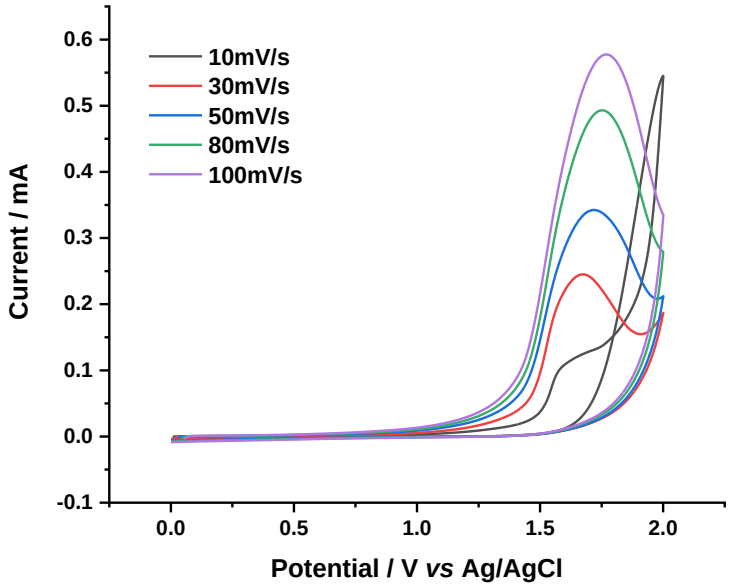


Figure 3.18: Scan rate effect on CV of tobramycin. CV of TOB in 0.1 M KCl with different scan rate by GCE

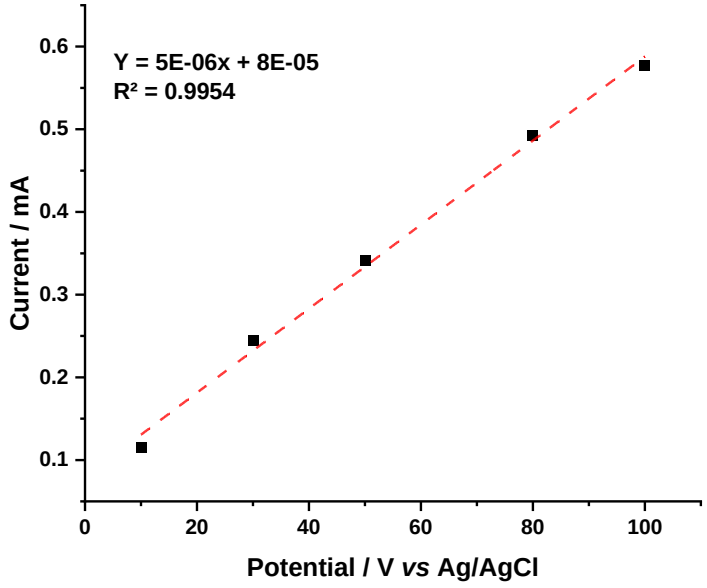


Figure 3.19: Relationship between peak current of TOB in a various scan rate versus scan rate

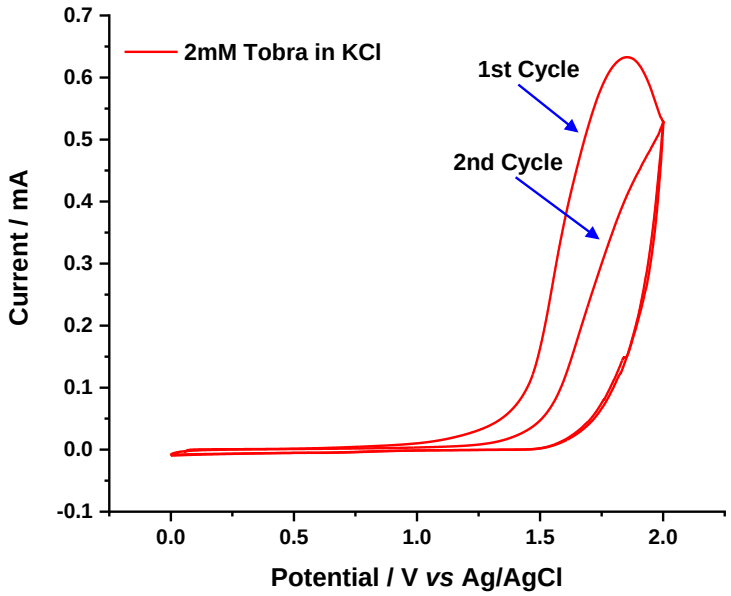


Figure 3.20: Repetitive CV of tobramycin in 0.1 M KCl solution at bare GCE at a scan rate of 50 mV s^{-1}

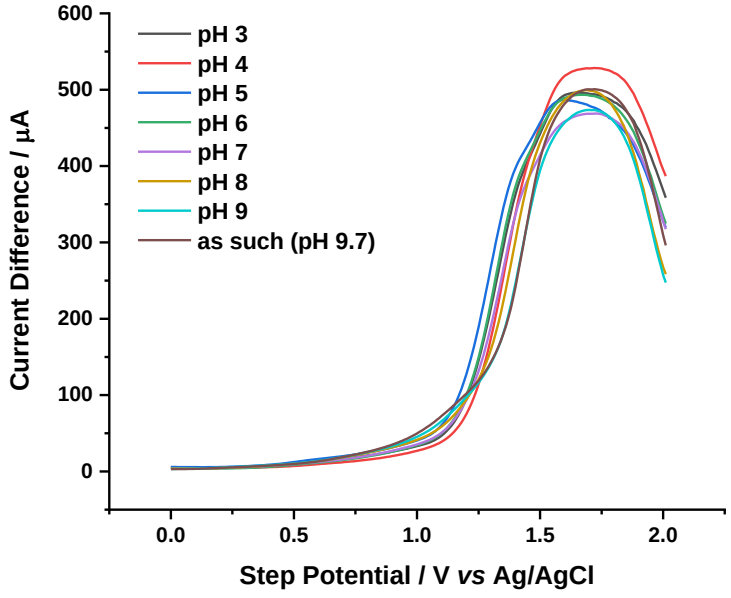


Figure 3.21: DPV of TOB in 0.1 M KCl at bare GCE with different pH of the solution ranges from 2 to 9.7

3.3. Summary

Electrochemical characterization of three antibiotics, amx, cip, and tob, were performed at bare electrodes. Electrochemical properties such as redox behavior, peak potential, mass transport, electron transfer mechanisms, choice of electrodes and suitable electrolytes were explored, which would be a reference for bio-electrochemical studies in Chapter 5 and 6. In future, various electrode modifications could be employed to meet different purposes. Nanoparticles modification, for example, could be performed to enhance the electrochemical signal and aptamer modification could be done for selective measurements.

CHAPTER 4: Electrochemical Characterization of a Novel Hybrid Compound

This chapter focuses on the electrochemical characterization of a recently developed antibiotic hybrid. The increase of drug resistance particularly in Gram negative bacteria is a major concern because of their quick adopting new resistance mechanism.[25] There are only few antibiotics in the pipeline which are effective against multi drug resistant Gram-negative bacteria.[4] Now researchers are developing combined molecules to combat this issue. For instance, Ceftazidime/avibactam, a combination of oxyimino cephalosporin with a diazabicyclooctane non-β-lactam β-lactamase inhibitor, is under clinical trials against multi drug resistant Gram-negative bacteria.[4] Reports show that some antibiotics show weak activity on their own, but making hybrid compounds augments their antibacterial activity.[3] Electrochemistry will be an appealing choice of technique for the assessment the novel hybrid compounds as potential antimicrobial agents. We analyzed the electrochemical properties of a ciprofloxacin-tobramycin hybrid. This work has been published in Electrochemistry Communications Journal in 2020 volume 119 Number 106825 titled "Electrochemical characterization of the antibiotic hybrid ciprofloxacin-tobramycin".

Contribution:

I performed experiments, analysed data, and wrote the manuscript. Dr Vikram Singh treated the data and wrote the manuscript. Derek Ammeter and Prof Dr Frank Schweizer designed and performed synthesis of CIP-TOB. Sabine Kuss designed the research, analysed data, wrote the manuscript and obtained research funding.

4.1. Research Article

Electrochemical characterization of the antibiotic hybrid ciprofloxacin-tobramycin

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4.2. Abstract

Electrochemical sensing of antibiotics is important to assess environmental contaminations. Due to the emergence of drug resistant bacteria alternative medication, such as hybrid drug compounds are being developed. The present study reports a comprehensive electrochemical characterization of the antibiotic hybrid Ciprofloxacin-Tobramycin. The electrochemical behaviour of the hybrid was monitored by cyclic voltammetry and differential pulse voltammetry, identifying a well-defined oxidation behaviour. Levels of detection and quantification were determined at bare glassy carbon electrodes. This is the first electrochemical characterization of an antibiotic hybrid drug, providing information about detection parameters and conditions towards the efficient electrochemical sensing of hybrid compounds *in vitro* and in the environment.

Keywords: Ciprofloxacin, Tobramycin, Hybrid Antibiotic, Electrochemical Sensing, Multidrug Resistance

4.3. Introduction

Multidrug resistance has become a global crisis, which poses a serious threat to the global public health.[46] According to the Centers for Disease Control and Prevention, approximately 2.8 million illnesses are subject to drug resistance each year in the United States alone, and 10 million deaths are attributed to drug resistance worldwide .[168] These numbers are expected to double by 2030, owing to the excessive use of antibiotics against various Gram-negative and Gram-positive bacteria. As a result, disease treatments against severe bacterial infections become ineffective.[169] International health organizations, including the World Health Organization, have called for the urgent development of effective treatment strategies to tackle drug resistance.[170] Antibiotics, such as Ciprofloxacin (CIP) and Tobramycin (TOB), emerged as revolutionary medicines in the health industry for treating a wide range of infectious diseases in both livestock and humans.[71,169,171] However, many pathogens have already developed resistance towards various antibiotics.[168,169,172] To this end, the design and development of hybrid antibiotics has received significant attention in the recent past.[21] The fundamental concept behind antibiotic hybrids is the linkage of two or more complementary pharmacophores within a single synthetic construct, which are known to offer antimicrobial effects. Literature has reported an enhanced effectiveness of the antibiotic hybrid adjuvant of Ciprofloxacin-Tobramycin (CIP-TOB) against drug resistant bacteria compared to its individual antibiotic constituents.[173,174] In particular, CIP-TOB was able to potentiate multiple classes of antibiotics and cancer drugs in combination therapy against wild-type and multidrug-resistant *Pseudomonas aeruginosa* isolates.[21,173]

Many antibiotics are excreted from the living body *via* urine and feces, ultimately contaminating both soil and water, and accelerating the spread of drug resistance.[175] Related effects, such as nephrotoxicity and ototoxicity are serious health issues present especially in developing countries. Therefore, a sensitive method to detect antibiotic contaminations in the environment is needed [2]. Various analytical techniques such as HPLC[176], gas chromatography[177] and colorimetric methods have been successfully used to detect antibiotics in soil, water and animal products.[50] Although these techniques have shown high sensitivity and selectivity, their application is time-consuming, expensive and not suitable for on-site detection. Electrochemical methods on the other hand offer high sensitivity and selectivity, cost effectiveness and a rapid result readout.[178]

In the presented study, and for the first time, an antibiotic hybrid molecule is fully characterized by electrochemistry. The compound CIP-TOB was analysed using cyclic voltammetry (CV), and differential pulse voltammetry (DPV) at the surface of bare glassy carbon electrodes (GCEs).

4.4. Result and Discussion

Ciprofloxacin-Tobramycin (CIP-TOB) consists of ciprofloxacin (CIP) and tobramycin (TOB) constituents, linked by 12-carbon groups (**Figure 4.1.A**), and was synthesized using a previously established multi-step synthesis protocol.[173,179] The electrochemical setup comprised of a 3 mm GC working electrode, an Ag/AgCl reference and a Pt counter electrode. Other information regarding materials and methods can be found in the electronic supplementary information (SI).

Initially, CV was performed in 1 mM CIP-TOB, dissolved in 0.1 M KCl to drive the electrochemical oxidation of the compound. As shown in **Figure 4.1.B and 4.5.A**, a pronounced oxidation peak and a smaller shoulder peak were observed during the anodic scan at peak potentials of 1.5 and 1.2 V *vs.* Ag/AgCl, respectively. The main oxidation peak is attributed to the oxidation of TOB present in the hybrid, whereas the shoulder peak is identified as Ciprofloxacin moiety. Controlled measurements were carried out in an electrolyte solution containing the individual constituents CIP and TOB. In the presence of 2 mM CIP, **Figure 4.5.B** shows an oxidative peak current at 1.4 V *vs.* Ag/AgCl, which was absent during CV without the addition of CIP (**Figure 4.5.D**). These results are in agreement with the literature, reporting the oxidation of individual CIP .[180] CV measurements performed in TOB revealed an oxidation peak at 1.7 V *vs.* Ag/AgCl, which confirms the origin of second peak in the hybrid compound CIP-TOB, as shown in **Figure 4.5.C**. Interestingly, a cathodic peak potential shift was observed for both CIP and TOB constituents of about 230 mV and 215 mV, respectively, during the oxidation of the hybrid (**Figure 4.5.A**). This indicates that the oxidation of both CIP and TOB is facilitated in the hybrid molecule at the unmodified GCE. No faradaic current was observed during the reverse scan (**Figure 4.1.B)** which indicates an irreversible electrochemical oxidation process for CIP-TOB at the unmodified GCEs. The effect of different electrolytes viz $NaNO_3$, KCl, HCl and phosphate buffer solution was assessed for both CIP and TOB individual compounds. As shown in **Figure 4.6**, TOB shows the most well-defined oxidation peak in KCl solution. Hence, 0.1 M KCl solution was confirmed as ideal electrolyte to further characterize CIP-TOB by electrochemistry.

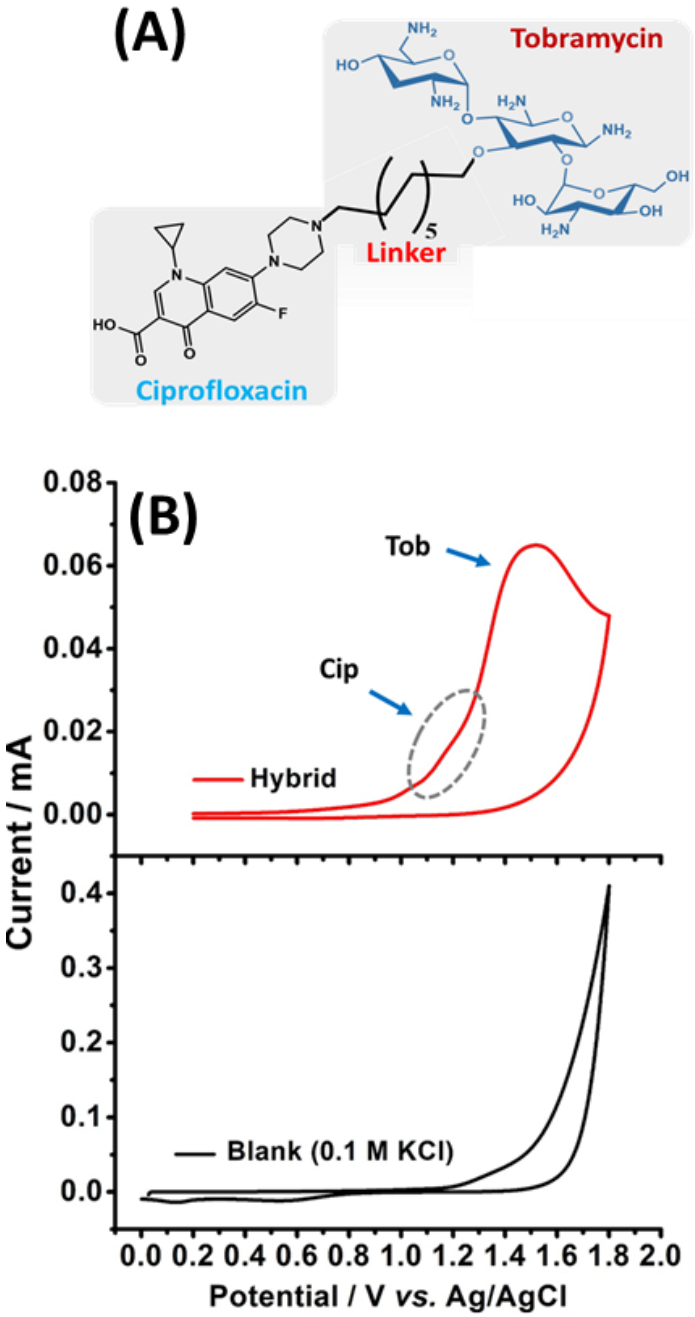

Figure 4.1: Electrochemical recognition of CIP-TOB. (A) Molecular structure of the CIP-TOB hybrid, and (B) its electrochemical oxidation during cyclic voltammetry (red) at 50 mV s^{-1} in 0.1 M KCl. A blank was recorded in 0.1 M KCl (black).

In order to assess the oxidation mechanism of CIP-TOB at the GCE surface, CV was performed at various scan rates ranging from 10 mV s^{-1} to 100 mV s^{-1} in 0.1 M KCl. As shown in **Figure 4.2.A**, an enhancement of peak current with increasing scan rate was observed. In fact, an increase in oxidation peak sharpness was observed with increasing scan rates, whereby the peak potential remained constant. A linear relationship was determined between the anodic peak current and the scan rate (**Figure 4.2.A inset**), which indicates an adsorption-controlled process. A scan rate analysis was also performed for both individual CIP and TOB by CV. **Figure 4.2.B** and **4.2.C** reports an increase in the peak current with increasing scan rate for both species. A linear relationship between peak current *vs.* square root of scan rate was observed (**Figure 4.2.B and C insets**), similarly to CIP-TOB and confirming an adsorption-controlled process. Furthermore, a deposition of the oxidation product of CIP-TOB was observed during CV. As shown in **Figure 4.7.A**, a pronounced decrease in peak current can be seen after the first cycle. Faradaic current contributions become negligible as the number of cycles increases (**Figure 4.7**).

The effect of the solution's pH on the electrochemical oxidation of CIP-TOB at GCEs was studied to demonstrate the feasibility of compound detection in varied environment. CV was conducted at various pH values ranging from acidic (2.0) to alkaline (10.0) conditions. As shown in **Figure 4.3.D** and **Figure 4.8,** no significant change in peak position and current was observed as the pH of the solution varied. This indicates that the electro-oxidation of CIP-TOB is not affected by the pH of the solution and is therefore a suitable candidate for onsite environmental monitoring.

Since the peak current during the oxidation of CIP-TOB is affected by the oxidation of the electrolyte, DPV measurements were performed. **Figure 4.3.A** presents DPV performed in 1 mM CIP-TOB in 0.1 mM KCl at a pulse height of 100 mV for 10 ms and a step height of 10 mV after every 100 ms. Two distinct oxidation peaks were observed, which correspond to the oxidation of both CIP and TOB and are in good agreement with the results of CV under identical experimental conditions. A concentration variation study was carried out for the electroanalysis of CIP-TOB to assess the quantitative detection of the hybrid compound. DPV at various concentrations of the hybrid, ranging from 0.007 mM to 1 mM, at pH 9.5 revealed two distinguishable peaks (**Figure 4.3.B**). Because electrolyte oxidation is significantly affecting the

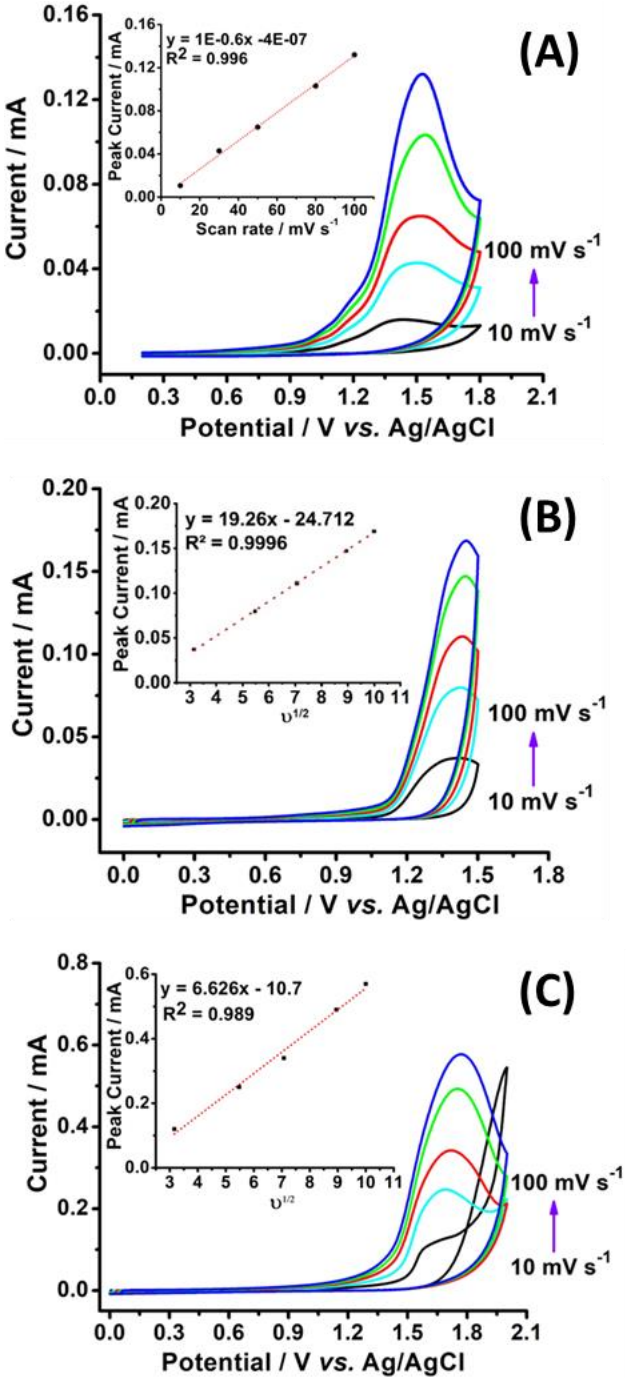

Figure 4.2: Electrochemical analysis of the CIP-TOB oxidation process. (A) Cyclic voltammogram of 1 mM CIP-TOB collected at scan rates ranging from 10 mV s^{-1} to 100 mV s^{-1}. CV in 2 mM of (B) CIP and (C) TOB. Insets showing peak currents as a function of scan rate.

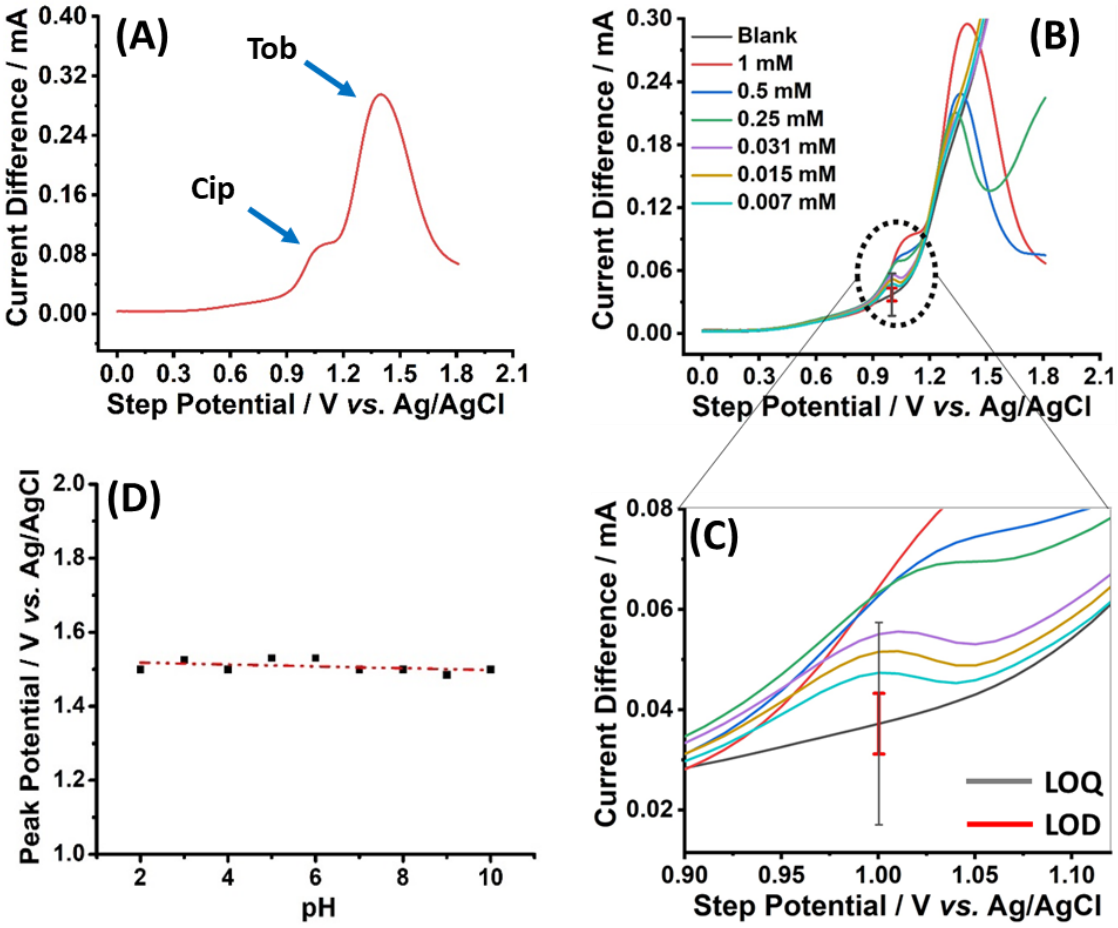

Figure 4.3: (A) DPV showing the well-defined oxidation peaks for both CIP and TOB moieties present in the CIP-TOB hybrid (1 mM CIP-TOB in 0.1 M KCl). Quantitative determination of CIP-TOB. (B) DPV collected at various concentration, (C) concentration study revealing (C) LOD and LOQ for CIP-TOB and (D) peak potential vs. pH for CIP-Top hybrid.

TOB oxidation current, only the CIP signal was considered for the quantitative analysis of CIP-TOB detection (**Figure 4.3.C**). A limit of detection (LOD) for CIP-TOB was determined. This represents the lowest concentration of hybrid, at which its oxidation yields in a signal-to-noise ratio of ≥ 3 during DPV measurements. An LOD of 7 µM was determined, which is in good agreement with the LOD for individual CIP, as shown in **Figure 4.9**. The limit of quantification (LOQ), representing a signal-to-noise ratio of ≥ 10, was found at 0.25 mM (**Figure 4.3.C**), which is in good agreement with the LOQ of individual CIP.

The quantitative electrochemical conversion of the hybrid during the oxidation process was also analysed by evaluating the surface coverage (Γ) at the electrode surface using equation (4.1)

$$\Gamma = \frac{Q}{nFA} \tag{4.1}$$

where, Γ stands for surface coverage, Q represents the integrated charge of the oxidation peak (in Coulombs), A is the electroactive area of the GCE and n denotes the number of transferred electrons during the process .[181,182] The surface coverage was calculated to be 0.78 nmol cm^{-2}.

4.5. Conclusions

In summary, the presented study demonstrates for the first time the electrochemical detection and characterization of an antibiotic hybrid drug. CIP-TOB was characterized by electroanalytical methods and revealed important parameters, such as an adsorption controlled oxidation, which is pH independent. LOD and LOQ values were determined. Because CIP-TOB can be detected at unmodified CGEs and various pH conditions, the presented study forms the bases of potential future CIP-TOB sensing applications. With straight forward methodologies, simple instrumentation and high sensitivity, electrochemistry offers a rapid, cost-effective and eco-friendly alternative for the determination of hybrid drug compounds in real samples. This has the potential to greatly impact the environmental detection of antibiotics in the fight against drug resistance.

4.6. Supporting Information

Electrochemical characterization of the antibiotic hybrid ciprofloxacin-tobramycin

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4.6.1. Experimental Section:

Materials and Chemicals

In the present study, Ciprofloxacin (CIP) and Tobramycin (TOB) were purchased from BioBaSic (750-950 µg/mg, Canada) and AK Scientific (>900 µg/mg, Canada) respectively. The Sodium nitrate ($NaNO_3$, ≥99.9%) was obtained from Sigma Aldrich (USA) and Potassium chloride (KCl, ≥99.9%) from Fischer Chemical (Belgium). All the chemicals were used as received.

For the electrochemical characterization, all solutions were prepared using Millipore water with a resistivity of no less than 18.2 MΩ cm at room temperature.

In order to understand the electrochemical behaviour of CIP, TOB and CIP-TOB, an electrolyte solution of 0.1 M of KCl was prepared and 2.0 mM of analyte (TOB or hybrid) was added. In case of CIP, 0.1 M $NaNO_3$ was used as electrolyte.

4.6.2. Electrochemical evaluation

All electrochemical measurements were carried out using a modular SP-200 potentiostat/galvanostat controlled with EC-lab software (BioLogic, France). All electrochemical measurements were carried out using a single chamber glass cell composed of a three electrodes set-up inside a faraday cage (Custom made by University of Santa Barbara, CA, USA) at 25 °C. A 3 mm glassy carbon electrode (BASI) was used as working electrode, a Pt wire functioned as counter electrode (CE) and a Ag/AgCl/3 M NaCl (BASI) was employed as a reference electrode (RE). Prior to each measurement, the working electrode was polished using alumina slurry (1.0,

0.3, 0.05 µm, Buehler, USA) on the nylon polishing cloths (Struers, Germany). After polishing, the GCE was sonicated for 2 minutes prior to electrochemical measurements.

Cyclic voltammetry (CV) and differential pulse voltammetry (DPV) were performed to characterize CIP, TOB and hybrid. For the electrochemical characterization of CIP, the potential window was chosen as 0.0 to 1.5 V *vs.* Ag/AgCl, whereas TOB measurements were performed at a range of 0.0 to 2.0 V *vs.* Ag/AgCl. The electrochemical behaviour of CIP-TOB was analyzed at a potential window of 0.0 to 1.8 V *vs.* Ag/AgCl. All DPV measurements were performed with a pulse height of 100 mV for 10 ms and step height of 10 mV after every 100 ms in their particular potential window. All electrochemical measurements were at room temperature (25 °C).

CIP exhibits a secondary amine group in its structure (**Figure 4.4**), which acts as a basic center with the availability of non-bonding electrons as donor. Therefore, it is suspected that the oxidation of CIP takes place at the –NH group **(Figure 4.4)** to form -N-OH.

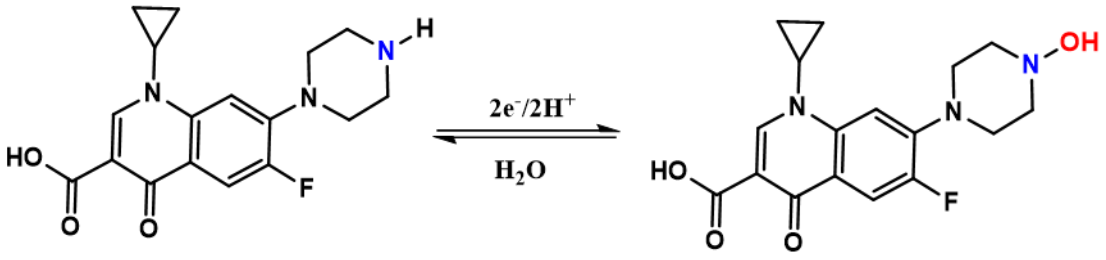

Figure 4.4: Mechanism for the electro-oxidation of CIP.

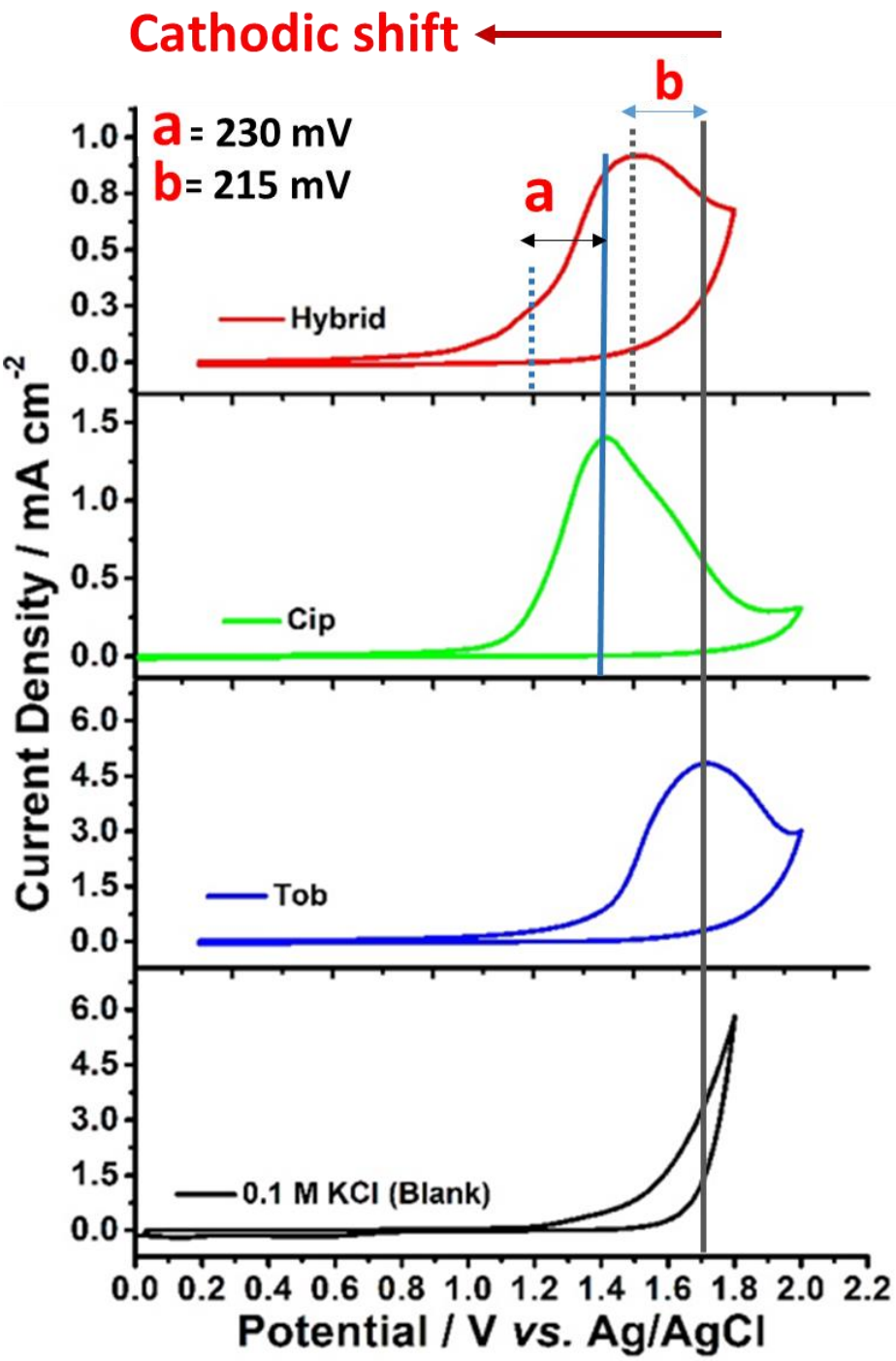

Figure 4.5: Cyclic voltammograms in 1 mM CIP-TOB (A), CIP (B), TOB (C) and KCl (D), showing a shift in oxidation potential of individual constituents of CIP-TOB. Measurements taken at 50 mV s^{-1}. The current was normalized with respect to the geometric area of the GC working electrode (3 mm).

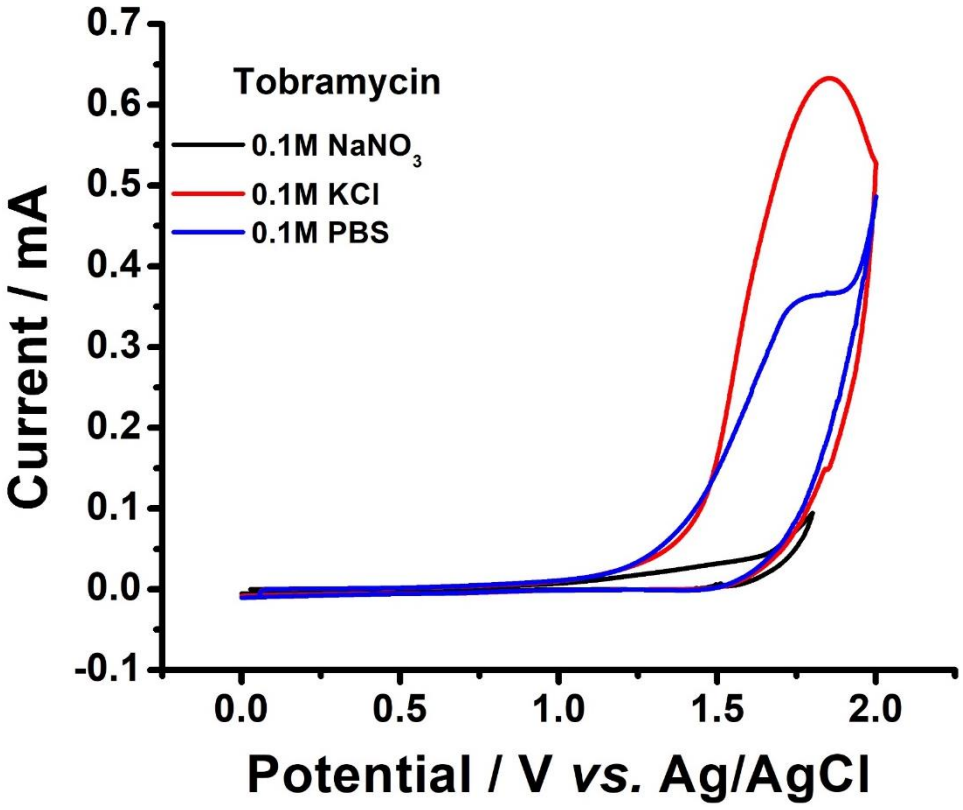

Figure 4.6: Cyclic voltammogram of 2 mM TOB at various electrolytes at 50 mV s-1.

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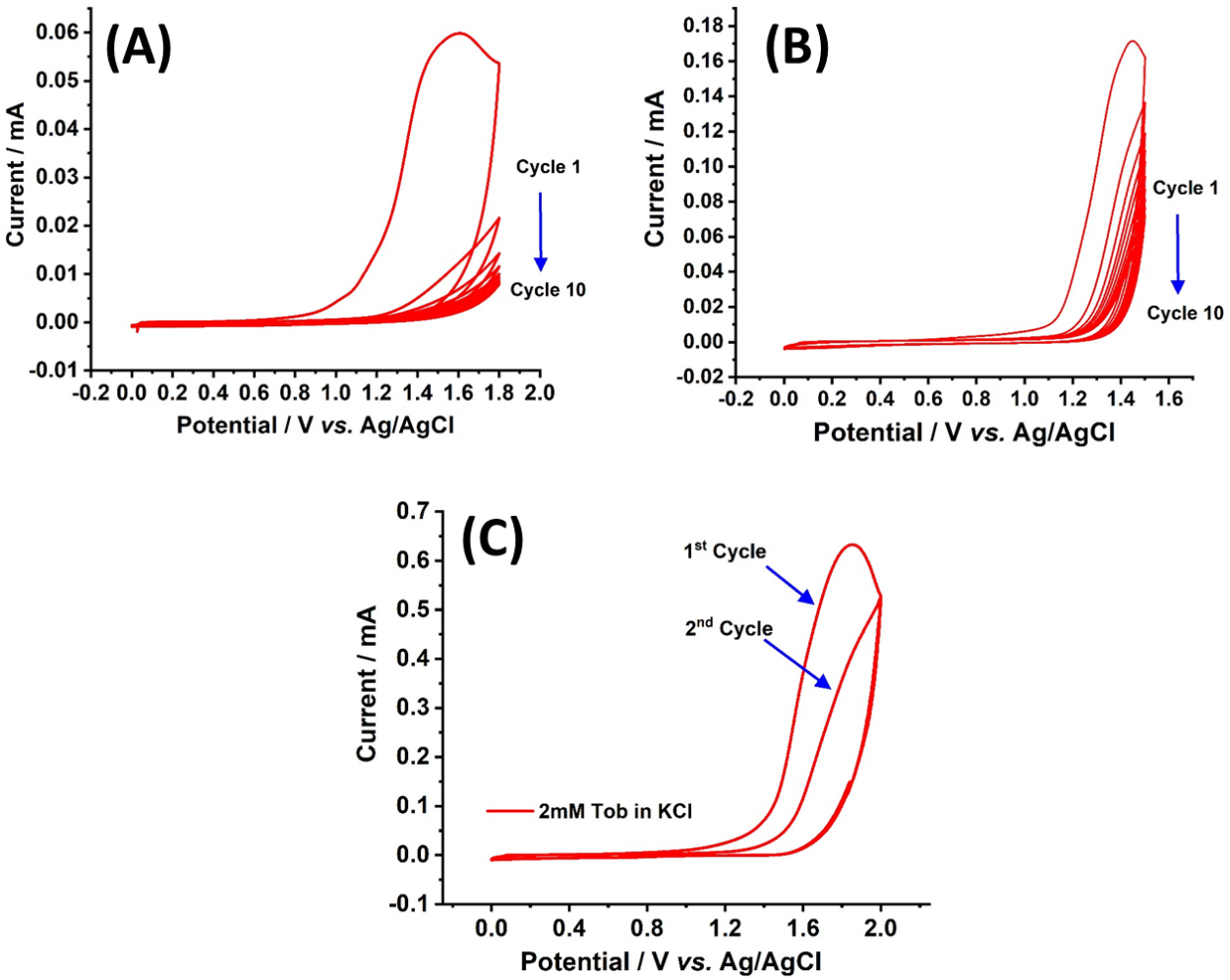

Figure 4.7: Cyclic voltammograms depicting the decreased oxidative current upon subsequent cycling for (A) CIP-TOB, (B) CIP and (C) TOB under identical condition at 50 mV s^{-1} in 0.1 M KCl at room temperature.

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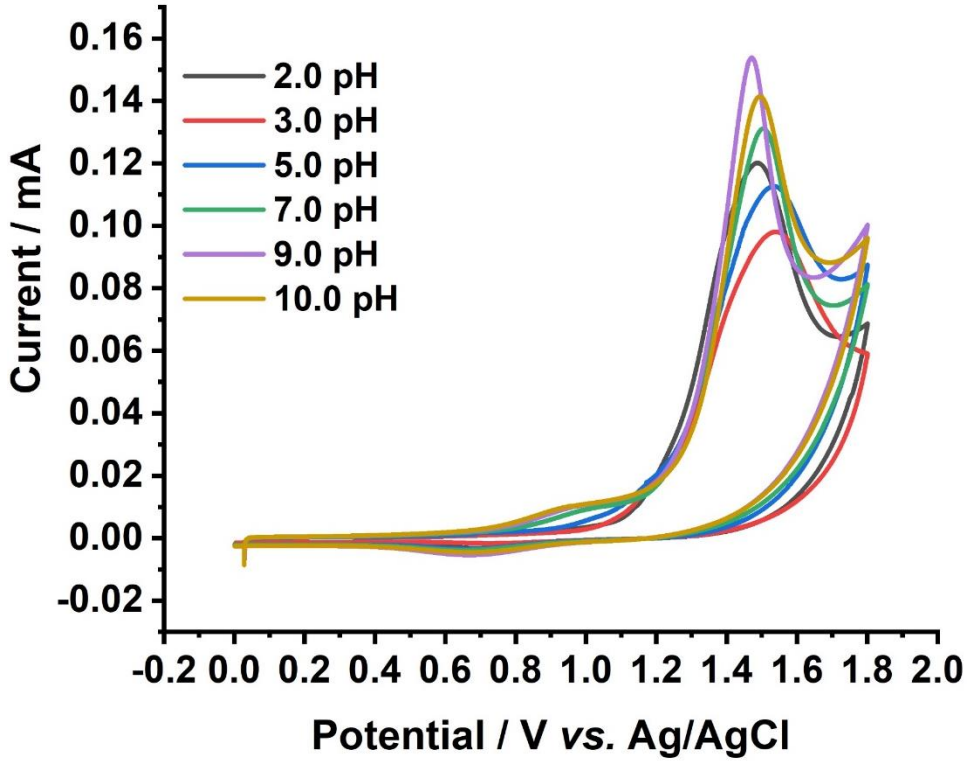

Figure 4.8: Cyclic voltammograms of CIP-TOB at 50 mV s^{-1} at various pH conditions at room temperature.

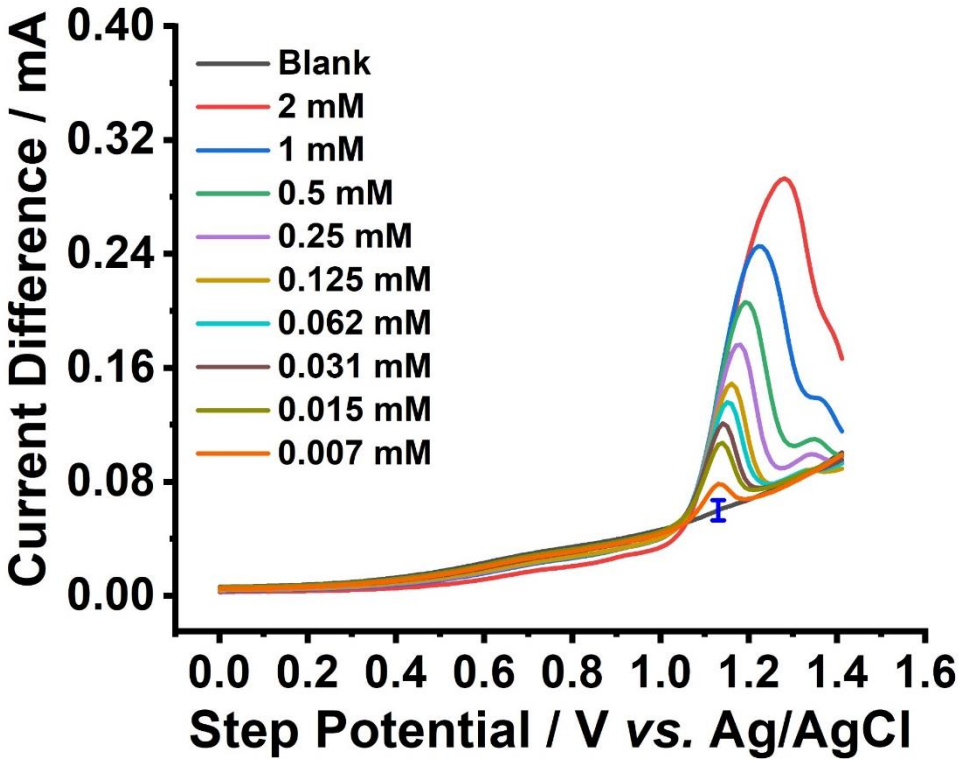

Figure 4.9: DPV of CIP at various concentrations at 50 mV s^{-1} in 0.1 M KCl

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CHAPTER 5: Quantification of Antibiotic Influx

The electrochemical characterization of common antibiotics (Chapter 3) and the hybrid drug ciprofloxacin-tobramycin (Chapter 4) provide the basis for the detection of antibiotic uptake in bacteria. Antibiotic influx quantification gives direct insight into the degree of antibiotic resistance, as most antibiotics have to go through the bacterial cell envelope to exert their antibacterial action.[30]

5.1. Introduction

To date most antibiotics have intracellular targets and they must enter the cytosol to exert their antibacterial effect.[31] The entry mechanism of antibacterial agents into the cell is crucial to unravel antibiotic resistance. Gram negative bacteria possess sophisticated cell envelop is containing outer and inner membranes, whereas Gram positive bacteria do not have an outer membrane (Figure 5.1).[33,183] The double-membrane cell envelope of Gram-negative bacteria acts as a selective barrier which facilitates the uptake of small molecules including nutrients and drugs, thus providing an extra layer of protection. This complex entry pathway of antibiotics to Gram negative bacteria makes them more resistant to multiple classes of antibiotics than Gram-positive bacteria.[31,184–187] High dose of antibiotics are used due to low permeability, which causes toxic side effects.

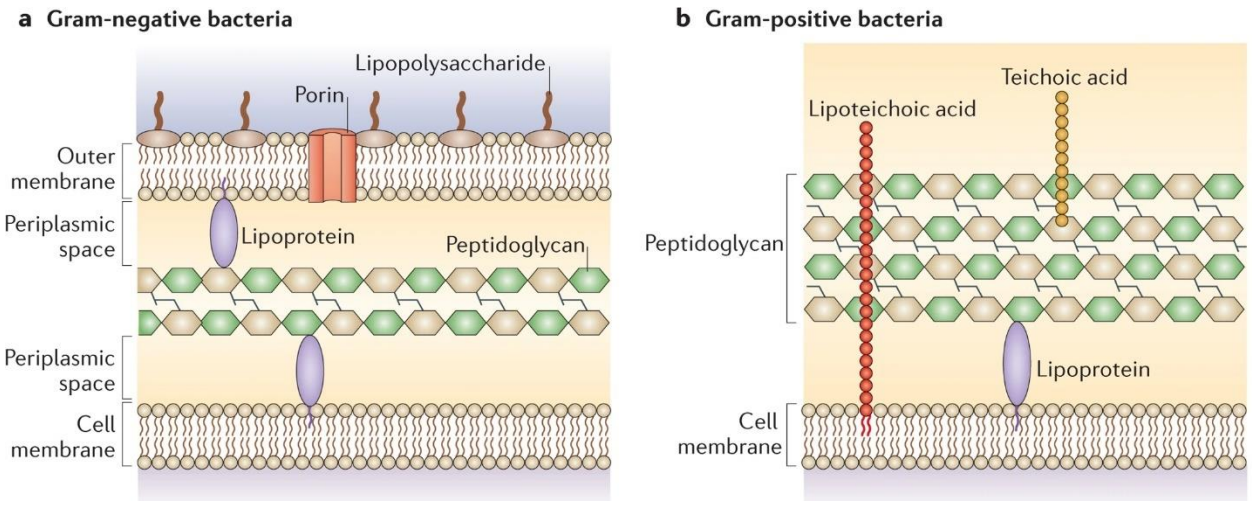


Figure 5.1: Structure of Bacterial Cell Wall[188]

Gram negative bacteria have a multifaceted cell envelope composed of a cytoplasmic membrane (inner membrane, symmetric phospholipid bilayer), a thin peptidoglycan layer and an outer membrane. The outer membrane is an asymmetric bilayer composed of an inner leaflet and an outer leaflet. The inner leaflet contains phospholipids, while lipopolysaccharides are predominantly present in the outer leaflet. Outer leaflet lipopolysaccharides consists of lipid A, Core oligosaccharides and O antigen.[31] In addition to those, the outer membrane contains large numbers of different types of proteins. For instance, murein lipoprotein (Lpp), OmpA and general diffusion porins are present. The core region, containing carbohydrate chains, plays important roles in providing the outer membrane its hydrophobic nature.[31,33,183] This hydrophobic nature of the outer membrane retards the passage of hydrophobic antibiotics such as aminoglycosides (Gentamycin, kanamycin), macrolides (erythromycin), rifamycin. Like a standard phospholipid bilayer, the outer membrane acts as a barrier for diffusion of hydrophilic molecules. Water filled β barrel channel structures (porins) are located on the outer membrane, through which necessary nutrients and metabolic substrates can penetrate. They are size exclusive and prefer hydrophilic charged molecules. Hydrophilic antibiotics such as β-lactams, tetracyclines, chloramphenicol, fluoroquinolones, pass through these porin channel proteins. The loss of porins, therefore, or any significant change due to mutation, lead to the development of antibiotic resistance. This has been reported in a large number of organisms: *E. coli, P. aeruginosa, Neisseria gonorrhoeae, Enterobacter aerogenes* and *Klebsiella pneumoniae.*[22,189,190]

5.2. Influx Quantification Methods in the Literature

The quantification of influx of antibiotics upon penetration is necessary to detect the development of antibiotic resistance. To date there is no rapid, robust method to quantify the influx of antibiotics.

5.2.1 Whole Cell Assays

Whole cell assays are possible strategies to quantify influx. In this technique, bacteria were incubated with antibiotics for a certain time frame. Then separation was performed to remove any drugs residue which was not taken up by the bacterial cells.[32,191] After separation, the internalised antibiotics concentrations are measured by a number of biophysical techniques. Standard approaches use radioactive labelled compounds. The sensitivity of the technique relies on the separation technique to remove the external antibiotics "sticking" on the cell surface from internalised antibiotics. Sticking antibiotics are those which have not been internalised by the bacterial cells, therefore they are present on the outer region of the bacterial cell envelope. The use of radioactive labelled compounds is very expensive. Now the MS spectrometer is another approach to quantify after separation. However, MS is not a direct quantitative technique as it depends on intensity of the ion peaks generated at a certain mass/charge ratio. Presence of contaminants impairs the intensity of peaks.[32,191]

5.2.2. Enzyme Assays

Enzyme assays are another approach to quantify the uptake in cells, is developed by Zimmermann and Rosselet.[188] This technique measures signals coming from the hydrolysis of antibiotics by bacterial enzymes, for instance hydrolysis of β-lactam by β-lactamase enzyme. This is limited to one kind of mechanism through which bacteria renders its resistance.[191]

Studying subcellular concentrations of antibiotics has been carried out by single cell Microscopy. The Pagès group and collaborators used deep UV autofluorescence microscopy and microfluorimetry to measure the uptake of antibiotics in single bacterial cells. A pellet was collected from the exponential phase of bacteria by centrifugation and resuspended in NaPi-MgCl2
buffer. Then the cells are mixed with antibiotics right before microscopy. Then the bacterial cells are suspended between two quartz coverslips and examined by a tunable deep UV light source.

Deep UV require quartz optics, quartz objectives, and number of control cells to separate the cellular autofluorescence and unwanted signals from drug signals. Deep UV can be lethal to the cells.[32,191]

5.2.3. Electrophysiology

Electrophysiology is another approach to measure the cellular accumulation of antibiotics. It measures the sub cellular accumulation of antibiotics through porin channels. However, antibiotic interactions with the porin channels can be affected by ions and water molecules. For kinetics, association and dissociation rates, ions concentration, pH of the solution are important parameters for the electrophysiology experiment. The current state of electrophysiology is unable to provide details on influx at the molecular level.[191]

5.2.4. Electrochemistry

Electrochemical techniques could be a potential analytical tool to quantify antibiotic influx, as it offers advantages over other techniques. Advantages include the detection of analytes at very low concentrations, such as at the femto-molar range, fast analysis, simple equipment setup and low cost. Differential Pulse Voltammetry is a highly sensitive technique and can detect in the nano-molar range. The waveform in DPV is a series of pulses where periodically potential pulses are applied over a linear ramp potential (**Figure 5.2**).[42,192] Current is sampled just prior to applying the potential pulse and potential then is stepped and current is measured again at the end of pulse. The difference between two current measurements is recorded and plotted versus applied step potential.[189] The way the current is recorded in DPV minimizes the background current contribution. This technique is extremely helpful to achieve high resolution peak shape with similar redox potential and peak separation compare to cyclic voltammetry.

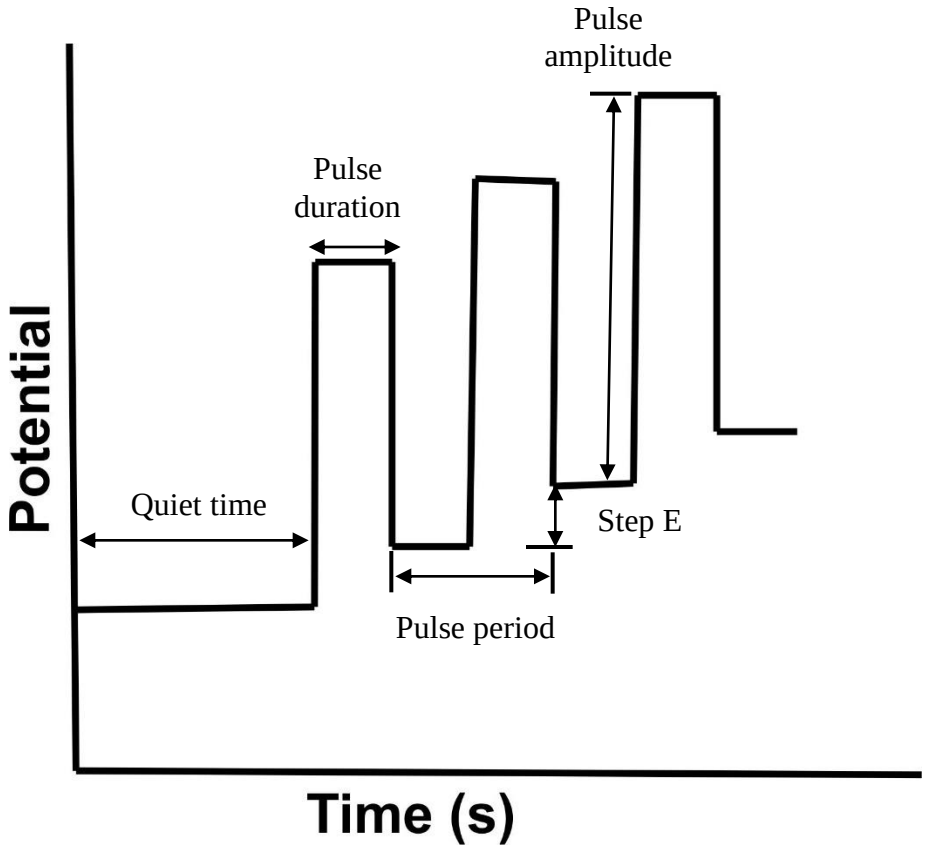


Figure 5.2: Differential pulse voltammogram in waveform

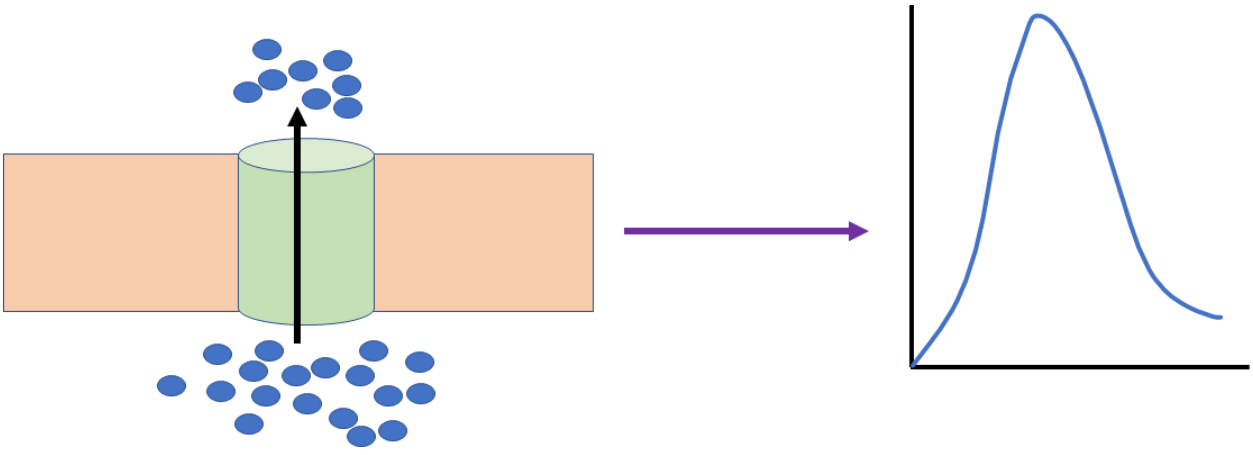


Figure 5.3: Schematic representation of electrochemical quantification of antibiotic influx in bacterial cells.

Pseudomonas aeruginosa was used for the bacterial study. *P. aeruginosa* is a ubiquitous Gram-negative bacterium that thrives in a wide range of environments including soil and aquatic habitats and can colonize in the plants, animals, and humans. *P. aeruginosa* is an opportunistic

pathogen and responsible for leading causes of nosocomial infections worldwide. It mainly affects immunocompromised individuals and cystic fibrosis patients. It is responsible for high morbidity and mortality. *P. aeruginosa* is considered one of the six bacterial pathogens commonly denoted ESCAPE due to their resistance to available antibiotics on the market. The treatment of *P. aeruginosa* infections becomes extremely challenging due to its intrinsic and extrinsic resistance mechanism. The intrinsic resistance mechanism includes low outer membrane permeability and expression of efflux pumps.[193]

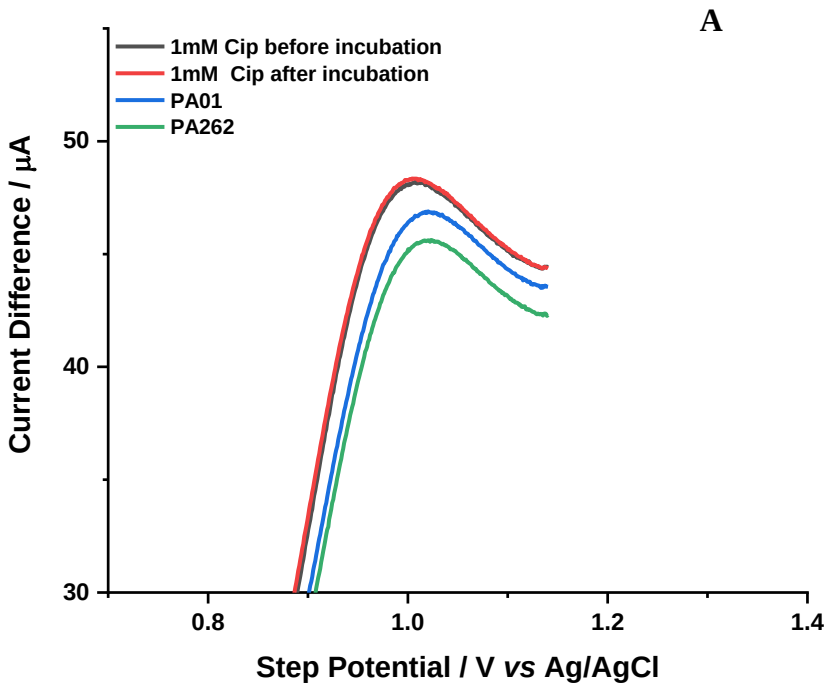
5.3. Experimental Approach

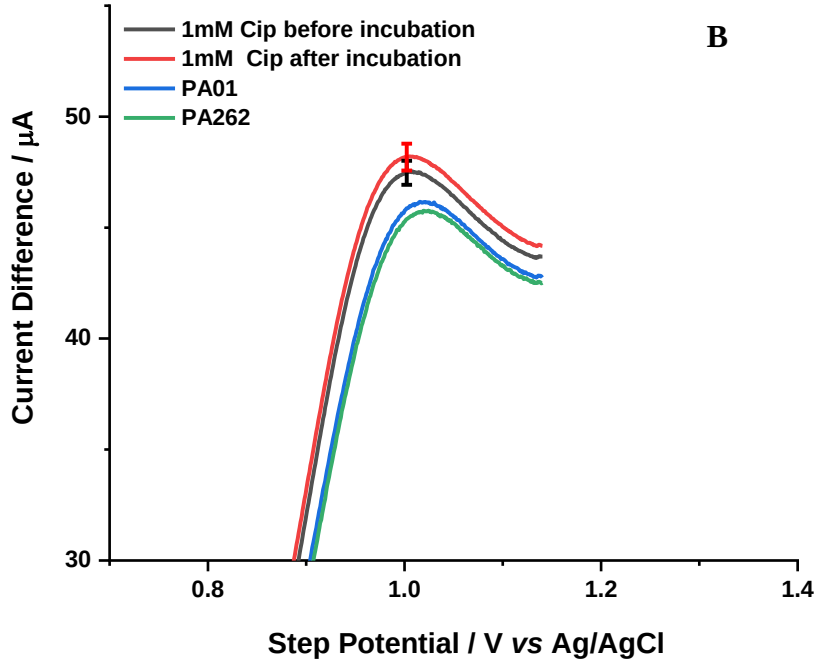
Pseudomonas aeruginosa culture was collected in Dr. Frank Schweizer's lab. Two strains, a sensitive strain PAO1 and a resistant strain PA262 were used for the experiments. The PAO1 strain is susceptible to CIP. The resistant strain PA262 exhibits an overexpression of efflux systems, which expel antibiotics from the cytosol to the exterior environment of the cell. This causes a decreased susceptibility and makes bacteria resistant to the applied antibiotics. Each vial contains cells in the range 10^6 to 10^8 per mL based on 0.5 McFarland (based on turbidity). Ciprofloxacin (2 mM) was prepared in PBS. CIP (5 mL, 2 mM) was diluted with 5 mL of 0.1 M PBS to make it a 1 mM CIP solution. pH was adjusted to 6.5. DPV measurements of 1 mM CIP were taken. CIP (5 mL, 2 mM) was added into both falcon tube containing 5 ml of bacterial cultures of sensitive and resistant strains. Then falcon tubes were incubated at 37ºC for different lengths of time. After incubation, centrifugation was performed at 4000 rpm for 10 min and supernatant was collected. Then DPV measurements were performed on supernatant solutions. Pellets containing bacterial cells and the CIP molecules that had entered the cells were discarded. So, the purpose was to analyse the difference between the supernatants of two strains. Due to low outer membrane permeability, resistant cells impair the entry of CIP molecules to the cytosol. Thus, more CIP molecules remain in the supernatant and will produce more current response in resistant cells (Figure 5.3).

5.4. Results and Discussion

The results (Figure 5.4) from DPV show two oxidation peaks of ciprofloxacin at 1.0 V *vs* Ag/AgCl and 1.25 V *vs* Ag/AgCl which can be associated with two electron transfers of the CIP moiety. No differences were observed between before incubation of 1 mM CIP and after incubation in the absence of bacteria, which indicates there is no degradation of CIP moiety over

the time due to the incubation process (Figure 5.4). The current response of the supernatant solution after 15 min of incubation showed no statistically significant difference between sensitive and resistant strains, which is shown in **Figure 5.4.A**. The analysis of after 25 min incubation samples showed a similar current response in both bacterial cells (Figure 5.4.B). The same electrochemical signal was observed after 35 min of incubation in both resistant and sensitive cells (Figure 5.4.C). In **Figure 5.4.D**, the incubation time was increased to 45 min to allow the bacterial cells more time to uptake CIP molecules. And it revealed no difference in electro-oxidation signal for both sensitive and resistant bacterial cells. Importantly, this study demonstrates that electrochemistry is able to detect the uptake of CIP by bacterial cells. Furthermore, this study indicates the resistance of the PA262 bacterial strain against CIP is not due to a decreased outer membrane permeability.


A
1mM Cip before incubation
1mM Cip after incubation
PA01
PA262
Current Difference / µA
50
40
30
0.8
1.0
1.2
1.4
Step Potential / V vs Ag/AgCl


B
1mM Cip before incubation
1mM Cip after incubation
PA01
PA262
Current Difference / µA
50
40
30
0.8
1.0
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Step Potential / V vs Ag/AgCl

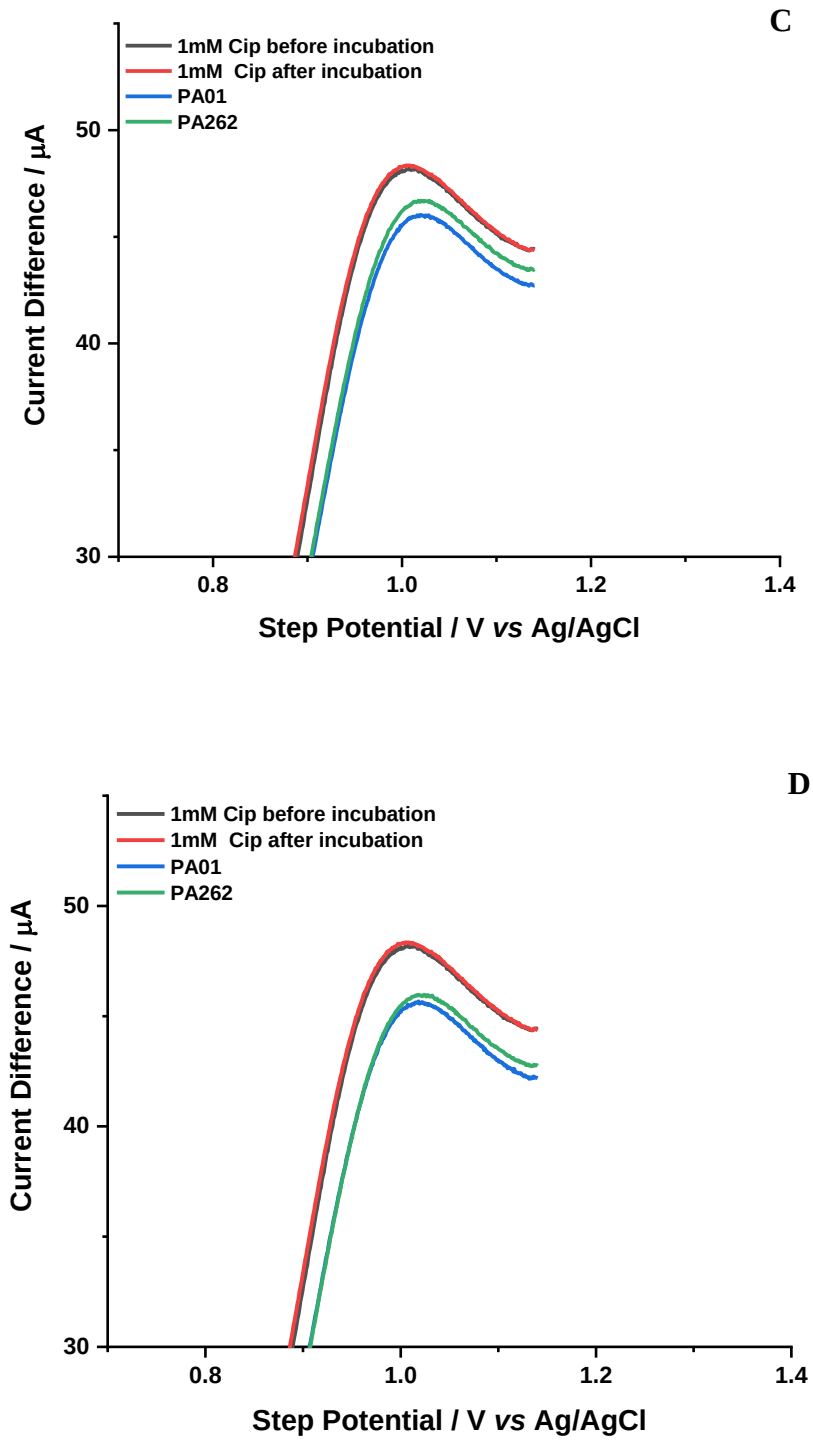

Figure 5.4: Influx quantification of ciprofloxacin *in P. aeruginosa*. 1mM CIP solution in 0.1 M PBS was used as a control before and after incubation without any bacteria. Blue and red line are

susceptible and resistant cells respectively incubated in presence of CIP with different incubation time. A) 15 minutes, B) 25 minutes, C) 35 minutes, and D) 45 minutes

In **Figure 5.5 and 5.6**, the effect of incubation time on the influx quantification was studied for sensitive and resistant bacterial cells. There is no difference observed in current response between sensitive and resistant bacterial cells, which demonstrated that this method is time independent. It portrays the important message that this method could be used to quantify drug influx from patient blood samples right away.

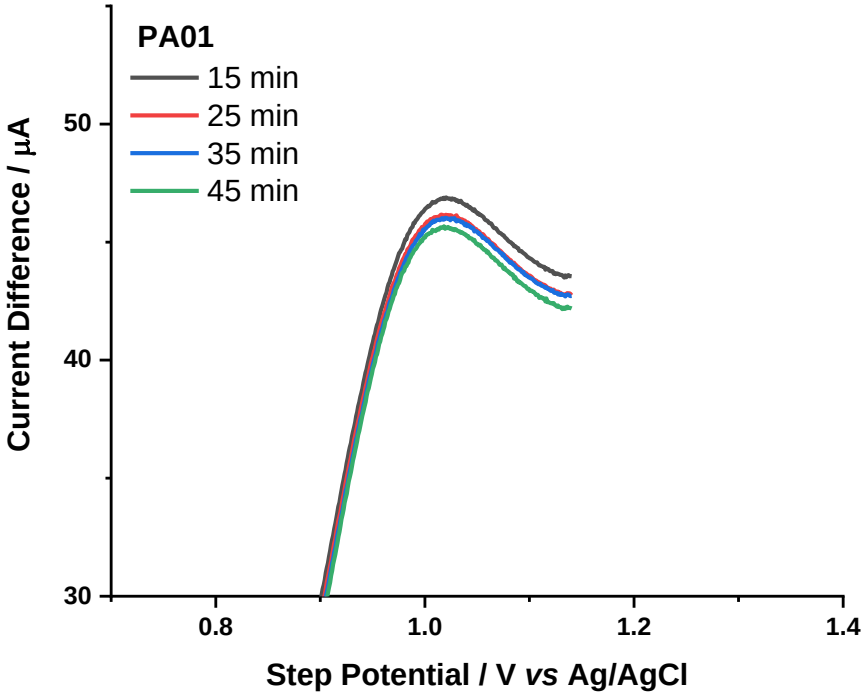

Figure 5.5: Time dependence study of *P. aeruginosa* susceptible cells by DPV. Cells were incubated with CIP in various time set to observe their response.

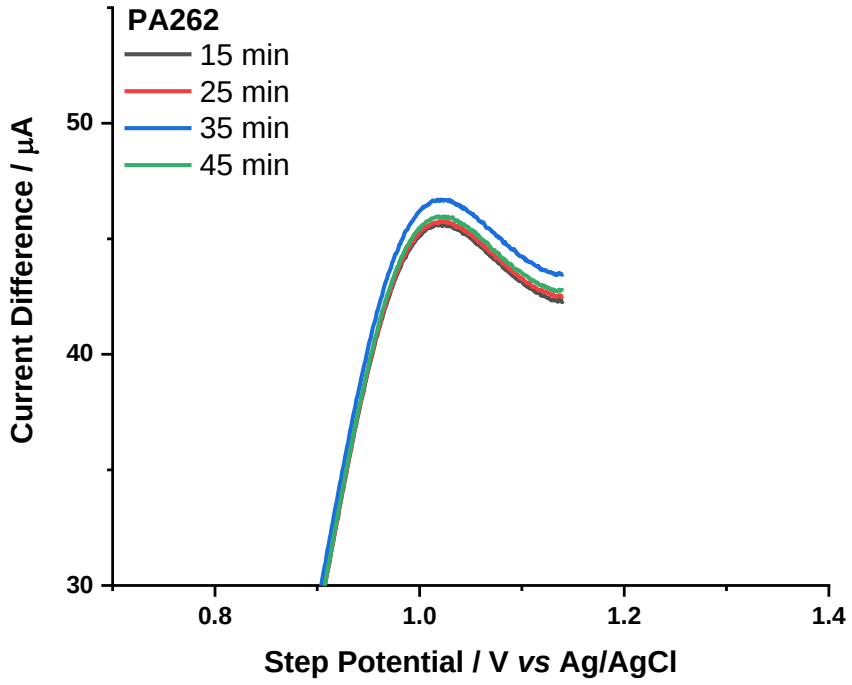

Figure 5.6*:* Time dependence study of *P. aeruginosa* resistant cells by DPV. Cells were incubated with CIP in various time set to see the difference in response.

An influx study was also performed with the CIP-TOB hybrid molecules. The DPV results of 1 mM CIP-TOB hybrid produced two oxidation peaks at 0.9 V and 1.43 V vs Ag/AgCl at GCEs (Figure 5.7). The first peak at 0.9 V vs Ag/AgCl is attributed to the oxidation of CIP molecules and the later one at 1.43 V vs Ag/AgCl is due to the tobramycin oxidation. The DPV of supernatant solution of resistant bacterial cells showed slightly higher current response, which is not significant enough statistically. This can be associated with the tested bacterial resistance mechanism. The resistant strain PA262 demonstrates the resistance mechanism by overexpression of efflux pumps which expel antibiotics from the cell, decreasing the susceptibility of the bacteria against the antibiotics. The experimental procedures could be improved for the better influx antibiotic quantification. Since the uptake of antibiotics and efflux pump systems function simultaneously, it is extremely important to know the accurate uptake time of individual antibiotics for the tested bacterial organism. The determination of exact uptake

time of antibiotics will help to remove the contribution from efflux in quantification of antibiotic influx.

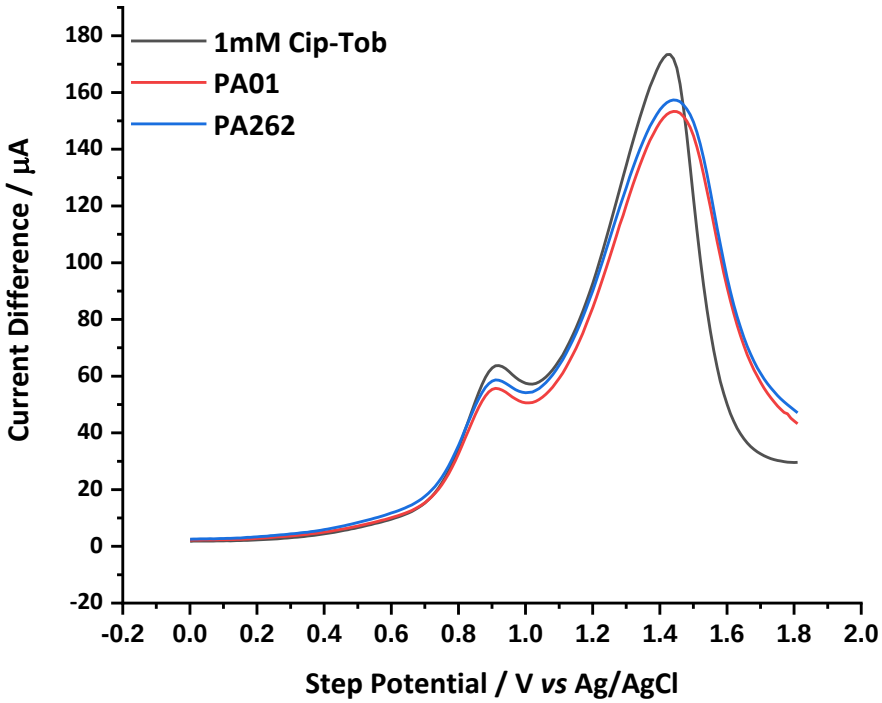

Figure 5.7: CIP-TOB hybrid influx quantification in *P. aeruginosa* by DPV. PA01 and PA262 are susceptible and resistant bacterial cells respectively. The black line is the control experiment which 1 mM CIP in 0.1 M PBS and no bacteria present in control.

CHAPTER 6: Quantification of Antibiotic Efflux

Cell membrane permeability provides a first line of defense for bacteria to tackle the antibiotics. However, low permeability demonstrated the resistance mechanism in synergistic action with the efflux pumps system of bacteria. Efflux pumps located in the cell membrane help to decrease the intracellular antibiotic concentration by expelling the antibiotics to the outer environment, which renders bacteria to be resistant. The efflux pumps system is one of the predominant resistance mechanisms used by multi drug resistant bacteria. Efflux quantification, therefore, is crucial to detect antibiotic resistance.[194,34]

6.1. Introduction

Efflux pumps, also known as drug transporters, are cytoplasmic membrane proteins present ubiquitously in all types of cells including bacteria and eukaryotic cells.[194,34] They are prominent in the extrusion of any waste: metabolites, harmful substrates such as dyes, chemicals, and antibiotics out of the cells.[36] The genes encoding the efflux pumps are located on chromosomes or plasmids. Efflux pumps are the prominent and one of the effective intrinsic bacterial resistance mechanisms due to their high efficiency of drug extrusion. Overexpression, mutation of genes encoding these transporters or asymmetric accumulation during cell division led to the development of resistance to antibacterial agents.[35194] Efflux pumps can be formed of single units or multiple units. Transmembrane efflux pumps composed of multiple units are exclusively present in Gram negative bacteria and Gram-positive bacteria mostly possess single polypeptide efflux pumps present on cytoplasmic membrane.[36,195]

6.2. Physiological Roles of Efflux Pumps

In addition to expel out antibiotics, efflux pumps have other physiological functions. They help in adaptation under stress conditions, in cell to cell communication, and pathogenesis of bacteria.[34,196,197]

6.2.1. Roles in bacterial pathogenicity and virulence

Active efflux system (AcrAB-TolC) can pump out the toxic bile salts, which helps to adapt the bacterial cells in the animal intestine. This has been reported in *E. coli, P. aeruginosa, S.*

Typhimurium. Bacterial efflux pumps facilitate the adaptation of bacterial cells in their ecological physiological habitat by expelling out host derived antimicrobial compounds.[34,196,197] Buckley *et al.* reported that mutant *S.* Typhimurium having a defective efflux pump did not persist in the chicken gut due to poor colonization. This indicates that the AcrAB-TolC system is required for the colonization of *S.* Typhimurium in the avian gut. In addition to host-pathogen interactions, colonization of bacterial cells is important for bacterial pathogenicity, as only survival cells can cause infection. Efflux pump systems also contribute to bacterial pathogenesis by exporting virulent determinants.[34]

6.2.2 Roles in cell-cell communication

Efflux pumps systems play an important role in intercellular communication to control bacterial behaviour in different environments. Intercellular Quorum sensing (QS) is one of the most important cell to cell communication mechanisms in response to extracellular chemical signals.[197] Autoinducers (chemical signaling molecules) are produced by the QS bacteria. They control various cellular activities including metabolic switch, motility, biosynthesis of antimicrobial peptides, polysaccharide synthesis and activation of many virulence factors. They are released and increased in concentration in response to cell population density. They can also act as transcription regulators once the threshold stimulatory level is reached. Transportation of these autoinducers into extracellular space is facilitated by efflux pumps.[34197]

6.2.3. Role in biofilm formation

A biofilm is a membrane like a protective layer produced by certain microorganisms which gives the resistance to antimicrobial agents. For example, *P. aeruginosa* is a well-known biofilm producer bacterium. Biofilm formation accelerates the spread of multidrug resistance bacteria by preventing antimicrobial agents from entering the microbial population. A study showed the impairment of biofilm formation by the deficiency of efflux activity. The presence of efflux inhibitors caused reduced biofilm formation as reported by Kvist et al. in *E. coli* and *Klebsiella pneumoniae*.[196]

6.3. Efflux pump Types

Bacterial drug efflux pumps have been classified into six families that contribute to efflux pathways (Figure 6.1).[198]

(1) the ATP-binding cassette (ABC) superfamily

(2) the major facilitator superfamily (MFS)

(3) the multidrug and toxic compound extrusion (MATE)

(4) the small multidrug resistance (SMR) family

(5) the resistance-nodulation-division (RND) superfamily

(6) proteobacterial antimicrobial compound efflux (PACE) family

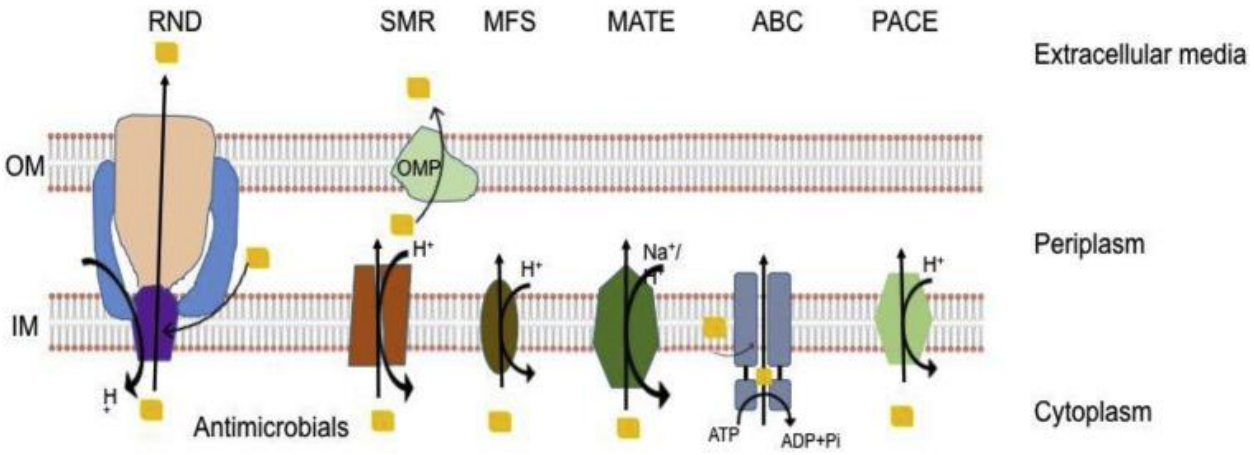

Figure 6.1: Structure of different efflux pump families[35]

Except the RND superfamily, all other five families ABC, MFS, MATE, SMR, PACE are ubiquitously located in both Gram-positive and negative bacteria. The RND superfamily is exclusively found in Gram-negative bacteria and associated resistance. In Gram-positive bacteria, the MATE family is mostly associated with resistance.[35,198]

6.4. Efflux Pump Quantification in the Literature

6.4.1. Drug Susceptibility Measurements

Drug susceptibility measurements (such as MIC) are a common method to detect the differences in efflux activity. The principle of this method is the comparison of bacteria with greater extent of efflux pumps and the bacteria with lesser extent of efflux pumps. The bacteria with greater

extent of efflux pumps export the drug more and make the bacteria less sensitive than the bacteria with lower expression of efflux pump. The sensitivity of this technique is limited as it can only detect the large differences in efflux activity.[199]

6.4.2. Direct Measurements

Cells are preloaded with fluorescent substrates being measured and incubated with the presence of efflux inhibitors which inhibit the transportation.[199] Then the fluorescence of the cell is measured. Initially the fluorescence will be high due to the accumulation of dye. Then glucose is added into the medium which allows the cells to pump out the dyes. Then fluorescence is measured over time as the dye is expelled out of the cells. The rates of efflux based on the fluorescence measurements are compared in different strains. Ethidium bromide, a DNA intercalating agent, is a common substrate. Finding a proper substrate is crucial for this assay. For example, ethidium bromide is a carcinogenic agent.[199]

6.4.3. Intracellular accumulation assay

This method involves the measurement of intracellular accumulation of substrates to detect the efflux level. A higher accumulation of substrates inside the cell indicates lower efflux activity. One approach is to use radiolabelled substrates. The level of radioactivity is used to measure the accumulated substrates. But, to avoid the use of radioactivity, fluorescent substrates or dyes are commonly used. In this assay, an initial fluorescence reading is measured in the absence of any dye or drug inside the bacterial cell. Then the dye is added, and accumulation of dye is increased over time as well as fluorescence until they reach a steady state. The cells with lower expression of efflux pumps will have higher steady state levels than the cells with high expression efflux system as they can not expel out the dye. This accumulation is an indirect method of efflux measurement and less sensitive than the direct method, because accumulation of substrates is also affected by other factors such as rate of influx.[199]

6.4.4. Other Techniques

In addition to these techniques, recently developed techniques to measure efflux include flow cytometry[200] which can measure accumulation of dye in a single bacterium cell. Quantitative real time polymerase chain reaction,[201] liquid chromatography coupled to tandem-mass

spectrometry[202] (LC-MS/MS) are also recent developments for the measurements of efflux activity. However, they are expensive and tedious.[199]

6.5. Experimental Method

Two strains of *Pseudomonas aeruginosa* were used for the efflux study and collected in Dr. Frank Schweizer lab. Two strains, sensitive strain PA01 and resistant strain PA262 were used for the experiment. Two falcon tubes containing sensitive bacterial cells (range 10^6 to 10^8 per ml based on 0.5 McFarland standard) and two falcon tubes containing resistant bacterial cells were taken. Ciprofloxacin (2 mM) was prepared in PBS. CIP (5 ml of 2 mM) was added to the falcon tubes containing 5 ml of sensitive and resistant bacterial cells respectively. PBS (5 ml of 0.1 M) was added to the other two falcon tubes containing sensitive and resistant bacterial cells. A positive control was prepared by using 1 mM CIP solution in PBS. All five falcon tubes were kept in an incubator at 37ºC for 25 minutes. After incubation, they were centrifuged at 4000 rpm for 10 minutes. Then the supernatant was discarded, and the pellet was resuspended into 5 mL of PBS. An electrochemical cell was prepared containing 0.1 M PBS. GCEs, working electrodes were polished on a polishing pad using an alumina slurry of 1 µ, 0.3 µ and 0.05 µ. Then GCEs were sonicated for 2 minutes to remove alumina slurry and air dried. Resuspended solution (4 µL) was drop casted onto the surface of GCEs and dried by N_2. The dried GCEs were immediately immersed into the electrochemical cell containing PBS solution. After immersing into the PBS, different lengths of time were used to allow the cells to expel the antibiotics and soon after that, the DPV measurements were taken. The theoretical principle is that the resistant bacterial cells will produce a higher current response as they have more efflux pumps to expel out the antibiotics than the sensitive bacterial cells which have lower expression of efflux pumps. **Figure 6.2** shows the schematic representation of the electrochemical technique employed to detect antibiotic efflux. Bacterial cells are drop-casted onto the surface of the electrode. Right after the solution has evaporated, the electrode was immersed into the electrolyte solution and given different time intervals to expel the antibiotics from the cells, which was followed by DPV measurements.

6.6. Results and Discussion

Two bacterial strains (PAO1 and PA262) without incubation with CIP and PBS showed no peak in DPV. In positive control, **Figure 6.3** showed anodic peak current at 0.75 V vs Ag/AgCl. And

equal current response was observed in sensitive bacterial cells after allowing the cells 5 seconds to expel the antibiotics. In resistant bacterial cells, lower current response was observed. This indicates 5 s was not sufficient for the cells to expel out the antibiotics.

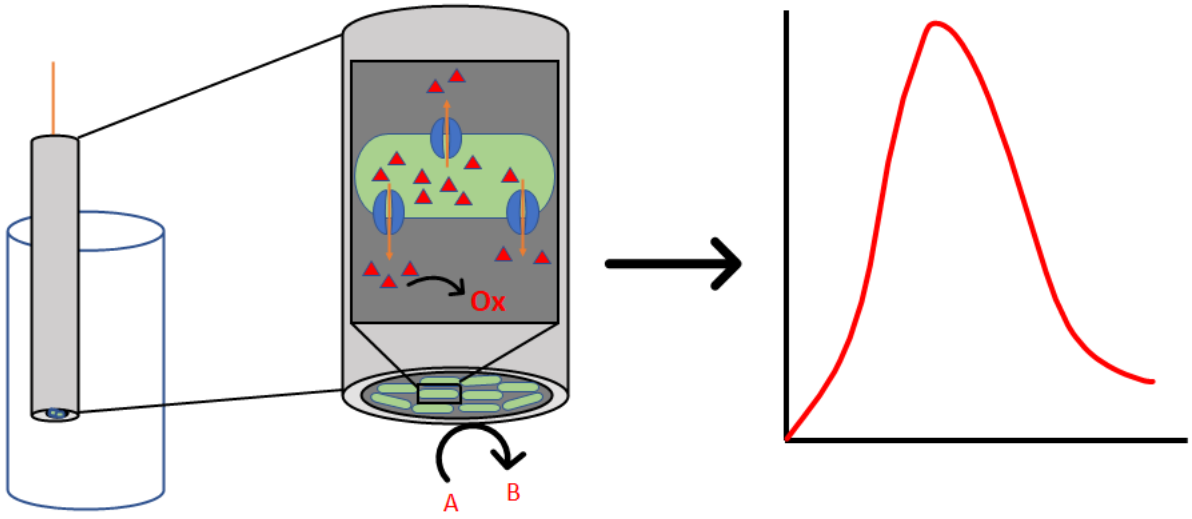

Figure 6.2: Schematic representation of detection antibiotic efflux by electrochemical techniques

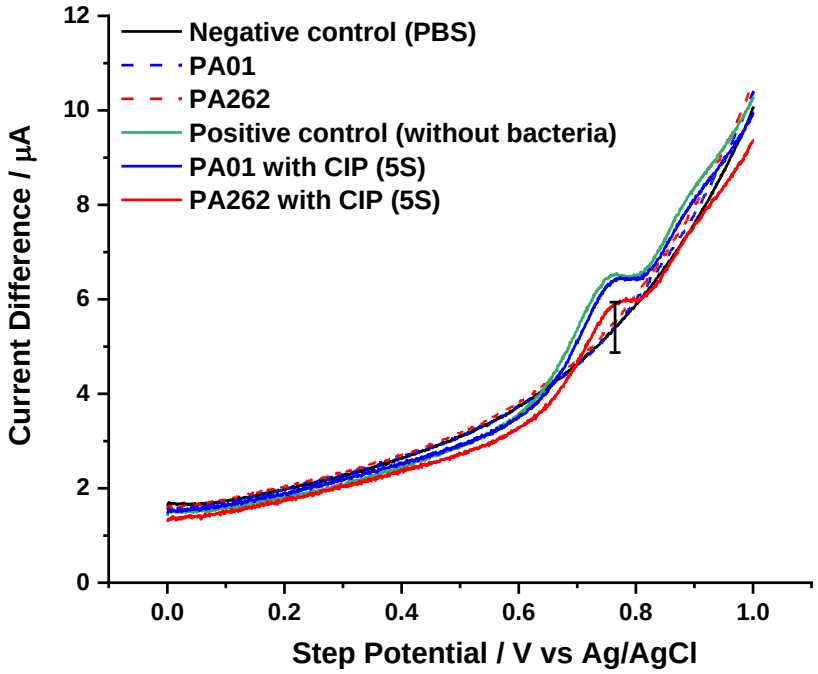

Figure 6.3: Efflux quantification by DPV in *P. aeruginosa*. Dotted line are bacterial strains without any antibiotics. Green full line is the CIP molecule dissolved in 0.1 M PBS and incubated without any bacteria. Black full line is only PBS as a negative control. And blue and red full line are the target analysis samples susceptible and resistant cell respectively.

The drop casted electrode was immersed into an electrochemical cell containing PBS for 10 seconds which provides cells more time to pump out antibiotics from the cells. Then DPV was measured. In PBS, PA01, PA262 samples there was no electrochemical reaction observed as expected because there is no ciprofloxacin molecule in the samples. Positive control samples (1 mM CIP) produced oxidation peak 0.75 V vs Ag/AgCl and sensitive bacterial cells produce little higher current response than the positive control samples (Figure 6.4). Resistant bacterial cells showed significant current response compare to the sensitive and resistant cells which is attributed to their high expression of efflux pump system.

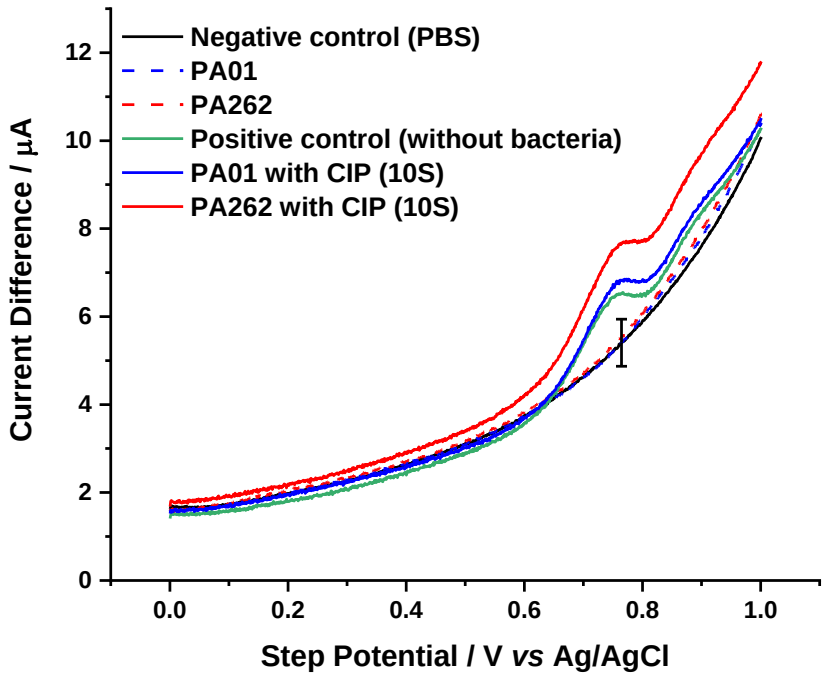

Figure 6.4: Efflux quantification by DPV in *P. aeruginosa* after 10 seconds. Black full line is as a negative control which is only 0.1 M PBS solution. Green full line is the CIP molecule dissolved in 0.1 M PBS and incubated without any bacteria used as positive control. Dotted line are bacterial strains without any antibiotics. And blue and red full line are the susceptible and resistant cell respectively incubated with CIP.

In case of 20 and 30 seconds, signals from the electrochemical oxidation reaction of CIP molecules were the same in **Figure 6.5** and **Figure 6.6**. Resistant bacterial cells produced the highest oxidation current signal at 0.75 V vs Ag/AgCl whereas sensitive bacterial cells showed low current signal as they have low expression of the efflux pump system to expel out the antibiotics. Positive samples produced the same current response as sensitive bacterial cells. The negative control and only bacterial cells produced no electrochemical signal.

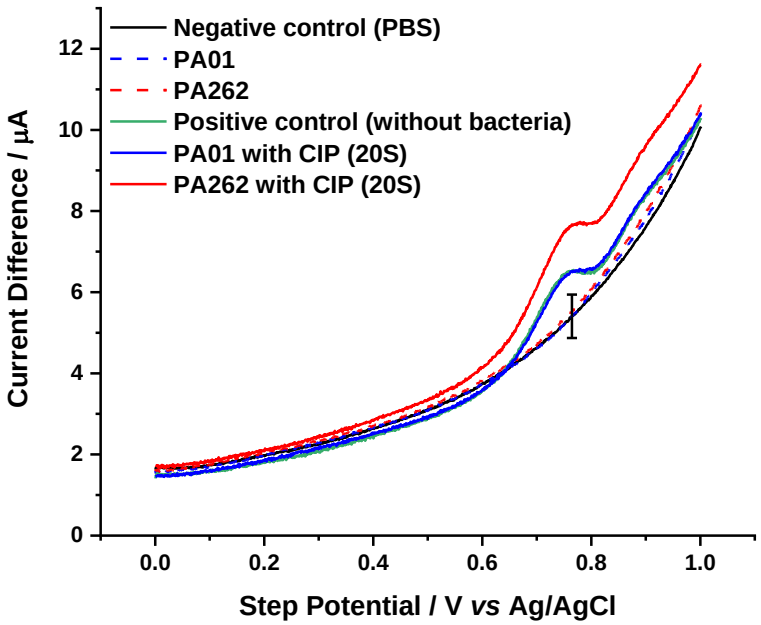

Figure 6.5: Efflux quantification by DPV in *P. aeruginosa* after 20 seconds. Black full line is as a negative control which is only 0.1 M PBS solution. Green full line is the CIP molecule dissolved in 0.1 M PBS and incubated without any bacteria used as positive control. Dotted line are bacterial strains without any antibiotics. And blue and red full line are the susceptible and resistant cell respectively incubated with CIP.

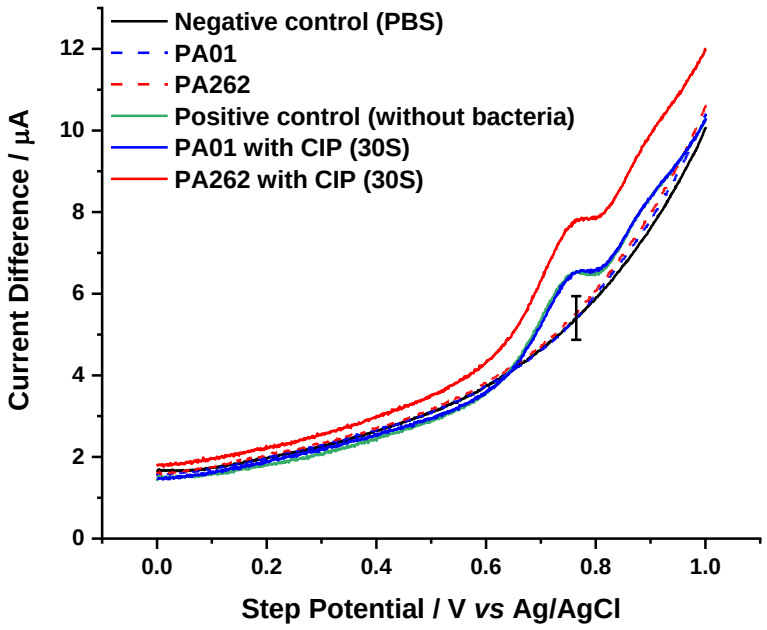

Figure 6.6: Efflux quantification by DPV in *P. aeruginosa* after 30 seconds. Dotted line are bacterial strains without any antibiotics. Green full line is the CIP molecule dissolved in 0.1 M PBS and incubated without any bacteria. Black full line is only PBS as a negative control. And blue and red full line are the target analysis samples susceptible and resistant cell respectively.

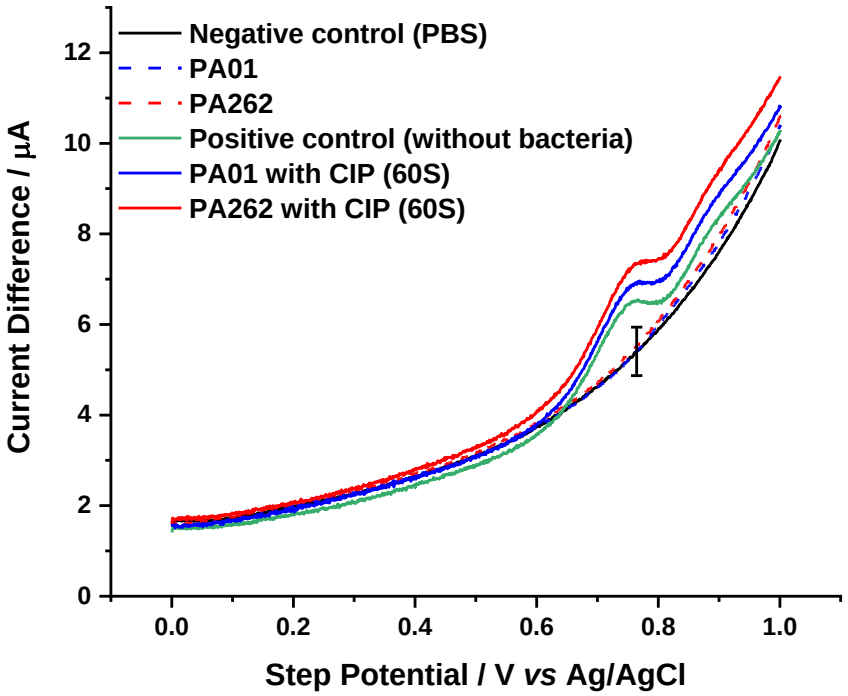

Figure 6.7: Efflux quantification by DPV in *P. aeruginosa* after 60 seconds. Black full line is as a negative control which is only 0.1 M PBS solution. Green full line is the CIP molecule dissolved in 0.1 M PBS and incubated without any bacteria used as positive control. Dotted line are bacterial strains without any antibiotics. And blue and red full line are the susceptible and resistant cell respectively incubated with CIP.

After 60 seconds, the highest electrochemical signal was found in resistant bacterial cells. Susceptible cells produced lower current signal than the resistant cells and higher than positive control samples (Figure 6.7). In **Figure 6.8** and **Figure 6.9**, the electrochemical signals of CIP produced at the surface of GCEs were shown in response to the time allowed for extrusion of antibiotics. Thirty seconds was considered an optimum time for the extrusion of antibiotics from the bacterial cells. Future data treatment is required to analyse the data further. Improvements could be made for future quantification, for example washing steps could be employed to remove

the contribution from the antibiotic solution in the efflux signal. The quantification study could also be performed in the presence of efflux inhibitor for comparison.

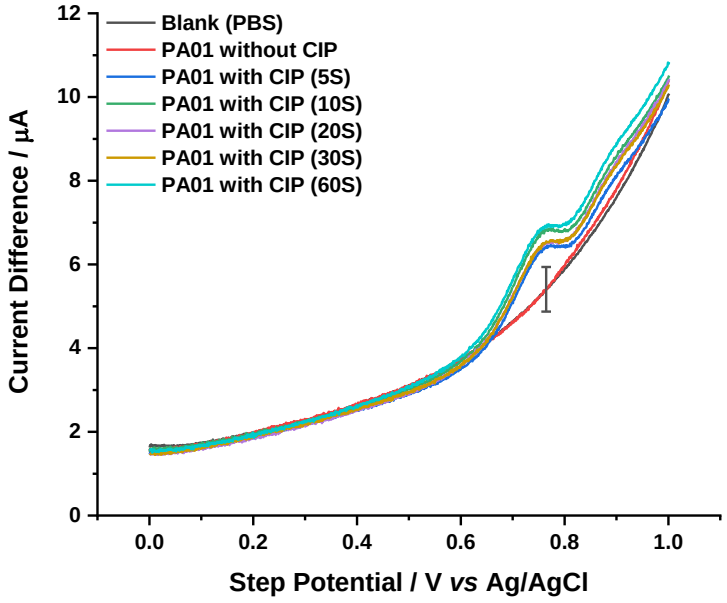

Figure 6.8: Measurements of efflux pumps of ciprofloxacin in susceptible bacterial cells. Two control were used which are black and red line. Blank line is 0.1 M PBS and red line is susceptible bacterial cells incubated without antibiotics.

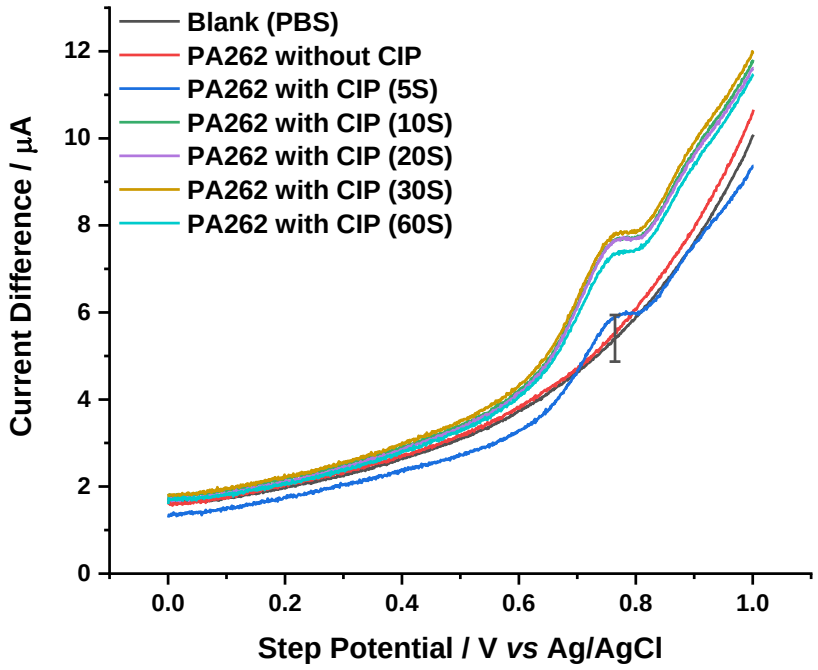

Figure 6.9: Measurements of efflux pumps of ciprofloxacin in resistant bacterial cells. Two control were used which are black and red line. Blank line is 0.1 M PBS and red line is resistant bacterial cells incubated without antibiotics.

CHAPTER 7: Discussion

7.1. Discussion and Prospects

Due to the evolving resistance power of bacteria, it has become a global challenge to find new antibiotics and alternative treatments to combat antibiotic resistance.[203–206] Some bacteria have become multidrug resistant such as the methicillin resistant *Staphylococcus aureus* (MRSA), *P. aeruginosa*, *Klebsiella penuamonaie*, *Vibrio Cholerae*, and *Mycobacterium*. These bacteria have the capacity to withstand lethal doses of various antibiotics. At the Shahid Motahari Hospital, in Tehran, 52 *K. pneumoniae* strains were isolated from burn patients, and screened. Out of 52 strains, 40 isolates revealed resistance to multiple antibiotics including ciprofloxacin, tetracycline, ceftazidime and gentamycin. Multidrug resistance is a severe concern in the health science sector[203,206,207]. A recent report states that a superbug could be responsible for 80% of deaths in intensive care units (ICUs) in Bangladesh.[173] Moreover, an antimicrobial resistant superbug could cause the death of 10 million people worldwide and devastate the global economy.[50]

It is a dire need to develop a rapid methodology to detect antibiotic resistance to help practitioners to prescribe antibiotics specific to individual needs of patients. Available traditional methods, such as antibiotic susceptibility tests, take a day or longer, depending on the incubation period of bacteria.[18] Electrochemical techniques can play an important role to develop a bioanalytical sensor because of their simplicity, cost effectiveness and rapid analysis.

In this thesis, electrochemical characterization of widely used antibiotics CIP, TOB, AMX was performed at bare electrode surfaces. The well-defined electrochemical properties of these antibiotics provide a basis for further exploration of antibiotic resistance.

Emerging multidrug resistant bacterial infections urge the researchers to develop new compounds to combat this issue. Studies showed the combination of two antibiotics can enhance the antibacterial efficiency.[21] Electrochemical investigation of CIP-TOB hybrid molecules in this thesis revealed the electrochemical properties of the CIP-TOB hybrid and showed the potential applicability of the method to assess any new electroactive antibiotics for their efficacy. The electrochemical investigation of hybrid compounds also shows that this method could be used to

detect two molecules simultaneously. The urge of developing new antibacterial agents against multidrug-resistant bacteria and the scarcity of available options drives scientists to consider a non-conventional approach to combat this alarming issue.[208] Developing hybrid molecules by combining existing antibiotics has become a great choice of strategy.[208] The electrochemical investigation of β-lactam hybrids will be a good choice of exploration in the future, because β-lactams are the most widely used class of antibiotics due to their effectiveness and safety.[209] Aminoglycoside-based hybrids will also be of interest for future electrochemical investigations.[167,210]

Low outer membrane permeability is an intrinsic antibacterial resistance mechanism. The outer membrane of Gram-negative bacteria also provides an external barrier to antibiotics to pass through.[186] Quantification of drug influx is one of the most important criteria to investigate in bacterial resistance. DPV has been used in this experiment to quantify antibiotic influx. The main challenge to quantify drug influx is the synergistic effect with efflux expression. For future studies, this method could be improved by using nanoparticles and modified electrodes to quantify the influx study.

The following potential improvement strategies are suggested:

Electrode modification: Electrode modification could be an important approach to improve the presented method. Electrode modifications using nanoparticles increase the surface area of the working electrode, which enhances the electrochemical signal in a great manner (Figure 7.1). This would lower the LOD and LOQ values. Electrode modifications can also be done using DNA aptamers, polymers, and antibodies.[168]

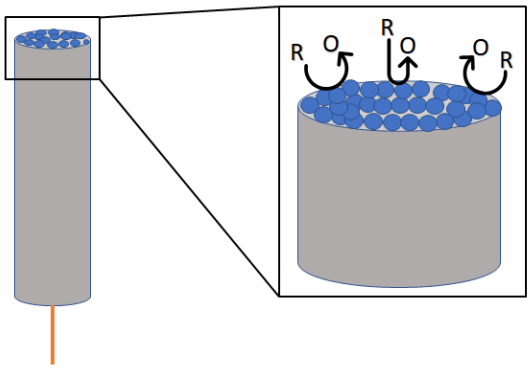

Figure 7.1: Electrode modification with nanoparticles.

Interference Study: The analysis in buffer solution does not replicate the analysis in complex biological samples and environmental samples. The clinical specimen is a complex sample where other inorganic and organic species exist. The analysis, therefore, in real samples such as blood samples is important, to study the effect of presence of any other substances on the electrochemical signal.

Determining the uptake time: For the quantification of antibiotic influx, it is crucial to determine the accurate diffusion time of antibiotics into the bacterial cell. This will eliminate the signal contribution due to efflux activity and make the influx quantification more accurate.

Washing: For efflux quantification, more washing steps could be employed before taking the DPV measurement. These washing steps will eventually remove any contribution in electrochemical signal coming from the antibiotic solution used for incubation of bacteria.

Using efflux inhibitors: Efflux inhibitors can be used to measure the response and will be helpful for comparison and effectiveness of the method. Specific efflux pumps can be revealed responsible for resistant phenotypes in bacterial strains.

Efflux pump systems are the most common antibiotic resistance mechanism found in both intrinsic and acquired resistance.[211] Overexpression of efflux pumps is associated with the development of multi-drug resistant bacteria. In Japan for instance, 50 clinical *E. coli* strains isolated from human clinical samples and dog feces were screened, which showed high-levels of fluoroquinolone resistance in all 20 multi-resistant strains associated with the overexpression of efflux pumps AcrAB.[211,212] The development of a rapid method for efflux drug quantification is necessary to investigate antibiotic resistance. In this project, electrochemical quantification of efflux pumps by DPV showed promising results to quantify antibiotic efflux. This study demonstrates that this electrochemical method can detect antibiotic efflux directly from bacterial cells. Based on the efflux quantification, susceptible and resistant bacterial cells should be differentiated. This study would provide a basis for future development of a bioanalytical sensor to detect antibiotic resistance.

For further investigation of efflux quantification in bacterial cells, scanning electrochemical microscopy (SECM) could be used to quantify drug efflux in single bacteria.

Bacteria can be patterned in specific areas on a plastic surface, using elastomeric membranes, which has been successfully done in the literature with cancer cells.[148] SECM can be used to analyze those immobilized bacterial cells. SECM is an electroanalytical technique, using a micro-or nano-electrode, which is scanned across the surface to analyze its electrochemical activity. The SECM technique also allows analyzing single cells. The SECM technique is highly sensitive, which allows the determination of the number of molecules released from a single cell per second. Efflux activity has been monitored in cancer cells.[149] SECM measurements can be performed at patterned bacterial cells using micro-or nano electrodes, biased at a potential where the dissolved antibiotic will be oxidized or reduced. The antibiotics oxidized or reduced at the electrode surface will be replenished by the efflux pump in resistant cells. A higher electrochemical signal in resistant cells is expected compared to susceptible cells. The faradaic current will be monitored during SECM imaging, which reveals information about topography and electrochemical activity of the underlying surface.

In addition to that, using an appropriate redox mediator, SECM could detect antibiotic efflux pumps expression. Kuss *et al*., used ferrocenemethanol ($FcCH_2OH$) as a redox mediator to differentiate between multi-drug resistant cancer cells and wild cells by SECM.[148]

This thesis focuses on detection of antibiotic resistance associated with low permeability and efflux pump systems. However, there are two more prominent resistant mechanisms, namely inactivating enzyme production, and drug target alteration. Therefore, the methods described in this thesis need to be explored for their applicability to detect antibiotic resistance associated with the production of bacterial enzymes that can inactivate the antibiotics or modify drug targets. The future prospects of these methods should focus on the development of a universal strategy to detect antibiotic resistance.

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