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Antibiotic Sensitivity Testing of Foodborne Bacteria Using Surface-Enhanced Raman Spectroscopy

Joshua Gukowsky
University of Massachusetts Amherst

ANTIBIOTIC SENSITIVITY TESTING OF FOODBORNE BACTERIA USING SURFACE-ENHANCED RAMAN SPECTROSCOPY

A Dissertation Presented

by

JOSHUA C. GUKOWSKY

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Approved as to style and content by:

_____
Lili He, Chair

_____
Matthew D. Moore, Member

_____
John Gibbons, Member

_____
Mingxu You, External Member

_____
Lynne A. McLandsborough, Department Head,
Department of Food Science

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ABSTRACT

ANTIBIOTIC SENSITIVITY TESTING OF FOODBORNE BACTERIA USING SURFACE-ENHANCED RAMAN SPECTROSCOPY

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JOSHUA C. GUKOWSKY, B.S.,
UNIVERSITY OF MASSACHUSETTS AMHERST

Ph.D., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Lili He

The spread of antibiotic resistant bacteria around the world has become a major public health issue, and it is essential that effective detection methods exist for identifying these organisms and preventing them from spreading throughout our food systems and into the environment. The goal of this research is to develop a novel analytical procedure that is capable of easily identifying antibiotic resistance in bacterial samples, and also provides more information about the biochemical characteristics of the bacteria and their responses to antibiotic exposure. Surface-enhanced Raman Spectroscopy (SERS), an analytical technique that uses light scattering to produce a spectrum based on the chemical composition of a sample, was used as the basis for this protocol. First, several different SERS-based procedures were evaluated for their effectiveness in this application, using ampicillin-sensitive and resistant *E. coli* O157:H7 as a model organism. These included a conventional method in which the bacteria were simply mixed with gold nanoparticles and

analyzed, as well as more novel approaches for analyzing the extracellular matrix liquid of the bacteria and using SERS-based filter mapping. Each of these methods were found to have potential advantages and disadvantages, but the latter two approaches were found to be particularly promising for future work. After these tests, we worked to develop a filter-based SERS protocol that could be used with a portable Raman spectrometer, which would be much more suitable for future practical applications. Additional antibiotics, including neomycin and chlortetracycline, were also evaluated, and our portable SERS method was found to be effective for evaluating bacterial sensitivity to each of these antibiotics. Our SERS procedure was also tested with bacteria samples isolated from ground beef, and was able to correctly assess their antibiotic sensitivity. Next, we worked to optimize an extracellular matrix liquid-based analysis method that could be used with a portable Raman spectrometer, which requires additional testing and optimization steps to design compared to the filter-based method. A variety of different experimental conditions were tested, which also provided valuable information about the origins of particular SERS patterns observed in the samples and the conditions required to observe them. The optimized portable SERS liquid analysis procedure would allow us to avoid several labor-intensive and time-consuming sample preparation steps that are required for the previously developed SERS approaches, and our method was be used effectively with a variety of different bacterial samples. Finally, we successfully tested our liquid-based portable SERS procedure with antibiotic resistant bacteria isolated from supermarket poultry samples. Our research demonstrates that SERS can be an efficient and accurate method for testing the antibiotic sensitivity. Potential future work includes testing more types of food samples to assess the potential differences in the SERS patterns of their bacteria isolates, as well as

conducting further in-depth research into testing mixed bacterial populations and analyzing the development of antibiotic resistance in a sample over time.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 1

INTRODUCTION

1.1. Background

Surface-enhanced Raman spectroscopy (SERS) is an analytical technique that makes use of inelastic light scattering to produce a spectrum, which contains peaks that are indicative of the composition of a sample and can be used to analyze various chemical and biochemical phenomena. SERS has become a topic of great interest for the research community, and over that past several decades SERS has been used to develop a wide variety of procedures for detecting various substances and characterizing many different kinds of samples (Langer et al., 2020). SERS has a number of advantages over other traditional analytical procedures- it is fast, relatively simple to perform, non-destructive, requires minimal sample preparation, and is extremely sensitive (McNay et al., 2011). There are also a variety of different types of equipment available for use in SERS analysis, including portable and handheld Raman spectrometers which are well-suited for practical applications and traveling to different locations for on-site analysis, and are less expensive than traditional benchtop laboratory equipment used in this application. The development of many novel SERS procedures in recent years, combined with the production of less expensive and more practical equipment for Raman analysis, has made SERS a more promising analytical technique than ever before.

One of the potential applications of SERS is the detection and analysis of antibiotic resistant foodborne bacteria. The development and spread of antibiotic resistance is a major global public health problem, and SERS could potentially be an effective way to monitor

these organisms and help prevent them from spreading throughout our food systems and into the environment. SERS has previously been used to develop a variety of procedures for detecting and characterizing pathogenic foodborne bacteria (Liu et al., 2017), and there have been a number of efforts to develop SERS-based procedures for testing antibiotic susceptibility (Galvan and Yu, 2018). However, there is a great deal of work that remains to done in order to accomplish the goal of developing an efficient and effective method for characterizing antibiotic resistant bacteria using SERS. There are a wide variety of ways in which SERS procedures for bacterial analysis can be optimized, including using various types of substrates, preparing the sample to minimize interference and obtain the clearest possible signal, and using different types of Raman equipment and experimental parameters. In order to develop the most effective possible SERS method for identifying antibiotic resistant bacteria, it is essential that more work be done to optimize the experimental conditions and improve our understanding of how SERS can be used to study these organisms.

1.2. Objectives

The ultimate goal of this project is to develop a reliable and efficient SERS-based method for assessing the antibiotic sensitivity of a bacterial sample and identifying antibiotic resistant organisms. This method would be an improvement upon existing procedures for analyzing bacteria and testing antibiotic sensitivity, and could be used for a variety of different types of food and environmental samples. It would also ideally be compatible with many different common strains of foodborne pathogenic bacteria, as well as with a number of different widely used antibiotics with a variety of mechanisms. In order

to achieve this goal, there are several more specific objectives which we aim to accomplish and are necessary for ensuring that our method meets these standards.

<u>Objective 1:</u> Test different SERS approaches for assessing antibiotic sensitivity in a bacterial sample. There are a number of different ways in which SERS can be used to test a bacterial sample, and we aim to determine which types of bacterial SERS approaches are able to be used successfully for testing antibiotic sensitivity. SERS approaches we will test include conventional SERS analysis of a sample of a bacterial culture, analysis of the extracellular matrix liquid surrounding the bacteria, and SERS filter mapping.

<u>Objective 2:</u> Adapt a SERS procedure for analyzing foodborne bacteria using a portable Raman spectrometer. Once we have established the preferred methods for assessing the antibiotic sensitivity of a bacterial sample with SERS, we will adapt a procedure for use with a portable Raman device. Using a portable device would be preferable in practical applications to benchtop Raman microscopes, but it can come with its own challenges. We will use this portable SERS method to test a number of different varieties of bacteria species, multiple antibiotics with different mechanisms of action, and isolated bacteria from real food samples.

<u>Objective 3:</u> Optimize the conditions required for testing bacterial antibiotic sensitivity using SERS. After we have tested different SERS procedures for this application using both the Raman microscopes and the portable Raman spectrometer, we will determine what type of protocol is best suited for testing antibiotic sensitivity and

further optimize the conditions required to successfully perform this type of analysis. Further optimization steps that can be performed include adjusting the concentration of the SERS substrate, testing different types of liquid media for incubating the bacteria and the antibiotics, and determining what part of the sample displays the clearest SERS signals. Through this testing, information can also be obtained about the origins of particular bacterial SERS patterns, and the conditions necessary to observe them. Once a method has been optimized, we will try to isolate antibiotic resistant bacteria from real food samples and use our method to assess their sensitivity to different antibiotics.

CHAPTER 2

LITERATURE REVIEW

2.1. Antibiotics and Antibiotic Resistance

2.1.1. Antibiotics: Uses and Mechanisms

Throughout history, one of the greatest potential threats to society has been the development of new types of pathogenic organisms. From the bubonic plague to the 1918 influenza outbreak, and to the COVID-19 pandemic in the present day, illnesses caused by these types of infectious agents can cause untold death and disruption to millions of people. Fortunately, many diseases which used to be gravely feared around the world can now be effectively treated with modern medicine. One of the key treatments which has been developed since the 20th century is the use of antibiotics, which have revolutionized the fight against bacterial illnesses. Antibiotics are a broad category of substances, containing many different types of chemical compounds with one unifying feature- the ability to inhibit the growth of bacteria or kill the organisms altogether (Davies and Davies, 2010). Antibiotic substances naturally present in the environment have been used by humans since ancient times, and modern research into antibiotics began in the late 19th century (Durand et al., 2019). This culminated with the famed discovery of penicillin by Alexander Fleming in 1928, and the introduction of the earliest commercially manufactured antibiotics in the 1930s. In the decades since the beginning of modern antibiotic usage, they have had a massive impact on our society. While antibiotics have made it far easier to treat many pervasive diseases throughout the world, we are just beginning to grapple with the potential

negative impacts of their overuse and the development of potentially untreatable antibiotic resistant disease-causing organisms.

Antibiotics have become an essential part of fighting diseases, making it dramatically easier to fight illnesses such as tuberculosis, typhoid fever, and leprosy which have caused widespread suffering and death throughout history. As a result of their unique ability to fight bacterial infections, they have become ubiquitous not only for treating diseases in humans but in animals as well. For decades, antibiotics have been widely used in animal agriculture for a number of different purposes, such as disease control and enhancing the feed-to-weight ratio in animals used for meat production (Marshall and Levy, 2011). Antibiotic usage in agriculture typically makes up a larger proportion of overall antibiotic usage than medical usage for humans around the world- in the United States, for example, this proportion has been as high as 80% in recent years (Martin et al., 2015). The global use of antibiotics in agriculture is expected to rise significantly in the coming decades, particularly in middle-income countries where larger scale agricultural operations are being introduced, which make use of more antibiotics than traditional farming practices (Van Boeckel et al., 2015). It is essential that we understand the global impact of the widespread and growing use of antibiotics in agriculture, and the potential consequences this trend could have for our society.

In order to adequately discuss issues relating to antibiotic usage, it is important to understand precisely how antibiotics are able to control microbial growth. There are a variety of classes of antibiotics with different mechanisms of action, which have been used in a number of applications in medicine, agriculture, and other industries. Understanding the mechanisms of how antimicrobials function is key to determining their effectiveness in

different applications, and whether or not they can be used as effective treatments against specific microorganisms. While there are more than 20 different classes of antibiotics (Coates et al., 2011), there are a relatively small number of targets within bacterial cells that these antimicrobials impact, and multiple classes of antibiotics often impact the same target organelles or biochemical processes within the cell. These antibiotic mechanisms include disrupting the synthesis of the cell wall, blocking the transcription of RNA or the translation of proteins, preventing the replication of DNA, and inhibiting key metabolic processes such as folate synthesis which are necessary for cells to successfully replicate (Morar and Wright, 2010; Levy and Marshall, 2004). The diverse but relatively small number of cellular processes which can be targeted by antimicrobials ensures that a wide range of treatments for different bacterial infections, but also poses the risk that large numbers of antibiotics could simultaneously become ineffective if bacteria evolve ways to survive these treatments.

2.1.2. Antibiotic Resistance

Antibiotics have become widely used around the world to treat bacterial illnesses, and are a fundamental part of modern medical practice. However, the widespread usage of antibiotics for much of the last century has led to a new problem which threatens the effectiveness of these essential treatments- the development of antibiotic resistant pathogens. There are a variety of mechanisms which bacteria can evolve to become resistant to specific types of antibiotics, such as using enzymes to break down or alter antibiotic molecules and using efflux systems to pump antibiotics out of the cell (Davies and Davies, 2010). Since many antibiotics are simply chemical compounds produced by

microorganisms naturally occurring in the environment, antibiotic resistance is not solely a modern phenomenon- antimicrobial resistance genes have been identified in samples which are thousands of years old, long before the use of these substances by humans (D'Costa et al., 2011). The massive increase the usage of antibiotics in the 20th century, combined with simultaneous improvements in transportation and the increased trade of goods around the world have led to a rapid increase in the natural development of antibiotic resistant organisms throughout the environment (Davies and Davies, 2010; Ventola, 2015).

The global development and spread of antibiotic resistance raises serious concerns about common antibiotics becoming ineffective for treating bacterial infections, making it more difficult to properly fight serious diseases and potentially reversing some of the progress that been made over the past century to make it easier to combat bacterial diseases around the world. Of particular concern are multi-drug resistant organisms, which are resistant to more than one antibiotic and can be able to withstand several different classes of antimicrobial treatments (Gall et al., 2020). Some of the more well-known multi-drug resistant pathogens include methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant enterococci, and strains of organisms such as *Acinetobacter baumanii* which have become resistant to all known antimicrobial agents (Siegel et al., 2017). These types of infections, which can be much more deadly and require more health-care resources to fight than other strains of the same bacteria which are sensitive to antibiotics, can wreak particular havoc in hospitals and other environments in which diseases spread particularly easily and have been found in all regions of the world (Siegel et al., 2017; World Health Organization, 2020). Additionally, these problems are compounded by a major decline in the development of new antibiotics compared to the

mid-20th century, when most of the currently used classes of antibiotics were discovered (Coates et al., 2011; Davies, 2006).

In recent years, some progress has been made to limit the usage of antibiotics in agriculture and help slow the development of antibiotic resistant organisms. Leading the world in these regulations has been the European Union, which has prohibited the usage of antibiotics as a growth promoter in animal feed since 2006 (European Commission, 2005). A number of European countries had previously taken action to limit antibiotic usage in agriculture as early as the 1980s (Sneeringer et al., 2019). In the United States, some specific antibiotics were prohibited for use with food-producing animals in the 1990s and 2000s, culminating in the implementation of GFI #213 in 2017 which banned the use of all medically-important antibiotics as growth promoters in agriculture and introduced other regulations on their overall usage (Sneeringer et al., 2019). However, while these limits may be a step in the right direction, the broader picture around the world points to overall antibiotic usage continuing to dramatically grow in the coming decades, particularly in developing areas in Asia, Africa, and South America (Van Boeckel et al., 2015; Klein et al., 2018; Blaskovich, 2018). While there may continue to be additional restrictions placed on the global usage of antibiotics in the future, it is clear that the issue of the widespread development of antibiotic resistant pathogens is here to stay, and will continue to be a problem of growing importance in the coming years

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2.2. Methods for the Detection of Antibiotic Resistant Organisms

In modern times, the ability to transport goods and travel easily across the world presents an even greater threat of disease-causing organisms spreading rapidly across the

world, and it is essential that we have effective detection methods for identifying antibiotic resistant organisms. A summary of the methods that have previously been developed for testing and identifying antibiotic resistant organisms is shown below in Table 1.

Table 1. Summary of existing techniques that have been developed for testing antibiotic susceptibility, and their respective advantages and disadvantages.

Techniques		**Advantages**	**Disadvantages**
Category	**Examples**		
Growth-based	Agar dilution	▪ Considered to be the "gold standard" ▪ Widely used for decades, established international guidelines for usage	▪ Time-consuming, can take days to get results ▪ Doesn't provide information about the mechanism of resistance or the genetic basis
	Broth dilution		
	Diffusion-based		
PCR-based	Traditional PCR	▪ High degree of accuracy ▪ Much faster than growth-based methods	▪ Only identifies presence of a gene sequence, not whether it is expressed or provides resistance ▪ Not useful for identifying new types of resistance with unknown target gene sequences
	Real-time PCR		
	Multiplex PCR		
DNA-based	DNA microarrays	▪ Can screen for large number of genes at once	▪ Like PCR, requires knowledge of specific gene sequences associated with antibiotic resistance
	Aptamer-based	▪ Simple to use, can be easily adapted into chips or microfluidic systems	▪ Aptamers can be difficult to develop, and be prone to degradation and other functionality issues
Antibody-based	ELISA	▪ Convenient, clearly indicates presence of proteins responsible for antibiotic resistance	▪ Antibodies can be expensive to develop and produce ▪ Requires knowledge of specific proteins required for different cellular resistance mechanisms
	Lateral flow immunoassays		

Mass spectrometry	MALDI-TOF	▪ Highly accurate, looks directly at the biochemical changes occurring in the cell	▪ Requires expensive equipment and extensive laboratory training

2.2.1. Growth-based Detection Techniques

The most widely used techniques for assessing the antibiotic susceptibility of bacterial samples are straightforward, culture-based procedures that test the ability of the bacteria to grow in the presence of specific antibiotics (Pulido et al., 2013). These include methods such as agar dilution and broth dilution, in which the bacteria are grown on agar plates or in tubes of broth supplemented with different concentrations of antibiotics (Schumacher et al., 2018). Also included in this category are diffusion-based techniques, in which paper discs or plastic strips containing antibiotics are placed on plates inoculated with bacteria, and they are subsequently incubated and assessed for the development of an inhibitory zone on the plate in which specific antibiotics or antibiotic concentrations were able to inhibit the growth of the bacteria (Schumacher et al., 2018). These methods, which have been used reliably for decades, are considered to be the "gold standard" for antibiotic susceptibility testing (Schumacher et al., 2018; Engelkirk and Duben-Engelkirk, 2008). The principal advantage of these procedures is how reliable and well-established they are, and there are widely accepted international guidelines for their standardization in laboratories around the world (European Committee on Antimicrobial Susceptibility Testing, 2020). However, these methods do have a number of drawbacks which limit some of their practical usefulness. In particular, they can be quite time consuming, traditionally taking at least 24 hours to obtain results and potentially several days, depending on the type of samples being tested (Pulido et al., 2013; Schumacher et al., 2018). This is not

particularly ideal for testing the antibiotic susceptibility in human infections, since obtaining this data as fast as possible allows doctors to be able to prescribe appropriate antibiotic treatments more quickly and helps patients to recover faster (Barenfanger et al., 1999). As a result, there have been significant efforts to develop faster, more efficient antibiotic susceptibility tests which are comparably effective to the traditional gold standard techniques.

2.2.2. PCR-based Detection Techniques

The most common alternatives to bacterial growth-based procedures are techniques based on the Polymerase Chain Reaction (PCR). PCR is a widely used procedure for amplifying specific sequences of nucleic acids, and it has been adapted over the past several decades for a large number of diagnostic applications (Yang and Rothman, 2004). On the most basic level, PCR can be used to identify antibiotic resistant organisms by determining whether specific gene sequences that are responsible for bacterial antibiotic resistance mechanisms are present in the sample. An example of this is the use of PCR to detect the *mecA* gene, which encodes a penicillin-binding protein associated with methicillin resistance, to identify MRSA (York et al., 1996). Traditionally, PCR procedures required additional processing post-amplification to visualize and assess the results, typically using gel electrophoresis. An alternative to these labor-intensive procedures is quantitative or real-time PCR, which allows for the detection of the target gene sequences during the amplification process by monitoring the concentration of DNA using fluorescent dyes (Yang and Rothman, 2004). Real-time PCR procedures have been adapted for the identification of a wide variety of microorganisms (Deepak et al., 2007). There have also

been techniques developed for antibiotic sensitivity testing based on multiplex PCR, in which several different DNA sequences are simultaneously amplified and can be used to identify multi-drug resistant organisms (Rathore et al., 2018). The most important advantage of PCR over traditional antibiotic sensitivity testing procedures is speed- the results can typically be obtained in under 2 hours, compared to over 24 hours for agar and broth-based testing methods (Schumacher et al., 2018). While PCR-based methods are fast and accurate for identifying the presence of specific gene sequences associated with antibiotic resistance, they do have some notable drawbacks as well. One issue with these procedures is that they are solely testing whether or not a sample contains a specific gene, when that is not the only factor that impacts whether or not the bacteria are resistant to an antibiotic- cells may contain a particular gene indicative of antibiotic resistance, but not express it to the level necessary for the bacteria to actually be resistant to the antibiotic in question (Pulido et al., 2013). This could lead to false positive results if using PCR to test for antibiotic resistance. In addition, these methods also require knowledge of what specific target gene sequence to look for in order to identify antibiotic resistance, which limits their use for detecting novel types of antibiotic resistance or mutations in known characteristic gene sequences.

2.2.3. Other Types of Detection Techniques

In addition to the traditional growth-based methods and PCR, there are a variety of more novel techniques for assessing antibiotic sensitivity that have been developed in recent years. One example is the development of techniques that make use of DNA microarrays to detect the presence of antibiotic resistance genes as an alternative to PCR

(Perreten et al., 2005; Frye et al., 2010). These procedures are based on the hybridization of specific DNA sequences, which have been labeled with a fluorescent dye, to oligonucleotide probes attached to a chip or slide. The most important advantage of using DNA microarrays is their ability to screen for large numbers of genes at one time- microarrays have been developed for testing hundreds of known antibiotic resistance genes at once (Frye et al., 2010). However, they still have some of the same drawbacks of PCR since they are simply looking for the presence of specific gene sequences, and are not necessarily measuring their expression or determining whether are not the cells in a sample are actually resistant to an antibiotic. There are also a variety of procedures that have been developed using various types of probes to capture bacteria or detect specific cell components of antibiotic resistant bacteria. One example is the use of antibodies for detecting proteins that are produced from antibiotic resistance genes and play a role in the cellular mechanisms of resistance. A common target of developed assays that make use of this mechanism is *mcr-1*, a gene sequence that produces a protein associated with resistance to colistin, which is a key antibiotic of last-resort for multi-drug resistant infections (Liu et al., 2016b). Antibodies for the MCR-1 protein have been used to develop a number of immunoassays designed for the detection of the protein, which can be used to identify resistance to colistin. These include enzyme-linked immunosorbent assays (ELISA) and lateral flow immunoassays (He et al., 2018; Volland et al., 2019). Both of these assays make use of antibodies, which can be conjugated to an enzyme or other tag can produce a color when it is bound to the target protein. These methods are convenient, since they can be visually observed with the naked eye and clearly indicate the presence of proteins directly responsible for antibiotic resistance. They do have drawbacks as well- the

procedures for ELISA can be labor-intensive, antibodies can be quite expensive to produce, and these methods, similar to PCR-based procedures, require knowledge of a specific target and are not useful for identifying new types of antibiotic resistance mechanisms. There have also been methods developed that use aptamers, which are specially designed oligonucleotides that can bind to a target, as an alternative to antibodies as a probe for antibiotic resistance. Aptamers have been used to identify several types of antibiotic resistant bacteria, and can be integrated into systems such as microfluidic chips to make them more practical for widespread use (Wang et al., 2019). These procedures can have similar limitations of antibody-based assays, in that they can be labor-intensive and challenging to develop, and aptamers can degrade easily and be prone to other functionality issues (Lakhin et al., 2013). Finally, another alternative to conventional detection methods is the use of mass spectrometry-based procedures for analyzing biochemical indicators of antibiotic resistance. For instance, MALDI-TOF, which is a technique that uses a matrix-based ionization process for analyzing molecules with mass spectrometry, has been used to assess the degradation of antibiotics in a bacterial sample which can indicate the activity of enzymes that confer antibiotic resistance (Hrabák et al., 2011). This is a useful procedure that directly looks at the mechanisms of antibiotic resistance, but compared to the other methods that have been previously described it is not as practical- the equipment required for typical mass spectrometry analysis can be very expensive, difficult to transport, and require specialized laboratory training. While all of these methods for detecting antibiotic resistance have potential uses and advantages, they also all have distinct drawbacks and none of them are perfectly suited for this application. As a result, it is still necessary to

continue developing new detection methods for analyzing antibiotic resistant bacteria and improve our ability to identify these organisms.

2.3. Raman Spectroscopy and SERS

2.3.1. Introduction to Raman Spectroscopy

One class of analytical methods which have gained interest in recent years as a potential alternative to traditional ways of detecting antibiotic resistant bacteria are procedures based on Raman spectroscopy, which is a technique that uses the scattering of light to produce a spectrum which provides information about the chemical composition of a sample. Along with infrared spectroscopy, Raman spectroscopy is one of the major spectroscopic techniques that is used to measure molecular vibrations to characterize chemical structures and identify substances (Smith and Dent, 2005). The theoretical basis of Raman spectroscopy is the inelastic scattering of photons, also known as Raman scattering. When photons in a beam of light (such as a laser) interact with a material, some of them may be absorbed and re-emitted in a different direction, which is referred to as light scattering. Most of the photons which are scattered are emitted with the same frequency and wavelength, which is referred to as elastic or Rayleigh scattering. This is the predominant form of light scattering that occurs when photons interact with matter, and Rayleigh scattering is responsible for a variety of observable natural phenomena such as blue color of the sky and the orange color of sunsets (Smith and Dent, 2005; National Weather Service). However, not all of the photons which are scattered from an incident beam of light are scattered elastically- sometimes, energy is transferred between the photons and the molecules of the substance. As a result, the emitted photons have a

different energy level than the incident photons, and consequently exhibit a change in wavelength and frequency (Laserna, 2014). This is referred to as inelastic or Raman scattering, and is the central phenomenon that is the basis for Raman spectroscopy.

There are two types of Raman scattering- Stokes Raman scattering, in which energy is transferred from the photons to the material, leaving the material in a higher energy vibrational state, and anti-Stokes Raman scattering, in which energy is instead transferred from the material to the photon (Smith and Dent, 2005). Raman spectroscopy techniques primarily make use of Stokes scattering since it is typically more intense than anti-Stokes scattering, but there are some types of Raman techniques which do make use of anti-Stokes scattering as well (Kauffman et al., 2019). The actual measurement which is used to construct a Raman spectrum is the Raman shift, which is calculated using the difference between the incident light wavelength and the wavelength of the scattered light. The Raman shift is typically expressed in units of wavenumbers, which are a measure of spatial frequency (Smith and Dent, 2005). What a Raman spectrum is showing is the intensity of the Raman scattering at different wavenumbers- the peaks in a spectrum indicate that the intensity of the Raman scattering is particularly intense at specific wavenumbers. The Raman spectrum is therefore measuring the characteristic way that a sample scatters the incident light, and these patterns be used to identify substances and study the chemical composition of the sample. Raman spectroscopy is quite versatile and can be used to study a wide variety of substances, and many Raman spectroscopy-based analytical techniques have been developed over the past century (Laserna, 2014). These methods have become a fundamental part of how we conduct chemical analysis, and Raman spectroscopy continues to be utilized in laboratories around the world.

2.3.2. Surface-enhanced Raman Spectroscopy (SERS)

While Raman spectroscopy is a versatile and useful analytical technique, it does have some drawbacks which limit its practical effectiveness. One of the major challenges with Raman spectroscopy is that Raman scattering makes up a very small portion of overall light scattering, and most of the light is instead scattered elastically through Rayleigh scattering (Laserna, 2014). In fact, only one out of every 10^6-10^8 photons which is scattered is scattered inelastically, which means that Raman signals are usually quite weak and difficult to detect (Smith and Dent, 2005). As a result, enhancing the intensity of the Raman signal is a major priority for research and is a necessity for these techniques to be used more effectively.

Surface-enhanced Raman spectroscopy (SERS) is a method which has been developed that solves this problem. SERS was discovered by accident in 1974, when it was found that the Raman scattering of pyridine was enhanced when placed on roughened silver (McQuillan, 2009). The basis of SERS is that the Raman signals of target molecules are dramatically enhanced when they are adsorbed onto roughened metal surfaces or combined with metal nanoparticles. The use of a substrate such as these can enhance the Raman scattering of light by factors up to 10^8 or even larger, which can be intense enough to analyze individual molecules (Langer et al., 2020). There has been much debate about the mechanisms responsible for this enhancement, but the modern consensus among SERS researchers is there is a combination of electromagnetic enhancement from plasmon excitation in the metal substrate, and chemical enhancement from the transfer of electrons between the target molecule and the metal (Langer et al., 2020). SERS has become widely

used as an alternative to traditional Raman spectroscopy, since it can provide much clearer and more intense signals and be used to analyze very low concentrations of substances.

There are a variety of experimental parameters which can impact the effectiveness of SERS analysis. One of the most important variables which can impact the results of SERS is the type of metal substrate which is used to enhance the signal. An effective SERS substrate should meet several standards- it should be able to provide strong enhancement, give a uniform enhancement across the entire substrate, and be able to be reproduced with consistent results for different experiments and samples (McNay et al., 2011). The standard choices for SERS are often gold or silver, which provide strong enhancement as well as being relatively stable and non-reactive (McNay et al., 2011). These metals can be used to make substrates in a variety of ways, such as preparing gold or silver nanoparticle suspensions that can be combined with a sample for SERS enhancement, or by using metal-coated slides or chips that a sample can be directly dropped upon for analysis. Using different types of substrates can result in different levels of enhancement and may also contribute background signals, which may interfere with the SERS analysis of the target depending on its corresponding spectral patterns. Different types of substrates may be also be better suited for various samples depending on their physical properties- for instance, substrates may be better suited for solid, liquid, or gas samples. Other parameters that can impact the effectiveness of SERS analysis include the power and wavelength of the laser, the exposure time, and the spot or pixel size used to obtain each spectrum.

2.3.3. Applications of SERS

SERS has been adapted for a large number of applications in a variety of different fields. The ability of SERS to easily characterize many different substances makes it well-suited for usage in forensic and analytical applications, and it also has many potential uses in the food industry for identifying chemical, physical, and biological contaminants. One of the major uses of SERS is in forensics and studying crime scenes- SERS can be used to detect DNA, drugs, explosives, fibers, and other substances of interest to investigators (Muehlethaler et al., 2016). SERS is can also be used by historians to study the chemical composition of objects and artifacts- for instance, it can be used to identify the pigments used in centuries-old paintings (Pozzi et al., 2014). Another application is confirming the authenticity of a sample by studying its components, such as using SERS to avoid counterfeiting by analyzing the chemical composition of the ink used in signatures (Zhou et al., 2020).

Of particular interest to us is the use of SERS to identify contaminants and adulterants present in the food supply, to help ensure the safety and security of our food systems. SERS has been used to test for a wide variety of food contaminants, and SERS-based procedures have been developed for analyzing chemical, physical, and biological contamination and adulteration in foods. SERS can be used simply to identify the presence of chemical additives in foods, such as aspartame (Peica, 2009) and MSG (Peica et al., 2007). It can also be used to test for potentially hazardous or illegal substances, such as melamine (Lin et al., 2008), pesticides (Pang et al., 2016), antibiotics (He et al., 2010), and banned food colorants (Gukowsky et al., 2018). SERS can also be used to analyze some types of physical contaminants and adulterants, such as titanium dioxide nanoparticles (Zhao et al., 2017) and microplastics (Lv et al., 2020).

In addition to chemical and physical contamination, SERS has been used to identify microbiological contamination in foods as well. SERS has been previously used to detect a variety of foodborne pathogens, such as *Escherichia coli*, *Salmonella*, *Staphylococcus aureus*, and *Listeria* (Liu et al., 2017). Bacterial detection with SERS can be done using chemical labels, in which a molecule with a strong and distinct SERS signal binds to bacterial cells to make them easy to detect (Pearson et al., 2017). Methods have also been developed using SERS for label-free bacterial detection, in which the chemical signatures of the bacterial cells are analyzed directly (Wang et al., 2016a; Gao et al., 2017). In addition to simply testing the cells and identifying them from a spectra, there have also been some more innovative approaches that have been developed for studying bacteria with SERS, such as analyzing the extracellular matrix liquid instead of the cells themselves (Premasiri et al., 2016), and using SERS filter mapping to create an image that can be used to identify specific peaks in a bacterial sample and visualize the data in a useful and accessible way (Gao et al., 2018). There are a variety of potential ways SERS can be used to study bacteria and identify foodborne pathogens, and there is much more work that needs to be done to further optimize these methods and make them more suitable for practical applications.

2.4. SERS Analysis of Antibiotic Resistant Organisms

One of the most important potential applications for SERS-based bacterial analysis methods is the identification and characterization of antibiotic resistant organisms. The speed and simplicity of SERS makes it well-suited for this type of application and it would have several advantages over existing detection methods, such as the ability to easily study the biochemical characteristics of a sample and that knowledge of a specific target, such as

a gene sequence for PCR primers, is not necessary. Indeed, in recent years there have been a number of studies that have used SERS-based analytical methods for studying and detecting antibiotic resistant organisms (Galvan and Yu, 2018). These methods can be broadly separated into two categories- direct detection methods which use chemical labels or label-free methods to identify antibiotic resistant organisms, and antibiotic susceptibility testing in which bacteria are exposed to an antibiotic and their responses to antibiotic exposure are analyzed with SERS.

2.4.1. Direct Detection of Antibiotic Resistant Organisms

Many of the studies which make use of SERS to analyze antimicrobial resistant organisms are based on simply trying to detect specific organisms using the SERS signal of the bacteria cells in a sample. These methods tend to be fairly straightforward, and can make use of a variety of substrates and detection mechanisms. For instance, Jones et al. used SERS to identify strains of carbapenem-resistant Enterobacteriaceae and multi-drug resistant *Klebsiella pnuemoniae* (Jones et al., 2016). This study used a label-free detection method, with gold nanoparticle attached graphene oxide as a substrate. Another study by Fan et al. used a similar gold nanoparticle-graphene oxide substrate for label-free detection of MRSA (Fan et al., 2013). These types of label-free SERS techniques can also be adapted for lab-on-a-chip devices, such as in Mühlig et al.'s study which used a lab-on-a-chip SERS system and silver nanoparticle substrate to differentiate between strains of mycobacteria, including multi-drug resistant strain of *Mycobacterium tuberculosis* (Mühlig et al., 2016). In all of these studies, specific biochemical features of the cells were detected via peaks in the SERS spectra, such as components in the cell wall. The spectra obtained from the

different strains of bacteria could then be differentiated using further statistical analysis, such as with Principal Component Analysis (PCA).

One way to streamline these methods further is to use biochemical labels to identify specific organisms. In these methods, specially designed labels with distinct SERS signals are used to target specific drug-resistant organisms, allowing them to be differentiated from other cells in a sample. One example of such a method is demonstrated in Khan et al.'s study, which used gold nanoparticles conjugated with antibodies to detect a specific strain of multi-drug resistant *Salmonella typhimurium* (Khan et al., 2011). In this procedure, the nanoparticles only bind to this strain of bacteria due to the antibodies, which gives the target cells a strong SERS signal that allows them to be differentiated from other types of *Salmonella* and other bacterial strains, which have a very weak signal compared to the target. Another type of biochemical label which could be used in this application are based on aptamers, which have been used in combination with gold nanoparticles modified with highly SERS active molecules to differentiate between strains of *Salmonella typhimurium* and *Staphylococcus aureus* (Zhang et al. 2015). Alternatively, methods such as dielectrophoresis can be used to separate different types of cells in a sample, which can be used to test specific strains of bacteria in a sample with SERS without using a label (Cheng et al., 2013).

2.4.2. Antibiotic Susceptibility Testing with SERS

The other type of SERS analysis which can be performed with antibiotic resistant organisms is antibiotic susceptibility testing, in which the bacteria in a sample are exposed to an antibiotic and the resulting SERS spectra is used to assess their response. These tests

could be used to identify the minimum inhibitory concentration (MIC) of an antibiotic for a particular bacterial sample, or simply to assess the biochemical changes that are occurring in the bacterial cells and determine whether or not they are sensitive or resistant to the antibiotic. SERS is an effective method for studying the effects of antibiotic exposure to bacteria- for instance, Wang et al. used SERS to analyze the effects of ampicillin and ciprofloxacin on *Lactococcus lactis*, and were able to clearly assess the impact these antibiotics were having on the cells and the biochemical changes that were occurring in the sample (Wang et al., 2016b). Similarly, Liu et al. used SERS to monitor antibiotic-induced changes in bacterial cells, and was able to use the spectra to determine the MIC of *E. coli* and *S. aureus* samples (Liu et al., 2009). SERS procedures have also been developed which can both assess the antibiotic susceptibility of a sample and determine the MIC (Liu et al., 2016a). An alternative to these approaches is using SERS to differentiate between live and dead bacterial cells, which could be used to identify resistant bacteria following exposure to an antibiotic (Zhou et al., 2015).

SERS has also been shown to be usable for antibiotic susceptibility testing in clinical samples. Premasiri et al. used SERS identify bacteria present in clinical isolates from urinary tract infections, and determine what antibiotics they would be susceptible to by comparing the SERS bacterial identifications to an established database of antibiotic sensitivity for different strains of bacteria (Premasiri et al., 2017). This type of analysis is an example of how SERS could be used in practical applications for quickly identifying what kind of antibiotic treatments would be most effective for fighting infections. More direct treatments involving antibiotic exposure have also been used in clinical applications, such as Han et al.'s study using SERS to determine the antibiotic susceptibility of bacteria

isolated from blood samples based on the spectral changes observed in response to antibiotic exposure (Han et al., 2020).

2.4.3. Challenges and Potential Future Research

While a number of SERS-based methods have been developed in recent years for identifying antibiotic resistant organisms and determining antibiotic susceptibility, there is still much work that needs to be done for refining these procedures and making them suitable for use in practical applications. There are a number of notable knowledge gaps regarding how to best develop a SERS procedure for antibiotic sensitivity testing, starting with basic aspects such as the sample preparation procedure. Nearly all SERS-based methods for this type of analysis involve simply dropping the cells on a metallic substrate or mixing them with SERS-active nanoparticles, and then scanning the sample to obtain spectra for further analysis. There are a number of alternative methods that have been developed in recent years for SERS analysis, such as analyzing the supernatant liquid instead of the cells themselves (Premasiri et al., 2016) and SERS filter mapping (Gao et al., 2018). These methods could have significant advantages compared to traditional techniques- for instance, analyzing the supernatant could reduce the interference and variation which can occur when testing the cells directly, and produce a more clear and consistent signal. SERS filter mapping would provide a useful way of visualizing the data and allow us to view the variation across a wider area of the sample, and potentially allow us to analyze individual bacterial cells. Additionally, there have been advances in Raman technology in recent years and techniques could potentially be developed using newer, portable systems that are more suited for practical applications. It is essential that more

work be done to develop improved and optimized SERS methods for testing antibiotic resistant organisms, and we must continue to work towards the goal of developing a quick, rapid procedure for testing these organisms that is an improvement upon existing methods and a step forward in the international effort to surveil and control the spread of antibiotic resistance around the globe.

CHAPTER 3

ASSESSMENT OF THREE SERS APPROACHES FOR STUDYING *E. COLI* O157:H7 SUSCEPTIBILITY TO AMPICILLIN

3.1. Introduction

Since the discovery of antibiotics in the early 20th century, antibiotic resistant organisms have become widespread around the world and threatened the effectiveness of these important treatments (Ventola, 2015; Davies and Davies, 2010; Levy and Marshall, 2004). Antibiotic resistant infections, and particularly multi-drug resistant strains of infections such as tuberculosis and MRSA, are already responsible for tens of thousands of deaths every year in the United States and the EU alone (Aminov, 2010; Laxminarayan et al., 2013). The dangers posed by antibiotic resistance are expected to continue to increase in the coming years, and they could have an enormous impact on public health and the global economy if action is not taken (O'Neill, 2016). In the short term, it is of the utmost importance that effective detection methods exist for identifying antibiotic resistant organisms to help prevent them from spreading. Being able to rapidly and easily identify antibiotic resistance in samples of bacteria would help to improve global surveillance of these organisms and make it easier to monitor at-risk locations such as water sources near hospitals, factories, and farms. It could also be of use in medical applications, and would reduce the unnecessary prescription of antibiotics by quickly identifying what treatments would be effective against a particular infection.

There are a variety of detection methods that currently exist for identifying antibiotic resistance, but the most widely used methods tend to have drawbacks that limit their usefulness and potential applications. Culture-based detection techniques, in which

the bacteria are grown in the presence of antibiotics to assess their susceptibility, are still considered the gold standard for clinical applications (Galvan and Yu, 2018). These methods typically involve growing bacteria in broth or on agar plates containing different antibiotics or varying antibiotic concentrations to determine which antibiotics inhibit bacterial growth, and they can be used to determine the minimum inhibitory concentrations (MIC) of antibiotics for specific organisms (Syal et al., 2017; Bouki et al., 2013; McLain et al., 2016). While these types of procedures have been performed for decades and provide reliable data on antibiotic sensitivity and resistance, they can be exceptionally time-consuming and costly- it takes at least 48 hours to obtain a result from this kind of testing, and depending on the type of sample being tested it can take over 7 days (Galvan and Yu, 2018). In order to provide a faster way to diagnose antibiotic resistant infections and identify effective treatments, there has been significant work done in recent years to develop faster detection techniques. One type of technique is the use of polymerase chain reaction (PCR) and other amplification techniques to identify specific DNA sequences associated with antibiotic resistance (Anjum et al., 2017). Examples of DNA amplification techniques that have been used to identify antibiotic resistant bacteria include real-time PCR (Volkmann et al., 2004), loop-mediated isothermal amplification (Mu et al., 2016), and recombinase polymerase amplification (Kalsi et al., 2015). DNA based methods have also been adapted into procedures that can be used to identify genes for more than one type of antibiotic resistance in a single sample, using techniques such as DNA microarrays (Call et al., 2003; Perreten et al., 2005) and multiplex PCR (Strommenger et al., 2003). Other types of bacterial detection techniques that have been applied to antibiotic resistant bacteria include ELISA (He et al., 2018) and various aptamer-based procedures (Jo et al., 2018;

Wang et al., 2019). While these existing rapid detection techniques may be much faster than the traditional culture based methods, they have their own drawbacks as well- for instance, any DNA amplification or aptamer based technique requires knowledge of a specific gene sequence that is associated with the particular type of antibiotic resistance that is to be detected in the target organisms. This reduces their usefulness for detecting new types of antibiotic resistant bacteria that have not been previously described, or for screening for a wide variety of potential genes for different resistance mechanisms that would each require an individual set of primers. In order to adapt to a world in which new types of antibiotic resistant bacteria are rapidly developing, it would be ideal to have a rapid detection method that can reveal whether or not an organism is sensitive or resistant to a particular antibiotic, without the need for prior knowledge of the genetic basis of the potential antibiotic resistance. Ideally, this detection method would be quick, simple to perform, and would provide detailed information about the biochemical composition of a bacterial sample, including the mechanism of antibiotic resistance. Developing a technique such as this would make it easier to identify these organisms in environmental samples and in therapeutic applications, and would make the diagnosis and treatment of antibiotic resistant infections substantially more efficient.

One potential technique that could be used to develop this kind of detection method is Surface-Enhanced Raman Spectroscopy (SERS). SERS is a procedure based on Raman Spectroscopy, an analytical technique that utilizes the interactions between light and molecules and measures the scattering that results to produce a distinct spectrum, which can be used as a fingerprint for identifying different chemical compounds (Rostron et al., 2016). SERS makes use of transition metal nanostructures to significantly enhance the

intensity of the Raman signal, and this technique has been utilized in a wide variety of chemical and biochemical applications (Schlücker, 2014; Huh et al., 2009; Hering et al., 2008). One such application of SERS is the detection and characterization of bacterial cells (Jarvis and Goodacre, 2008; Premasiri et al., 2005; Sengupta et al., 2006). SERS procedures that have been developed for analyzing bacteria utilize a range of different substrates and mechanisms, including both label and label-free techniques (Galvan and Yu, 2018; Liu et al., 2017), and a number of studies have been performed that involve the characterization of bacterial responses to antibiotics using SERS (Liu et al., 2009; Wang et al., 2016b; Athamneh et al., 2014). These techniques can subsequently be applied to analyze antibiotic resistant bacteria and attempt to differentiate between different strains of an organism (Galvan and Yu, 2018). Studies that have utilized Raman and SERS techniques for detecting antibiotic resistant bacteria typically utilize one specific analytical procedure to characterize different organisms or responses to different antibiotics (Liu et al., 2009; Germond et al., 2018; Cheong et al., 2017a; Cheong et al., 2017b; Walter et al., 2011), and the SERS procedures used in these studies involve simply placing a drop of the bacterial sample on a substrate surface (Liu et al., 2009; Cheong et al., 2017a; Cheong et al., 2017b). While these previous studies have demonstrated that a range of different bacteria species and antibiotic resistance mechanisms can be identified with conventional SERS methods, there are also a number of less conventional SERS techniques for bacterial analysis that could potentially be used to detect antibiotic resistant organisms, such as the analysis of the extracellular matrix liquid (Premasiri et al., 2016) and SERS filter mapping (Gao et al., 2018). There are significant knowledge gaps regarding how effective these novel techniques would be for characterizing antibiotic resistant bacteria, and how they

compare to the conventional SERS procedures in this application. Having an improved understanding of how effective different SERS procedures are for analyzing antibiotic resistant bacteria would allow researchers to develop improved detection techniques, and would also provide valuable information about the strengths and limitations of different SERS-based analytical methods.

In this study, three different SERS approaches were used to analyze strains of *E. coli* O157:H7 that are sensitive or resistant to ampicillin, a beta-lactam antibiotic that functions by inhibiting the synthesis of the cell wall (Cho et al., 2014). Ampicillin is considered to be highly important in human medicine and is also utilized in a variety of agricultural applications (Marshall and Levy, 2011), increasing the potential risk of ampicillin resistance and the threats that it poses. This study assessed the effectiveness of different SERS approaches for differentiating between ampicillin sensitive (AmpS) and resistant (AmpR) *E. coli* O157:H7 based on their responses to antibiotic exposure and the effect that these responses have on the SERS spectra. To directly compare the two types of bacteria, a strain of AmpS *E. coli* was transformed with a plasmid encoding a beta-lactamase enzyme, which provides the bacteria with ampicillin resistance by changing the structure of the antibiotic molecule and inactivating it (Livermore, 1998). Previous research has shown that Raman spectroscopy can be used to differentiate *E. coli* cultures transformed with a plasmid containing an AmpR gene (Walter et al., 2011), suggesting that SERS would also be effective in this application. The techniques that were utilized in this study include a conventional SERS procedure in which in a drop of the culture is placed on a slide for analysis, as well as a procedure for analyzing the bacterial matrix liquid and a SERS filter mapping technique. AmpS and AmpR *E. coli* were exposed to ampicillin and

analyzed with each of these SERS techniques, and the effectiveness of each technique for differentiating between the two types of bacteria were assessed.

3.2. Materials and Methods

3.2.1. Bacteria and Chemicals

AmpS *E. coli* O157:H7 (ATCC® 43888™) was obtained from the ATCC, and the bacteria was cultured overnight at 37°C for 16 hours in tryptic soy broth (TSB) to obtain a concentration of approximately 10^9 CFU/mL. For the antibiotic solution, ampicillin was obtained from Thermo Fisher Scientific (Waltham, MA, USA). 100 mg/mL stock solutions of ampicillin were prepared in sterile deionized water and subsequently diluted for the experiments. To prepare the antibiotic resistant bacterial culture, the antibiotic sensitive cells were transformed with pUC19 DNA using a TransformAid Bacterial Transformation Kit (both obtained from Thermo Fisher Scientific) to introduce an ampicillin resistance gene. The instructions from the kit were followed to grow the culture and transform the cells with the DNA, and the resulting AmpR bacteria were plated on a tryptic soy agar plate containing 50 μg/mL ampicillin to select for transformed cells containing the pUC19 plasmid. Conventional antibiotic susceptibility testing was also performed with the AmpS and AmpR *E. coli* to validate their sensitivity to ampicillin, and the methods and results of these tests are shown in Figure 1.

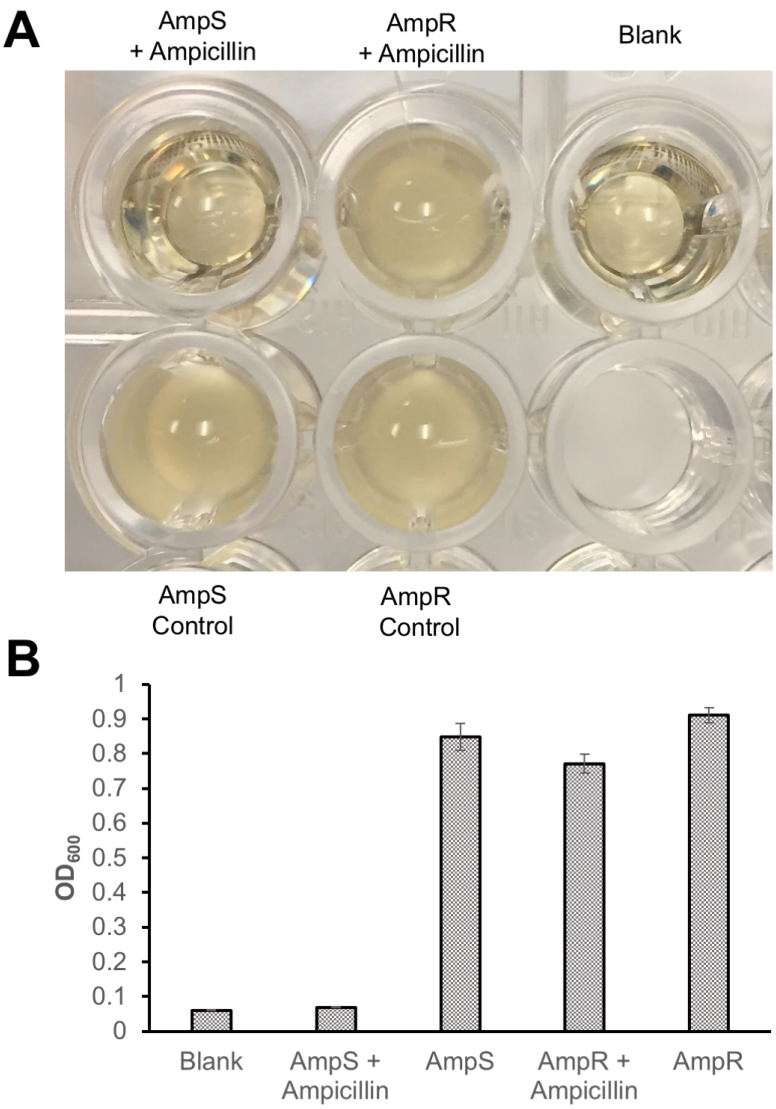

Figure 1. Antibiotic sensitivity testing of AmpS and the transformed AmpR *E. coli.* To prepare these samples, cultures of each *E. coli* strain were grown to an optical density of 0.1 at 600 nm. 2 µL of the culture was added to 498 µL of either TSB or TSB containing 0.1% ampicillin. A blank sample was also prepared using 498 µL of TSB and 2 µL of water. The samples were incubated overnight and checked the next day for growth. An image of the samples in transferred to a well plate are shown in (A), and optical density readings at 600 nm are shown in (B). Three independent samples were prepared and averaged to obtain the results in (B). The optical density readings were obtained using a SpectraMax M2 UV-vis spectrophotometer (Molecular Devices, San Jose, CA).

3.2.2. AuNP Synthesis

The synthesis procedure for the AuNPs used in this study based on the protocol previously described by Qu and He (2020). For synthesizing the AuNPs, chloroauric acid tetrahydrate ($HAuCl_4$) and sodium citrate were purchased from Sigma Aldrich (St. Louis, MO, USA). In a thoroughly cleaned and dried Erlenmeyer flask, 2 mL of 1% $HAuCl_4$ were mixed with 200 mL ultrapure water and heated on a stir plate (350 rpm) at 310°C. Once the $HAuCl_4$ solution reached a boil, 1.4 mL of 1% aqueous sodium citrate solution was added. The mixture continued to be heated and stirred until the color of the solution had turned a deep brick red color, approximately 30 minutes. The resulting AuNPs in the solution had an average diameter of 55 nm. The AuNP solution was then diluted to 200 mL to reach a final concentration of 0.1 mg/mL, and was cooled and stored in a refrigerator until further use.

3.2.3. Conventional Drop Method

The first SERS approach was the analysis of a drop of the bacterial culture that had been combined with the antibiotic and subsequently washed. This procedure is partially based on the analytical method previously used by Wang et al. to assess bacterial responses to antibiotics with SERS (Wang et al., 2016b). To prepare the samples, 2 separate 1 mL aliquots of the AmpS overnight *E. coli* culture were placed in separate tubes. The ampicillin stock solution was added to one of the tubes for a final concentration of 1 mg/mL. An equal volume of sterile deionized water was added to the other tube. The two tubes were incubated at 37°C for 90 minutes to allow the bacteria to interact with the antibiotic. After the incubation period, the two tubes were centrifuged at 3000x g for 1 minute to concentrate

the cells at the bottom of the tube, and then the resulting supernatant was removed and discarded. 1 mL of 154 mmol/L aqueous NaCl solution was added to each tube and mixed by pipetting up and down to wash the cells. The centrifugation and washing procedure with the NaCl solution was repeated two more times to remove any remaining traces of the antibiotic or growth media. Afterwards, 20 µL of the washed bacteria samples were each combined with 80 µL of the AuNPs in a separate tubes, and were incubated for 30 minutes at room temperature to allow the bacteria and the AuNPs to interact. An additional tube containing 80 µL of the AuNPs and 20 µL of the aqueous NaCl solution was prepared as a control sample for the background signal. Following the 30 minute incubation period, 5 µL drops of each sample mixture were placed on an aluminum foil-wrapped glass side and dried prior to SERS analysis. This procedure was subsequently repeated using the AmpR overnight *E. coli* culture. Three independent replicates were prepared and tested for each *E. coli* strain.

3.2.4. Extracellular Matrix Analysis

The second approach was the analysis of the extracellular matrix liquid. This procedure is partially derived from the method used by Premasiri et al. to analyze the bacterial supernatant (Premasiri et al., 2016). The initial sample preparation was similar to the conventional drop method- two 1 mL samples of the AmpS overnight *E. coli* culture were placed in separate tubes, one of which had ampicillin stock solution added to a concentration of 1 mg/mL ampicillin, and the other had an equal volume of sterile deionized water added. Once again, the tubes were incubated at 37°C for 90 minutes and then centrifuged at 3000x g for 1 minute. The supernatant was then removed and replaced

with 1 mL of sterile deionized water, and the cells were resuspended by gently pipetting up and down. The tubes were then centrifuged again at 3000x g for 1 minute and the supernatant was removed. 250 µL of sterile deionized water was added to each tube, and the cells were again resuspended by pipetting up and down. The two samples were then each filtered through Durapore PVDF filters (13 mm diameter, 0.22 µm pore size, obtained from MilliporeSigma, Burlington, MA, USA) using a 13 mm polycarbonate syringe filter holder (obtained from Cole-Parmer, Vernon Hills, IL, USA). 20 µL of the filtered liquid from each sample was combined separately with 80 µL of AuNPs, and a control sample was prepared by combining 80 µL of the AuNPs with 20 µL of sterile deionized water. The mixtures were incubated at room temperature for 30 minutes, and then 5 µL drops of each sample were placed aluminum foil-wrapped glass slide and dried prior to SERS analysis. This procedure was then repeated using the AmpR overnight *E. coli* culture, and three independent samples were prepared and tested for both the AmpS and AmpR *E. coli*.

3.2.5. SERS Filter Mapping

SERS filter mapping was the third approach tested, and the initial steps of the sample preparation were similar to the previous techniques. This procedure was partially based on the filter mapping procedure previously described by Gao et al. (2018). Two 1 mL samples of the AmpS overnight *E. coli* culture were placed in separate tubes, ampicillin stock solution was added to one tube for a final concentration of 1 mg/mL ampicillin, and an equal volume of sterile deionized water was added to the second tube. The two tubes were then incubated at 37°C for 90 minutes. Following the incubation period, each sample was filtered through a Durapore PVDF filter using a 13 mm polycarbonate syringe filter

holder. 1 mL of sterile deionized water was then filtered through each sample to wash the cells and remove any remaining antibiotic or growth media. 1 mL of the AuNPs was then filtered through each sample, and the filter membranes were removed from their holders and placed on a slide for SERS analysis. A control for the background signal was prepared by simply filtering 1 mL of AuNPs through an additional filter, and this control filter was also placed on the slide for SERS analysis. The same procedure was then performed for the AmpR overnight *E. coli* culture, and three independent filter replicates were prepared for both AmpS and AmpR *E. coli* analysis.

3.2.6. Data Acquisition and Analysis

For the conventional drop method and the matrix analysis, a Thermo Scientific DXR Raman microscope (Thermo Fisher Scientific, Waltham, MA, USA) with a 20x magnifying objective was used to obtain the SERS spectra from each sample. A laser power of 4 mW and a grating of 400 lines/mm were used, with an excitation wavelength of 780 nm and an exposure time of 1 second. Spots from the "coffee ring" of concentrated AuNPs in the dried samples were chosen for analysis, and spectra from 15 spots on each sample were obtained using a spectral range of 2000 to 400 cm^{-1}. TQ Analyst software was utilized to obtain the average spectra from the three sample replicates and perform Principal Component Analysis (PCA), a statistical technique that separates the data into orthogonal variables which can be used to analyze and visually demonstrate the variation within a sample.

For the SERS filter mapping procedure, a Thermo Scientific DXRxi Raman imaging microscope with a 20x magnifying objective was utilized. 250 μm^2 SERS maps

were created from random locations on each filter, using a laser power of 4 mW, an excitation wavelength of 780 nm, an exposure time of 0.002 seconds, and a step size of 2 μm. OMNICxi Raman imaging software was used to analyze the maps and obtain the average spectra. Since this software does not allow spectral maps to be averaged together, a representative map was chosen from the three replicates to present, and the average spectra from all three replicate maps were used to obtain the average SERS spectrum for each sample.

3.3. Results and Discussion

3.3.1. Conventional Drop Analysis

The results of the conventional drop analysis are shown in Figure 2. There was a significant increase in the overall intensity of the SERS spectrum when ampicillin was added to the AmpS *E. coli* (Fig. 2A), with particularly notable increases in the intensity of the 733 cm^{-1} peak and in the range between 1200 cm^{-1} and 1700 cm^{-1}. There were also intensity increases in the range of 850 cm^{-1} to 1050 cm^{-1} and between 600 cm^{-1} and 700 cm^{-1}, as well as specific, less prominent peaks observed at 1570, 1415, 1370, 1320, 1278, 1240, 1114, 1004, 964, 890, 680, and 508 cm^{-1}. These intensity changes were not observed when comparing the transformed AmpR *E. coli* samples with and without ampicillin (Fig. 2C-D)- the average SERS spectra obtained from the two samples were nearly identical, suggesting that the antibiotic did not have a significant effect on the AmpR bacterial cells.

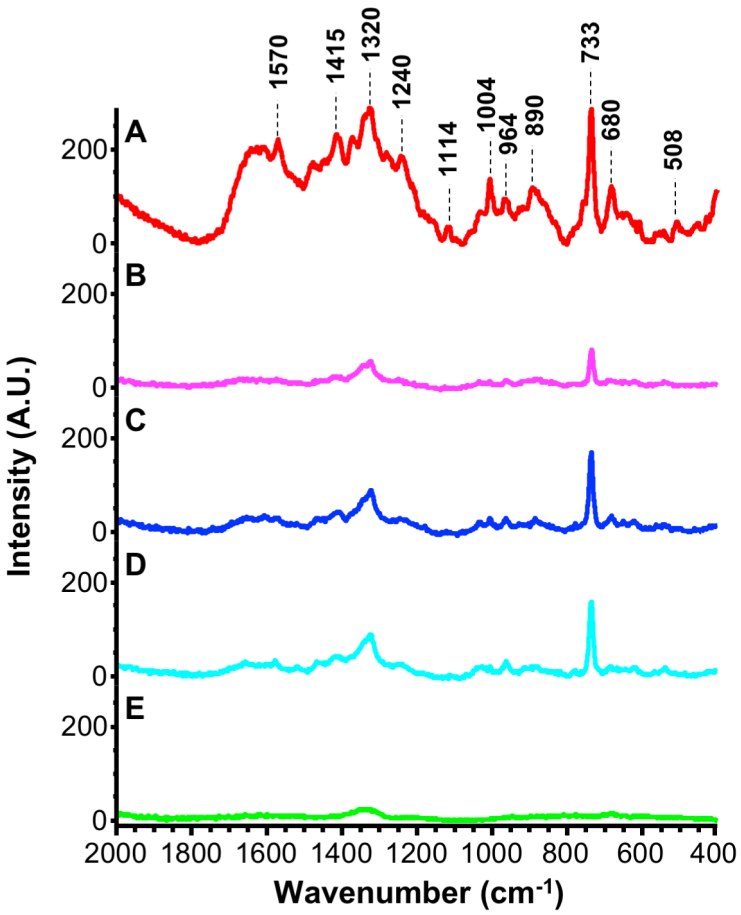

Figure 2. Average SERS spectra of AmpS and AmpR *E. coli* tested using the conventional drop method with AuNPs, shown at a common scale. A) AmpS *E. coli* combined with 0.1% ampicillin. B) AmpS *E. coli* control. C) AmpR *E. coli* combined with 0.1% ampicillin. D) AmpR *E. coli* control. E) AuNP control with no bacteria present.

The spectra obtained from these tests were subsequently analyzed using PCA to assess how easily the different samples could be distinguished based on the SERS spectra. The 3D PCA results are shown in Figure 3. All of the spectra from every sample except the AmpS + Ampicillin sample were clustered together and overlapped, showing they could not be statistically distinguished from one another. This was consistent with the results from Fig. 2, in which the average spectra from the AmpR samples and the AmpS

without ampicillin were all quite similar. However, as seen in the PCA, the spots representing the spectra from the AmpS + Ampicillin sample were much more spread out than the spectra from the other samples, suggesting more variation in the results from this sample and significantly different results from the AmpR bacteria in some of the spectra. There were a few spectra from the AmpS + Ampicillin *E. coli* that overlapped with the other samples, indicating that the effect of the antibiotic could not be seen as clearly in some spots that were tested.

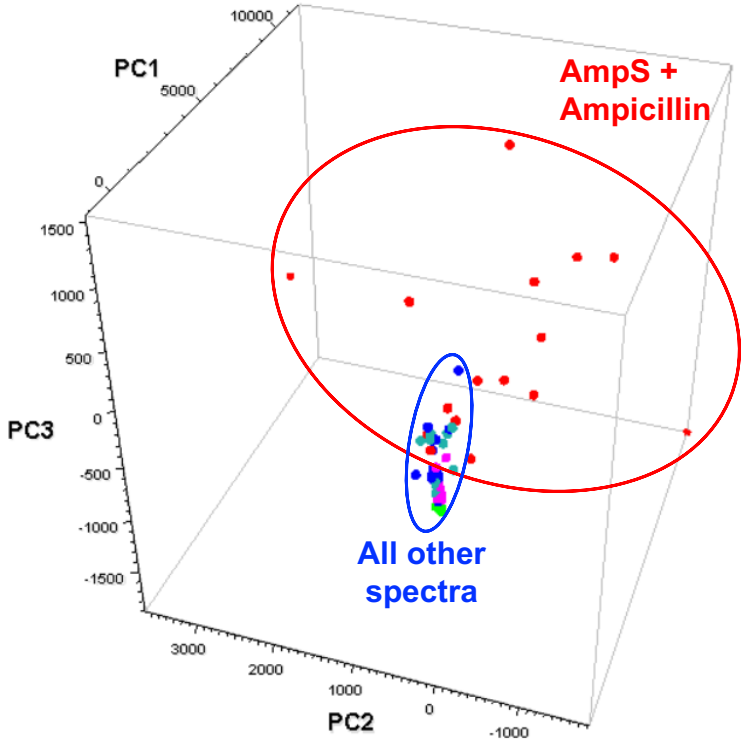

Figure 3. 3D Principal Component Analysis of the SERS spectra obtained from the conventional drop testing of AmpS and AmpR *E. coli*, which were used to make the average spectra shown in Figure 2. The spectra obtained from the AmpS bacteria exposed to ampicillin are distinguished from all of the other spectra, which includes the AmpS control, AmpR control, AmpR exposed to ampicillin, and the AuNP control. PC1, PC2, and PC3 explained 88.95%, 4.36%, and 2.43% of the variation, respectively.

3.3.2. Extracellular Matrix Analysis

The average spectra obtained from the analysis of the bacterial matrix liquid are shown in Figure. 4. Once again, the average spectra from the AmpR samples and the AmpS *E. coli* (Fig 4B-D) without the antibiotic present were largely identical, and the most prominent peak in each spectrum was at 733 cm^{-1}. The AmpS + Ampicillin spectrum (Fig. 4A) could be clearly differentiated from the other samples, although its average spectrum was considerably different than the corresponding sample from the conventional drop technique in Fig. 2A. In the matrix liquid sample, the intensity of the 733 cm^{-1} peak increased significantly when ampicillin was added to the AmpS *E. coli*, to an extent not seen in the conventional drop samples of the bacterial culture. For comparison, the intensity of the 733 cm^{-1} peak is more than five times greater in the extracellular matrix sample (Fig. 4A) than in the bacterial culture sample (Fig. 2A). Unlike the conventional drop testing, the 733 cm^{-1} peak was enhanced to a much greater degree than the rest of the spectrum in the matrix AmpS + Ampicillin sample. There was still some enhancement of the other peaks in the average spectrum compared to the AmpR samples, including the peaks at 1465, 1403, 1320, 1240, 1047, 964, and 621 cm^{-1}. Additionally, there was some minor variation in the background spectrum of the AuNPs when comparing Fig. 4E to Fig. 2E, since the AuNPs in the conventional drop sample control were combined with NaCl solution, causing them to aggregate, while the extracellular matrix sample control AuNPs were simply combined with deionized water. Deionized water was used for the extracellular matrix and the filter mapping approaches so that the resulting SERS spectra could be more accurately compared to the results obtained by Premasiri et al. (2016), while the conventional drop method results were intended for comparison with Wang et al.

(2016), who made use of NaCl solution instead of deionized water to wash the bacterial cells.

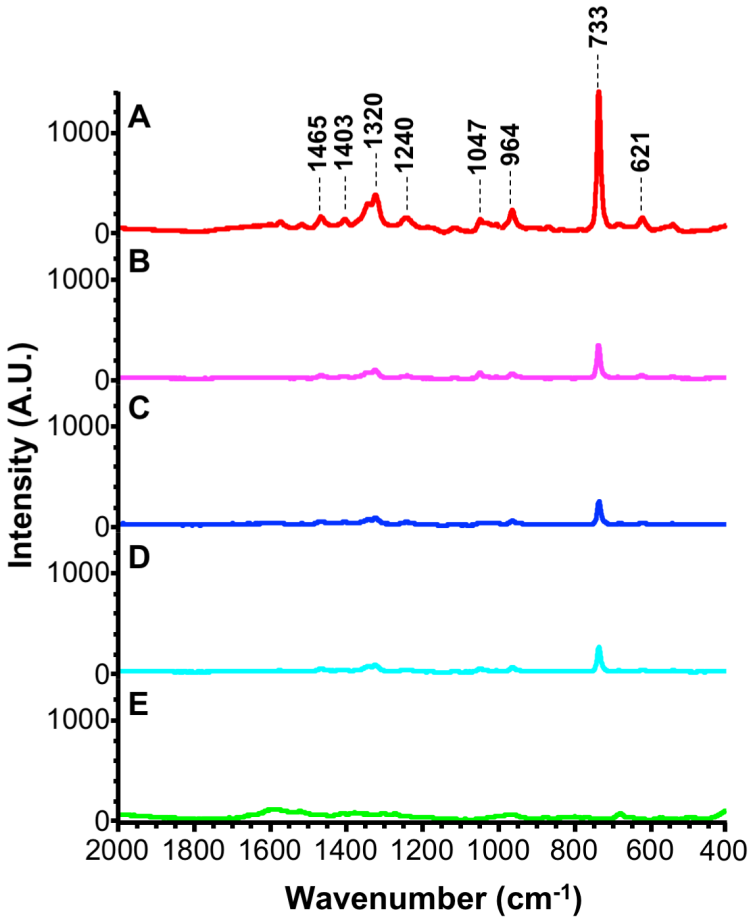

Figure 4. Average SERS spectra of the extracellular matrices obtained from samples of AmpS and AmpR *E. coli*, shown at a common scale. A) AmpS *E. coli* combined with 0.1% ampicillin. B) AmpS *E. coli* control. C) AmpR *E. coli* combined with 0.1% ampicillin. D) AmpR *E. coli* control. E) AuNP control with no bacteria present.

The corresponding 3D Principal Component Analysis of the spectra obtained from the extracellular matrix samples is shown in Figure 5. Similar to the conventional drop testing PCA from Fig. 3, the spectra obtained from the AmpR samples and the AmpS *E.*

coli without ampicillin all overlapped and could not be clearly differentiated from one another. However, in the matrix samples the AmpS + Ampicillin spectra were entirely separated from the other samples, indicating that they could be more clearly differentiated than in the conventional drop testing in which some spectra overlapped. The spectra from the AuNP background could also be clearly differentiated from the bacterial samples. The results from the PCA show that the SERS spectra obtained from the extracellular matrix analysis could be used to differentiate between AmpS and AmpR *E. coli* based on their response to the antibiotic, and the two types of bacteria could be distinguished more clearly with this procedure than the conventional SERS approach in which a drop of the bacterial culture is tested.

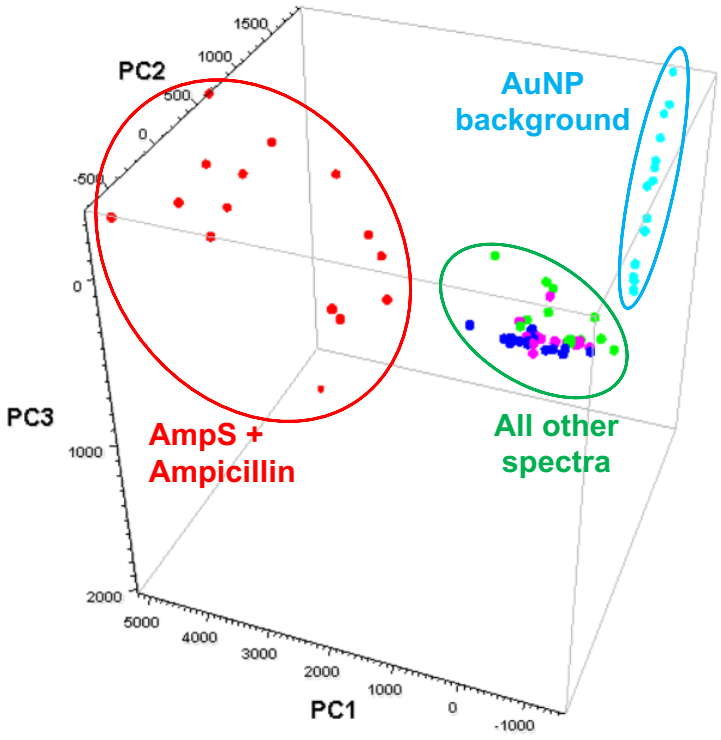

Figure 5. 3D Principal Component Analysis of the SERS spectra obtained from testing the extracellular matrices, which were used to make the average spectra shown in Figure 4. The "All other spectra" group includes the spectra obtained from AmpS *E. coli*, AmpR *E. coli*, and AmpR *E. coli* combined with ampicillin. PC1, PC2, and PC3 explained 86.50%, 9.14%, and 1.74% of the variation, respectively.

3.3.3. SERS Filter Mapping

The SERS filter maps obtained from the bacterial samples are shown in Figure 6, all at the same color scale representing the intensity of the 733 cm^{-1} peak. In the AmpR *E. coli* samples and the AmpS *E. coli* without the antibiotic (Fig. 6B, D, and E), nearly all of the pixels in the maps are blue or green, indicating a weaker SERS signal from the bacteria. In the AmpS + Ampicillin sample (Fig. 6C), however, there are a substantial number of

visible red, orange, and yellow pixels. These indicated a more intense SERS signal from the bacteria, and allowed the filter map of the AmpS + Ampicillin sample to be easily differentiated from the other maps. These results show how the SERS filter maps can be used to differentiate between the AmpS and AmpR *E. coli* based on their response to the antibiotic. The results also indicate one of the advantages of SERS mapping over simply picking a smaller number of individual spots to analyze, as the other approaches do. The bacteria and AuNPs are not distributed evenly or predictably across the surface of the filters, unlike in the other two approaches in which the sample droplets dry on a slide to form a concentrated "coffee ring" of AuNPs and bacteria which consistently has the most intense SERS signal. For the conventional drop method and extracellular matrix analysis, it is possible to reliably choose a smaller number of spots from the area that consistently has the strongest signal. On the filters, the concentration of bacteria or AuNPs and the intensity of the signal is much less consistently distributed across the sample. As a result, picking a smaller number of random spots on the filter to analyze with SERS could lead to significantly more variation than would be present in the other two approaches. Using SERS mapping on the filters instead of picking individual spots allows us to assess the broader patterns in a sample and reduces potential concerns that individual spots are not representative of the sample as a whole.

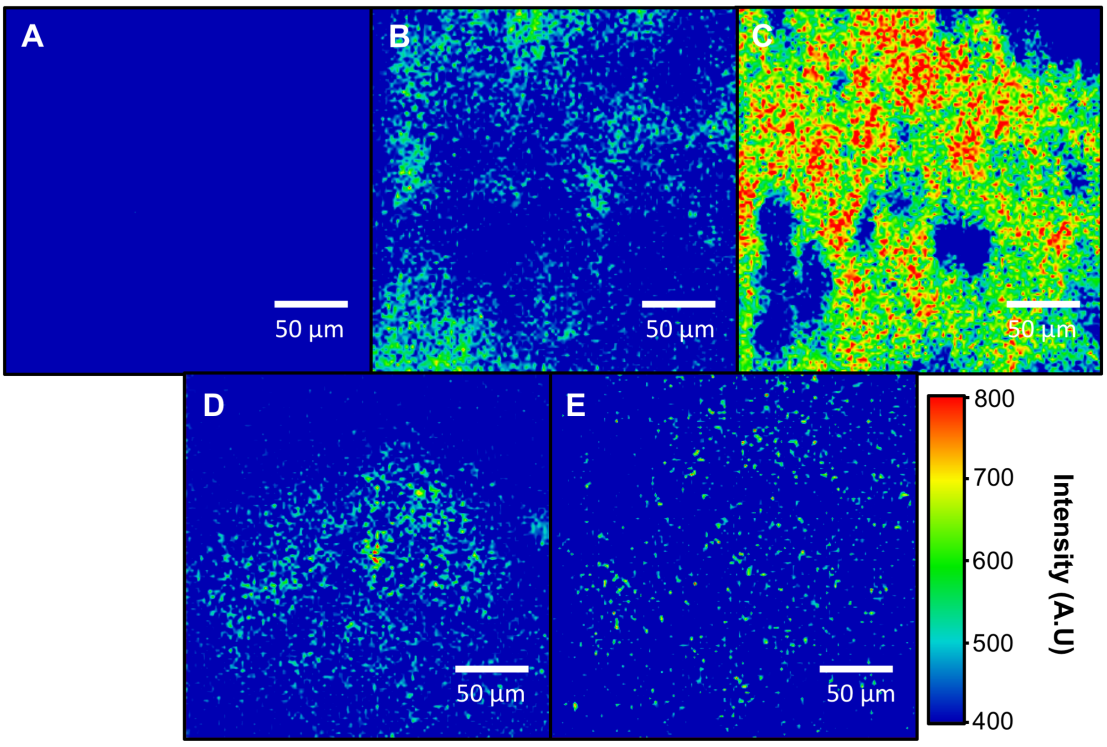

Figure 6. SERS filter mapping of AmpS and AmpR *E.coli*. A) is a control filter with only deionized water and AuNPs filtered through, without any bacteria. B) is the AmpS *E. coli* control, C) is AmpS *E. coli* combined with 0.1% ampicillin, D) is the AmpR *E. coli* control, and E) is AmpR *E. coli* combined with 0.1% ampicillin. The color scale on the bottom right indicates the SERS intensity of the 733 cm^{-1} peak in the maps.

The underlying spectra used to create the maps can also be separately analyzed. All of the individual spectra from each sample map were averaged together, and the resulting average spectra are shown in Figure 7. The intensity of these spectra was generally comparable to the bacterial culture tests using the conventional drop method in Fig. 1, although there was not a notable increase in the intensity of the entire spectrum in the AmpS + Ampicillin sample (Fig. 7A) as there was in Fig. 2. The 733 cm^{-1} peak did increase

a bit in intensity when ampicillin was added, as reflected in the corresponding filter map, but the intensity increase was less apparent than in the conventional drop tests or the supernatant analysis. There are a few potential factors that could explain this- for example, the average spectra for the map of each bacterial sample include a substantial number of blue spots containing a weak bacterial signal, since the bacteria are not distributed in a perfectly even fashion across the surface of the filter. When these negative spots are averaged in, it results in the average spectrum of the bacterial signal being weaker. Additionally, the filter mapping procedure uses a much shorter exposure time to obtain each individual spectrum than the other techniques, which could also result in a weaker overall signal in comparison. Another feature of the spectra is that the background signal of the AuNPs (Fig. 7E) is noticeably more intense than it was when tested with the other techniques, because a larger volume of the AuNPs was concentrated on the filter. This is partially reflected in the bacterial spectra as a broad increase in intensity between 1100 and 1700 cm^{-1}, although this could also be attributed to the concentration of bacteria on the filter.

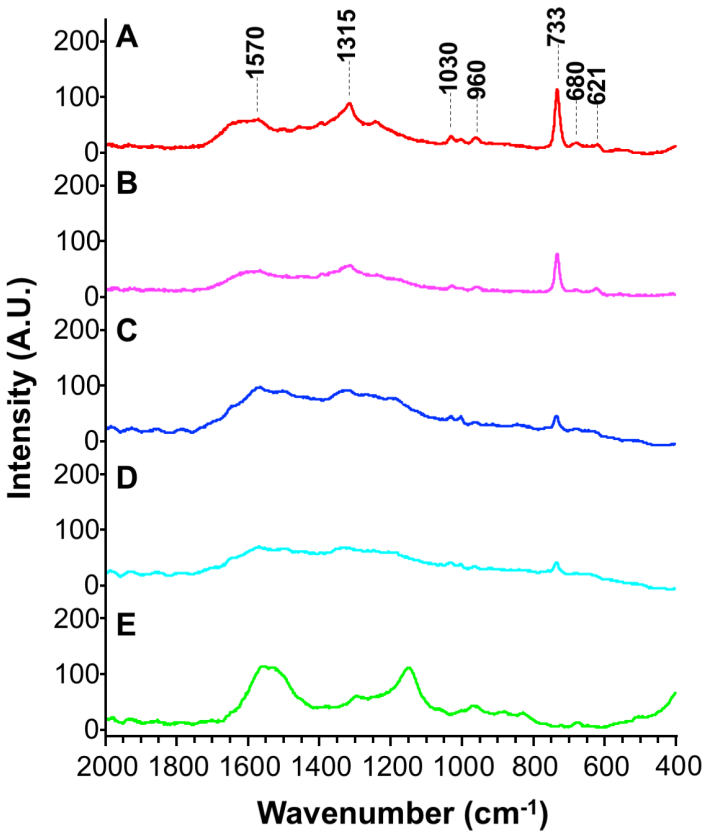

Figure 7. Average SERS spectra obtained from each of the corresponding maps in Figure 6. A) is AmpS *E. coli* combined with 0.1% ampicillin, B) is the AmpS *E. coli* control, C) is AmpR *E. coli* combined with 0.1% ampicillin, D) is the AmpR *E. coli* control, and E) is the control filter with only deionized water and AuNPs filtered through, without any bacteria present.

3.3.4. Data Interpretation

All three of the analytical approaches showed some consistent peaks and patterns in their resulting SERS spectra in response to ampicillin, indicating that there were distinct biochemical responses from the *E. coli* that could be detected with SERS. Although the concentration of bacteria in the AmpR samples would be higher than the AmpS samples

since they can survive exposure to ampicillin and continue to replicate, the AmpS bacteria exposed to the antibiotic consistently had a more intense signal than the AmpR bacteria. This suggests that other biochemical changes are occurring in the sample that impact the SERS spectra, rather than the signal being entirely based on the concentration of bacteria. The most consistent spectral change that occurred was an increase in the intensity of the 733 cm^{-1} peak in the AmpS *E. coli* spectra following ampicillin exposure, and this intensity change did not occur in the corresponding AmpR *E. coli* spectra. This intensity increase was observed to the greatest extent in the extracellular matrix, in which this peak was by far the most intense in the AmpS *E. coli* spectra (Fig. 4). Additionally, it was found that no additional correction or normalization was required to distinguish the SERS spectra from different samples from one another.

Since the SERS signal of a biological sample reflects the presence of specific chemical compounds with distinct spectral signatures, we can analyze the results to determine the biological origin of the SERS patterns observed in the AmpS and AmpR *E. coli* samples. Based on the results, the biochemical changes responsible for this intensity shift are occurring to a greater extent in the liquid matrix outside of the cell, but they are also present in the cells themselves since the 733 cm^{-1} peak were still observed in the cells trapped on the filter (Fig. 7). This peak has previously been associated with N-acetyl-D-glucosamine (NAG) and N-acetylmuramic acid (NAM), which are major components of peptidoglycan in the cell wall (Wang et al., 2016b; Premasiri et al.; 2016). However, our results suggest that the cell wall cannot be solely responsible for this peak, since it was considerably more intense in the extracellular matrix in which the cells had been filtered and separated out.

Another potential explanation for this peak is that it arises as the result of cellular metabolic processes and stress responses in response to antibiotic exposure. Previously, Premasiri et al. (2016) similarly found a distinct peak in this area and observed that this peak and other peaks in the bacterial spectra were very similar to the SERS spectra of some specific purine compounds, including adenine and adenosine monophosphate (AMP). They subsequently confirmed the presence of these compounds in the matrix liquid using mass spectroscopy. The presence of these compounds outside of the cell was attributed to the stress response of the bacteria to the conditions they are placed in during the sample preparation procedure, which increased the concentration of purine compounds in the cells' exogenous metabolome. In our results, the 733 cm^{-1} peak was observed in all of the bacterial samples, regardless of whether antibiotics had been added or not, which suggests that this peak is indeed related to an innate bacterial response to the sample preparation conditions. For comparison, we have included a SERS spectrum of adenine (Figure 8), which is shown to exhibit an intense characteristic peak at 733 cm^{-1}. The significant and consistent increase in the intensity of this peak in the AmpS + Ampicillin sample indicates that these purine compounds are present to a much higher degree in the extracellular matrix of this sample. While the media containing the compounds released directly during antibiotic exposure is removed during the washing procedure, the changes induced by the antibiotic are still having an effect on the cells which is visible in the SERS spectra of the final extracellular matrix liquid. One possible explanation for this is that exposure to ampicillin is causing a significant enhancement of the stress responses that occur when cells are resuspended in water, which were previously described by Premasiri et al. (2016). Since this peak and the corresponding intensity increase in the AmpS + Ampicillin sample

was observed to a much greater degree in the extracellular matrix than in the bacterial cells themselves, our results appear to confirm the conclusion of Premasiri et al. that these signals are from extracellular compounds and not from the components of the bacterial cell wall. It is also possible that debris from the lysed bacterial cells which make it through the filtration process could contribute to the bacterial signal. While the SERS patterns could be studied further in the future to gain additional understanding of how these mechanisms work, it is clear from the results that the SERS spectra can be used to differentiate between the AmpS and AmpR *E. coli*.

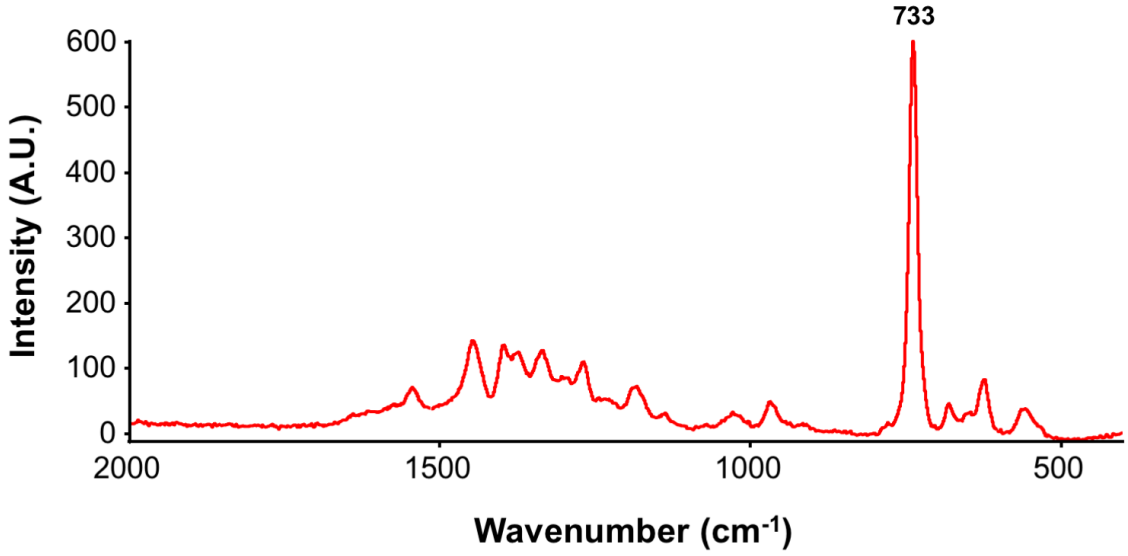

Figure 8. SERS spectrum of adenine. To obtain this spectrum, a 1% aqueous solution of adenine was prepared and 20 µL of the adenine solution was combined with 80 µL of AuNPs. 5 µL drops of the mixture were placed on an aluminum foil-coated slide and dried, and the spectrum was obtained using the DXR Raman microscope.

We can also assess information about the metabolic processes occurring in the AmpS and AmpR *E. coli* samples based on their SERS spectra and the relative intensity of

the adenine signal. Premasiri et al. (2016) previously examined the metabolism of purines in several different *E. coli* strains by examining their extracellular matrix liquid, and more detailed information about these metabolic pathways can be found in the KEGG database (Kyoto Encyclopedia of Genes and Genomes, 2020). Premasiri et al. found that the SERS spectra of *E. coli* strains that produced the enzyme adenosine deaminase, which coverts adenosine to inosine, were dominated by the compounds xanthine and hypoxanthine. These compounds are the byproduct of inosine metabolism, and could be directly observed in the extracellular matrix. However, when the *E. coli* was mutated to not produce adenosine deaminase, the resulting SERS spectra was dominated by adenine because the adenosine was solely converted into adenine instead of inosine. The SERS spectra from our extracellular matrix samples were very similar to the spectra from the mutant *E. coli* strain obtained by Premasiri et al., but were considerably different than the *E. coli* strains containing xanthine and hypoxanthine. The domination of both our AmpS and AmpR *E. coli* SERS spectra by adenine, without a clear contribution from xanthine or hypoxanthine, indicates that the *E. coli* samples we tested were primarily metabolizing adenosine into adenine instead of inosine. This suggest that the activity of adenosine deaminase in the *E. coli* strain we tested was significantly reduced or the enzyme was simply not produced.

3.3.5. Comparison of SERS Approaches

Comparing the results obtained from the three different SERS techniques provides valuable insight into the effectiveness of each approach for detecting ampicillin resistant *E. coli*, and allows us to assess the potential advantages and disadvantages of each

technique for detecting antibiotic resistant bacteria in general. A summary of these observations is shown in Table 2.

Table 2. Comparison of the advantages and disadvantages of each SERS approach for analyzing antibiotic susceptibility in bacteria.

SERS Approach	Advantages	Disadvantages
Conventional Drop Method	• Direct analysis of bacterial cells • Least amount of sample preparation required	• Potential interference from cellular compounds • Weaker signal from purine compounds (~733 cm^{-1} SERS peak)
Extracellular Matrix Analysis	• Clearest differentiation between AmpS and AmpR E. coli • Strongest signal observed from purine compounds	• May be less useful for antibiotics that affect interior of cell and not cell wall
SERS Filter Mapping	• More spectra can be obtained in a shorter amount of time • Can provide a broader view of the variation within a bacterial sample • More possibilities to adjust experimental parameters for different samples	• SERS signal can be weaker due to shorter exposure time • Increasing exposure time or decreasing step size can substantially increase collection time • May miss compounds in supernatant that were filtered through the membrane

The conventional drop SERS method provides some advantages compared to the other techniques, but also has some significant drawbacks that should be taken into account in future research. Our results showed that the SERS spectra of the AmpS *E. coli* combined with ampicillin obtained using this technique (Fig. 2A) contained more distinct, small peaks that could be used to differentiate between the AmpS and AmpR *E. coli* and characterize the bacterial sample. For example, there were small peaks observed at 1570, 1415, 1278, 1114, 1004, and 890 cm^{-1} that in the AmpS + Ampicillin spectrum with the conventional drop method that were not observed using the other techniques. This suggests that this type of conventional SERS method may be preferred for analyzing antibiotics that have less significant biochemical effects on the cell or only affect the interior of the cell, since the bacterial cells can be directly analyzed with this technique. This procedure also requires slightly less sample preparation than the other techniques. However, our testing revealed some of the problems that can be encountered when using a conventional SERS approach such as this procedure for analyzing bacteria. In particular, the intensity of the SERS signal was relatively weak, even in the AmpS + Ampicillin sample which had a stronger signal compared to the other samples. As demonstrated in the PCA in Fig. 3, there was also a significant amount of variation in the signal from the AmpS + Ampicillin. This is not necessarily a surprising observation, since there are several possible factors that impact how the antibiotic affects different cells within a sample that could lead to variation within the results. Beta-lactam antibiotics, such as ampicillin, specifically affect cells that are undergoing peptidoglycan biogenesis following cell division (Cho et al., 2014). The antibiotic would not have the same effect on cells in later stages of their life cycle, and based on our results would be expected to have different SERS spectra in response to the

antibiotic's effect. As a result, it is possible that different cells within the sample were in different stages of their life cycle when the antibiotic was added, and a range of responses can be observed in the SERS spectra which caused more variation in the overall results. Additionally, it is possible that there may be some cells in the sample which independently developed mutations or new mechanisms of resistance that would cause the SERS spectra in some areas to appear different than the rest of the sample. This variation, combined with the relatively low intensity of the spectra, makes it more difficult to differentiate between the AmpS and AmpR *E. coli*. These observations should be considered when evaluating the potential future effectiveness of this kind of conventional drop SERS technique for analyzing other types of antibiotic resistant bacteria. It should additionally be noted that there are a number of potential alterations to this procedure, including using other types of substrates and adjusting the experimental parameters and sample preparation techniques, that could be performed in the future to try to enhance the intensity of the SERS signal and reduce the variation within the sample. There are also aspects of the sample preparation procedure which could result in additional differences between the approaches- for instance, the washing liquid in the conventional method contains salt while the other two approaches simply use distilled water. This allows it to be more directly compared to the previous work done by Wang et al. (2016), but could also lead to potential differences between the conventional method and the other techniques.

The results from the extracellular matrix analysis procedure were the most clear in terms of being able to consistently differentiate between AmpS and AmpR *E. coli*. The significantly enhanced 733 cm^{-1} peak in the AmpS + Ampicillin sample allowed the AmpS *E. coli* to be easily differentiated from the AmpR *E. coli* based on their response to the

antibiotic, and this is reflected in the corresponding PCA (Fig. 5) in which the AmpS + Ampicillin spectra are entirely separated from the other bacterial samples, unlike the results from the conventional drop method. As discussed previously, this 733 cm^{-1} peak has been attributed to adenine present in the extracellular matrix due to purine metabolism occurring a bacterial response to stress (Premasiri et al., 2016). The significant and consistent increase in the intensity of this peak in the AmpS + Ampicillin sample indicates that adenine compounds are present to a much higher degree in the extracellular matrix of this sample- even though the media containing the antibiotic that was used to incubate the cells was removed after washing, the changes induced by the antibiotic are still having an impact on the resulting concentration of adenine compounds in the surrounding liquid when the cells are resuspended in water. This suggests that ampicillin exposure has an impact on the subsequent bacterial stress responses that occur when the cells are resuspended in water, causing a higher concentration of adenine compounds to be released. This could potentially be due to the damage that ampicillin causes to the cell wall (Wang et al., 2016b), allowing more intracellular compounds to be released during subsequent washing steps. An additional point that should be noted is that while the matrix liquid analysis procedure may have been the most effective for differentiating between AmpS and AmpR *E. coli*, this may not be the case if this technique is applied in the future to other types of antibiotics and bacteria. In particular, this approach could be less useful for analyzing the effect of antibiotics which affect the interior of the cell and have less of an impact on the exterior of the bacterial cells or the extracellular matrix. Solely analyzing the extracellular liquid could result in missing specific cellular signals in the SERS spectra that are not present in the

matrix, and this potential drawback should be considered in future experiments in which this technique is applied.

The SERS filter mapping approach was also found to be effective for differentiating between AmpS and AmpR *E. coli*, but it has some unique advantages and disadvantages compared to the other techniques. Considering that it is possible to obtain thousands of spectra from a single sample using our SERS mapping technique, it would likely be preferable for evaluating the degree of variation within a sample compared to the more conventional SERS techniques in which only a few spectra are selected from random spots. This in itself has limitations- the mapping procedure we utilized in this study, while extremely rapid to perform, only looks at sections of the sample on the filter. In practical applications, it would likely be preferable to evaluate as large of a section of the filter as possible, since the bacteria may not be distributed perfectly evenly across the filter surface and there may be variation in the signal within the bacteria in the sample. Unlike the conventional drop samples, in which the bacteria were reliably concentrated in the "coffee ring" around the edge of the sample droplets as they dried, the bacteria in the filtered samples were not as reliably distributed throughout the sample, leading to more variation in the intensity of the SERS signal in the maps. The maps obtained in our study (Fig. 6), while able to show the difference between AmpS and AmpR *E. coli*, demonstrate some of the issues with only testing random smaller sections of the filter- there are varying numbers of pixels in each map containing the bacterial SERS peak, and within the maps there is considerable variation between the intensity of the bacterial peaks (particularly in the AmpS + Ampicillin sample), which indicates that the bacteria is not evenly distributed across the surface of the filter. Analyzing the entirety of the filter using the same parameters

that this study used, such as the exposure time and step size, would take several hours. While this may still be a useful way of evaluating all of the bacteria in a sample, it reduces the convenience of this technique as a faster and more efficient alternative to other SERS techniques. Adjusting the parameters by decreasing the exposure time for each spectrum or increasing the step size would speed up the mapping process, but would reduce the intensity of the resulting spectra and decrease the quality and resolution of the final map.

These issues do not mean that SERS filter mapping is not worth pursuing further as a detection method for antibiotic resistant bacteria- in fact, SERS mapping may ultimately end up being much more important than other SERS techniques in this application and for bacterial analysis in general. SERS mapping is a relatively nascent field of research, and as the quality of Raman mapping technology continues to improve it will likely be possible to obtain clearer and higher quality results in shorter periods of time. To our knowledge there have not been any other SERS filter mapping procedures previously developed for differentiating between antibiotic sensitive and resistant bacteria, so there is ample ground for further research in this topic. There are a great deal of ways to adjust the experimental procedure to try to obtain better results for different samples, including the use of other types of substrates, adjusting the substrate concentration, and using other kinds of filter membranes made from different materials. Considering the promising results obtained in this study, it would be useful to continue to investigate this area to improve the detection procedure and develop new filter mapping techniques for antibiotic resistant bacteria. It may also be possible to apply other types of existing SERS mapping procedures to antibiotic resistant organisms, including the analysis of biofilms and the detection of compounds used in intracellular signaling (Bodelón et al., 2016). Since SERS mapping is

capable of analyzing a larger number of bacterial cells that the other SERS approaches, it could also be used to study the variation in antibiotic susceptibility within a sample, as well as the development of antibiotic resistance in a sample over time.

3.3.6. Comparison to Previous Studies

The results from the conventional drop testing are comparable to our previous study by Wang et al. (2016), which examined the response of *Lactococcus lactis* to ampicillin using a similar SERS technique in which the bacterial culture was combined with AuNPs and analyzed following exposure to the antibiotic. There were some differences between the peaks observed when comparing the response of *L. lactis* to ampicillin to our results obtained with *E. coli*- in particular, there were peaks at 1270, 1146, and 1078 cm^{-1} that became quite prominent in *L. lactis* after 90 minutes of ampicillin treatment which were either quite small or not detected in *E. coli*. We previously associated these three peaks with amides, C-C/C-N bonds, and C-O-C stretching, respectively. All three of these features are found in both NAM and NAG, the two components of the peptidoglycan layer in the bacterial cell wall. Gram-positive organisms, such as *L. lactis*, have a much thicker layer of peptidoglycan in their cell walls than Gram-negative organisms like *E. coli* (Silhavy et al., 2010). This could explain the lack of these peaks in the *E. coli* spectra, since *L. lactis* contains much more peptidoglycan in its cell walls which could interact with the AuNPs as the cells lyse in response to ampicillin. We also previously attributed the ~733 cm^{-1} peak to peptidoglycan, which we now recognize was likely incorrect. In comparison, Premasiri et al. (2016) concluded that the cell wall components do not contribute to the peaks in the bacterial SERS spectra at all, which are instead from purine compounds in the

extracellular metabolome. Our results suggest that the truth may be somewhere in between- in response to ampicillin, some of the SERS peaks are from the extracellular matrix (such as the 733 cm^{-1} peak) and some may be related to the cell wall components, such as the peaks observed in Gram-positive *L. lactis* but not Gram-negative *E. coli*. An additional point to note is that while there were a few differences in SERS peaks between the results from this study and Wang et al.'s tests with *L. lactis*, the overall spectral patterns in response to the ampicillin exposure were actually quite similar- both *E. coli* and *L. lactis* displayed broad enhancements in intensity between 1200 and 1700 cm^{-1} as well as a significant increase in the intensity of the ~733 cm^{-1} peak. Comparing the two studies suggests that the effect of ampicillin exposure on the SERS spectra of AmpS bacteria is relatively consistent between different organisms, and it may be additionally possible to differentiate between Gram-positive and Gram-negative organisms based on the appearance of additional peaks in the SERS spectra.

It is known that using different SERS substrates can significantly influence the characteristics of SERS spectra. Our study found that the sample preparation method and instrumentation parameters also had a major impact on the SERS spectra, despite the substrate remaining consistent in all of the experiments. Similar findings have been observed in other studies. For instance, Premasiri et al. (2016) and Cheong et al. (2017a) both utilized similar AuNP-coated silicon substrates to analyze various strains of *E. coli* but the resulting spectra from each study look quite different. In particular, the ~733 cm^{-1} peak in the *E. coli* spectra obtained by Cheong et al. was much less prominent than in the spectra obtained by Premasiri et al., or than in studies that simply combined AuNPs with the *E. coli* such as this study or the experiments performed by Wang et al. (2016). This

discrepancy deserves further study- it is possible that it is related to the experimental parameters, since Cheong et al. utilized a considerably higher laser power and longer exposure time than the other three studies. It should also be noted that different studies can utilize different methods for processing the SERS spectra, which can lead to variation in the final intensity of the peaks and can lead to differences between the results obtained from different studies. In any case, given the importance of this peak in differentiating between AmpS and AmpR *E. coli* it is essential that it shows up clearly in the SERS spectra when they are used for this application. If a SERS procedure is ultimately used to a practical application to characterize different kinds of bacteria, it is of the utmost importance that the substrate and the experimental parameters remain constant for all of the samples tested. More work can be done to further optimize the analytical procedure, but the results obtained in this study show that the AuNP substrate we utilized is sufficient for differentiating between AmpS and AmpR *E. coli*. It could be useful to test a diverse range of substrates for analyzing antibiotic resistant bacteria to evaluate their effectiveness and determine what kind of substrate is the most useful for this purpose. An additional consideration for future studies is the survival of bacteria after incubation with the antibiotic and mixing with AuNPs- it may be necessary to assess how many cells survived these treatments and how this impacts the SERS signal. While this step was not necessary for simply differentiating between antibiotic sensitive and resistant bacterial samples, it could be useful for quantitative analysis of the bacterial cells, such as the study done by Gao et al. (2018).

Significant differences could also be observed when comparing Raman and SERS for studying bacterial susceptibility to antibiotics. For example, Walter et al. (2011)

examined *E. coli* transformed with a pDrive plasmid encoding ampicillin resistance using micro-Raman spectroscopy. Similar to our study, Walter et al. exposed the *E. coli* to ampicillin and analyzed the bacteria with Raman spectroscopy to try to differentiate and characterize the different types of *E. coli*. Comparing the results of Walter et al.'s study to this one, it is clear that simply using Raman spectroscopy instead of SERS results in very different Raman spectra. For instance, Walter et al. did not observe a significant peak at 733 cm^{-1}, and there was very little signal at all in this area. This could be due to the fact that Walter et al. used different excitation wavelengths (244 nm and 532 nm) than this study (780 nm). A similar discrepancy was observed by Premasiri et al. (2016), in which different peaks appeared in the spectra when different excitation wavelengths were used. Regardless of the experimental parameters, it is clear when comparing the results of this study to Walter et al.'s that the SERS techniques we utilized more clearly show the impact of ampicillin exposure on the Raman spectra, which can be used to differentiate between AmpS and AmpR *E. coli*. In our SERS results, the AmpS *E. coli* exposed to ampicillin can be clearly differentiated from the other *E. coli* samples by simply looking at the average spectra, while in the Raman analysis from Walter et al. the antibiotic has a much more subtle effect on the spectra and requires further statistical analysis to clearly differentiate between the samples.

3.4. Conclusions

This study demonstrates that three different types of SERS techniques are able to differentiate between AmpS and AmpR *E. coli* O157:H7 based on their response to antibiotic exposure. SERS could be performed on either the bacterial culture or the filtered

extracellular matrix liquid to identify changes in the resulting spectra that could be used to tell apart the two varieties of bacteria. SERS-based filter mapping could also be successfully performed on AmpS and AmpR *E. coli* to visualize the intensity of specific SERS peaks in each sample, and the visible differences between the maps could be used to similarly differentiate between the bacterial samples. While each technique could be successfully applied to AmpS and AmpR *E. coli*, the results indicated the potential advantages and disadvantages of each technique for analyzing different types of antibiotic resistant bacteria in future studies. Analyzing the matrix liquid was more effective for differentiating between the bacterial responses to ampicillin than the conventional SERS analysis of the bacterial cells themselves, but the conventional techniques may be more useful for analyzing the effects of antibiotics that have less of an impact on the extracellular matrix. Our results also show that SERS mapping deserves further attention for its potential uses in this application, and can be a useful way to rapidly obtain information about variation within a population of bacterial cells. The SERS spectra we obtained can also be compared to results obtained from previous studies, and indicate that differences could be observed between Gram-positive and Gram-negative organisms and that the results could be significantly affected by the experimental parameters and the substrate that was utilized. SERS is a promising technique for the fast and simple analysis of antibiotic resistant organisms, and the SERS approaches used in this study are a potential foundation for a wide range of future research in this area.

CHAPTER 4

DEVELOPMENT OF A PORTABLE SERS PROCEDURE FOR DETECTING FOODBORNE ANTIBIOTIC RESISTANT BACTERIA

4.1. Introduction

The development of antibiotic resistant organisms around the globe poses a significant risk to public health. Over the past half-century, antibiotics have become widely used as fundamental treatments in human medicine, and they have revolutionized the way in which we fight bacterial infections (Davies and Davies, 2010). However, as antibiotic usage has increased around the world, there has been a simultaneous increase in the discovery of bacteria which are resistant to these treatments and pose a greater threat of inflicting antimicrobial resistant infections (Bell et al., 2014). This is a particularly major concern for agriculture and food systems, because a large proportion of antibiotics are used in agricultural applications such promoting growth in animals and as a prophylactic to prevent diseases (Van Boeckel et al., 2015; Isaacson and Torrence, 2002). The overuse of antibiotics in agriculture and food production has been associated with an increase in antibiotic resistance in foodborne pathogens such as *Salmonella*, and these organisms can easily spread antibiotic resistance to other, even more dangerous species of bacteria (Goldman, 2004). These trends are predicted to continue in the next few decades, as antibiotic usage increases in the developing areas of the world (Van Boeckel et al., 2015). As antibiotic usage continues to increase, antibiotic resistance infections are expected to become an even greater menace to public health around the world, potentially resulting in millions of deaths and causing significant damage to the global economy in the coming years (O'Neill, 2016). It is essential that we continue to take steps to fight the development

and spread of antibiotic resistance, so that we can ensure that these key treatments remain effective and avoid the worst-case scenarios to prevent needless death and devastation from these illnesses.

One of the most important steps that we can take to prevent the development and spread of antibiotic resistant organisms is to have effective detection methods, which are capable of rapidly identifying these organisms and able to help prevent them from spreading into our food supply, the environment, and ultimately to consumers. There are variety of methods which are currently used to identify antibiotic resistant bacteria, but all of these methods have various kinds of drawbacks which limit their practical usefulness. The most widely used techniques are based on measuring the ability of a bacterial sample to grow in the presence of an antibiotic (Pulido et al., 2013). These methods are able to determine if specific antibiotics inhibit the growth of bacteria, and they can also be used to determine the minimum inhibitory concentration (MIC) for different antibiotics. Examples of these types of methods include agar dilution, broth dilution, and disk diffusion. These techniques have the advantage of having been used for decades, and have internationally established procedures for their use which help to ensure reliable and accurate results (European Committee on Antimicrobial Susceptibility Testing, 2020). However, they do have some significant disadvantages as well. In particular, they can be quite time-consuming- it can take over 48 hours to obtain a result from this kind of testing. Since more rapid identification of antibiotic susceptibility can make it easier to treat bacterial infections (Barenfanger et al., 1999), it would be preferable to have detection methods that were capable of more quickly identifying antibiotic resistant organisms.

As an alternative to the traditional growth-based techniques, a number of more rapid detection methods have been developed for antibiotic resistant bacteria. The most common in clinical settings are methods based on PCR, which is able to amplify specific DNA sequences associated with antibiotic resistance (Schumacher et al., 2018). PCR-based methods are much faster than traditional techniques- results can be obtained in under 2 hours, and it is an accurate and efficient analytical procedure. PCR does have some major drawbacks as well- in particular, it requires knowledge of specific target gene sequences that are present in the bacteria sample. As a result, these methods are not useful for identifying new types of antibiotic resistance that have not previously been studied. Additionally, PCR only identifies the presence of genes- some gene sequences associated with resistance may be present but not actually expressed by the cell, and the bacteria are actually sensitive to the antibiotic despite being identified as resistant by PCR (Pulido et al., 2013). Other rapid methods that have been developed include DNA microarrays (Perreten et al., 2005; Frye et al., 2010) and antibody-based methods such as ELISA (He et al., 2018), and these methods have similar drawbacks to PCR- they require knowledge of specific cellular targets. Ideally, it would be best to have a detection method without these drawbacks, which could more rapidly identify any type of antibiotic resistant bacteria without the need for primers or antibodies and would be able to provide additional characterization of the biochemical processes in the cell and confirm that the cells are resistant to specific antibiotics.

Our goal was to create a new procedure for identifying antibiotic resistant organisms that did not have the same drawbacks as growth and PCR-based detection methods. This procedure will make use of surface-enhanced Raman spectroscopy (SERS),

which is an analytical technique that allows to characterize the chemical and biochemical composition of a sample. SERS is based on Raman spectroscopy, which makes use of the light scattering that occurs when a laser is shot at a sample to produce a spectrum that contains distinct peaks, which indicate the presence of specific chemical compounds or biochemical phenomena (Laserna, 2014). Raman signals tend to be quite weak, and metallic substrates such as roughened surfaces or nanoparticles can be used to enhance the intensity of the signal (Langer et al., 2020). This is the basis of SERS, which can be used to analyze a wide variety of potential samples in different applications. In this study, we will use SERS to differentiate between antibiotic sensitive and resistant bacteria samples. SERS analysis is often done using expensive benchtop laboratory equipment, which is not ideal for practical applications and transport to different location. We intend to develop a SERS procedure using a portable Raman spectrometer, which can fit into a briefcase and is more practical and less costly than more conventional equipment. Once we have tested our portable SERS technique with antibiotic sensitive and resistant bacterial samples, we will use it to analyze bacteria extracted from real food samples and determine how effective it is in real-world applications.

4.2. Materials and Methods

4.2.1. Chemicals and Bacteria

Antibiotic sensitive *E. coli* O157:H7 was obtained from the ATCC (ATCC® 43888™). The bacteria was cultured overnight at 37°C for 16 hours in tryptic soy broth (TSB) to obtain a concentration of approximately 10^9 CFU/mL to be used in the subsequent experiments. Ampicillin, neomycin, and chlortetracycline we obtained from Sigma Aldrich

(St. Louis, MO, USA) for the antibiotic sensitivity testing. 100 mg/mL stock solutions of the three antibiotics were prepared and diluted to the necessary concentrations for each experiment. To transform the *E. coli* and confer antibiotic resistance, plasmids were purchased from Carolina Biological (Burlington, NC, USA) and a TransformAid Bacterial Transformation Kit (obtained from Thermo Fisher Scientific, Waltham, MA, USA) was utilized. The plasmid pKan was used to confer resistance to aminoglycosides, and the plasmid pBR322 was used to confer resistance to tetracyclines and ampicillin. Following the testing of *E. coli*, strains of *Bacillus cereus* (ATCC® 14579™) and *Salmonella enterica* (ATCC® 51962™) were also tested to assess the effectiveness of this portable SERS method with other species of bacteria.

4.2.2. Substrate Synthesis

AuNPs were synthesized based on the procedure utilized previously by Qu and He (2020) and in the prior study in Chapter 3. In a clean and dry Erlenmeyer flask, 2 mL of aqueous 1% chloroauric acid tetrahydrate ($HAuCl_4$) solution was mixed with 200 mL ultrapure water and heated at 310°C on a stir plate at 350 rpm. 1.4 mL of 1% aqueous sodium citrate solution was added to the flask once the $HAuCl_4$ solution reached a boil. The contents of the flask were then heated and continually mixed until they turned a deep red color, which took approximately 30 minutes. The AuNPs in the resulting solution had an average diameter of 55 nm, and were diluted to 200 mL to obtain a final concentration of 0.1 mg/mL. The AuNP solution was then cooled and stored in a refrigerator until used in subsequent experiments. The chemicals used in this protocol were obtained from Sigma Aldrich (St. Louis, MO, USA)

4.2.3. Bacterial Sample Preparation

To expose the bacteria to the antibiotics and prepare the bacterial samples for SERS analysis, 1 mL volumes of the overnight bacterial culture were combined with 0.1% concentrations of each antibiotic. A control sample containing no antibiotics was also prepared by combining 1 mL of the bacterial culture with water, adjusting the volume of water so that the total volume is identical to the samples combined with the antibiotics. The samples were then incubated in a shaking incubator at 37°C for 90 minutes at 100 rpm. After the incubation period, the samples were centrifuged at 3000x g for 1 minute and the supernatant liquid was removed and discarded. To wash the cells, the pellets were each resuspended in 1 mL of water and centrifuged again at 3000x g for 1 minute. The supernatants were again removed and the pellets were resuspended in 250 mL of water. The samples were held at room temperature for 15 minutes, and then filtered through 13 mm Durapore PVDF filter membranes with a 0.22 μm pore size (obtained from MilliporeSigma, Burlington, MA, USA) using a 13 mm polycarbonate syringe filter holder obtained from Cole-Parmer (Vernon Hills, IL, USA). 500 μL of the AuNPs were then filtered through each sample, and the filter membranes were then removed and placed on an aluminum foil-coated microscope slide for SERS analysis. This procedure was performed for each of the three antibiotics, using both the antibiotic sensitive and resistant *E. coli,* as well as the other two species of bacteria. Control samples of the filter background were also prepared by filtering 500 μL of the AuNPs through one of the membranes, without any bacteria. This membrane was then tested to assess the background SERS signal of the filter membrane and the AuNPs.

4.2.4. Data Acquisition and Analysis

Once the bacterial samples were prepared on the filters, the SERS spectra were obtained using a TSI ChemLogix portable Raman spectrometer (Shoreview, MN, USA). The laser was focused on the sample, and spectra were obtained from three different randomly selected locations on the filters. For these experiments, a power setting two-fifths of maximum was utilized. The spectra were then saved and transferred onto a separate computer and analyzed using OMNIC software to characterize the samples and assess the intensity of the SERS peaks. T-tests were used to determine the statistical significance of the differences between the data sets.

4.2.5. Coliform Extraction from Ground Beef

Coliform samples were extracted from ground beef and identified using a procedure based on the solid medium method for coliform detection as described in the Bacteriological Analytical Manual of the U.S. Food and Drug Administration. 90% lean ground beef for testing was purchased from a local supermarket. Violet red bile agar (VRBA) was obtained from Hardy Diagnostics (Santa Maria, CA, USA) and plates were prepared using the manufacturer's instructions. 25 g of ground beef was placed into a bag with 225 mL of peptone water and placed in a Stomacher for two minutes. A series of tenfold serial dilutions were then prepared in peptone water using the stomached liquid, down to 10^{-5} g/mL. 1 mL of each dilution were plated on VRBA plates. Once the liquid soaked into the agar, additional VRBA was poured on each plate to cover the surface. Once the overlaid VRBA had solidified, the plates were incubated at 32°C for 48 hours. Afterwards, coliform colonies were identified based on their respective color and a colony

was used to grow an overnight culture in TSB and the same portable SERS analysis procedure used previously for the *E. coli* was performed. Separately, conventional antibiotic sensitivity tests in which the bacteria were grown in the presence of each antibiotic to confirm the sensitivity of the coliform samples to each antibiotic. This procedure is outlined in Figure 14.

4.3. Results and Discussion

4.3.1. Antibiotic Sensitive and Resistant E. coli

The average spectra obtained from the SERS testing of the antibiotic sensitive bacteria with the three antibiotics can be seen in Figure 9. The sensitive *E. coli*, when tested without any antibiotic added to it, had a distinct peak around 738 cm^{-1}. This peak was not observed in the filter background, which did not have any significant SERS signals. This 738 cm^{-1} peak was the only clearly distinct peak that was observed in the bacterial samples when tested with the portable Raman.

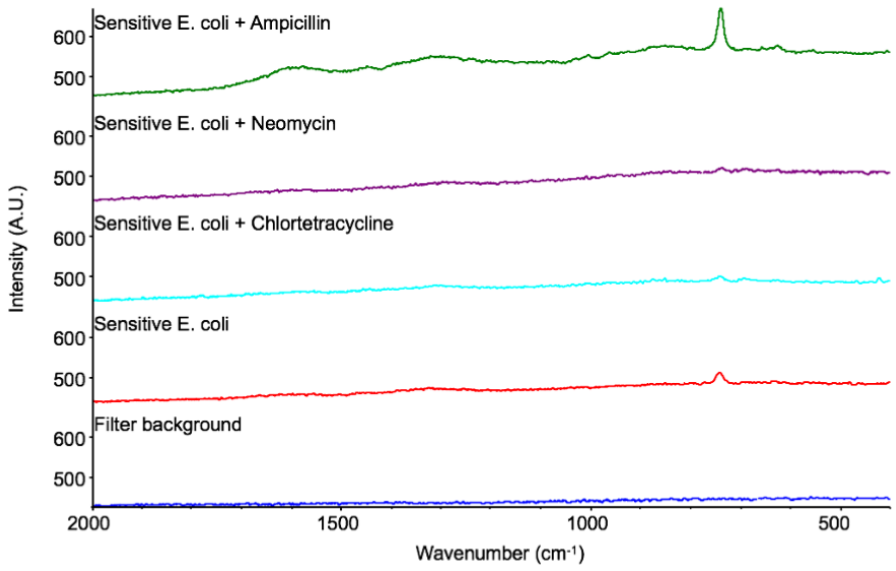

Figure 9. Portable SERS spectra of antibiotic sensitive *E. coli* combined with ampicillin, neomycin, and chlortetracycline.

There were noticeable changes which occurred in the SERS spectra after the antibiotics were added to the bacterial samples, primarily in the intensity of the 738 cm^{-1} peak. The changes in the intensity of this peak are quantified in Figure 10. When ampicillin was added to the *E. coli*, there was a substantial increase in the 738 cm^{-1} peak intensity that can be clearly observed in Figure 9, and can be seen to be statistically significant in Figure 10. There was also a modest increase in the overall intensity in the area of the spectrum between 1200 cm^{-1} and 1700 cm^{-1}, compared to the other bacterial samples. The other two antibiotics yielded considerably different results than ampicillin- both neomycin and chlortetracycline caused a decrease in the intensity of the 738 cm^{-1} peak, instead of an increase. As shown in Figure 10, the average intensity of this peak in the samples containing neomycin and chlortetracycline was significantly lower than the control bacterial samples without any antibiotics added. The 738 cm^{-1} peak intensity in these samples was instead more comparable to the filter background control sample, although the average intensity of this peak was still a bit higher than the background signal in the bacterial samples.

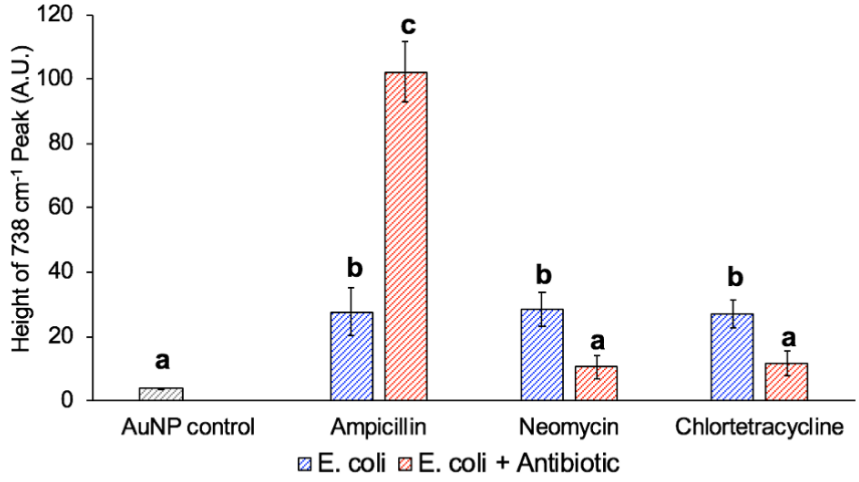

Figure 10. Height of the 738 cm^{-1} SERS peak in the antibiotic sensitive *E. coli* samples combined with ampicillin, neomycin, and chlortetracycline. Error bars represent the standard deviation of the peak heights from the Raman spectra obtained from each sample.

The spectra obtained from the transformed antibiotic resistant *E. coli* strains are shown in Figure 11. Similar to the sensitive *E. coli*, the most distinct peak that was observed in all of the bacterial samples was at 738 cm^{-1}. Unlike the sensitive *E. coli*, however, there was no significant change in the intensity of this peak when any of the antibiotics were added, which can be observed clearly in Figure 12. This pattern was consistent in both of the transformed *E. coli* strains, and can be contrasted with the notable changes observed in the sensitive *E. coli* as seen in Figure 9 and Figure 10.

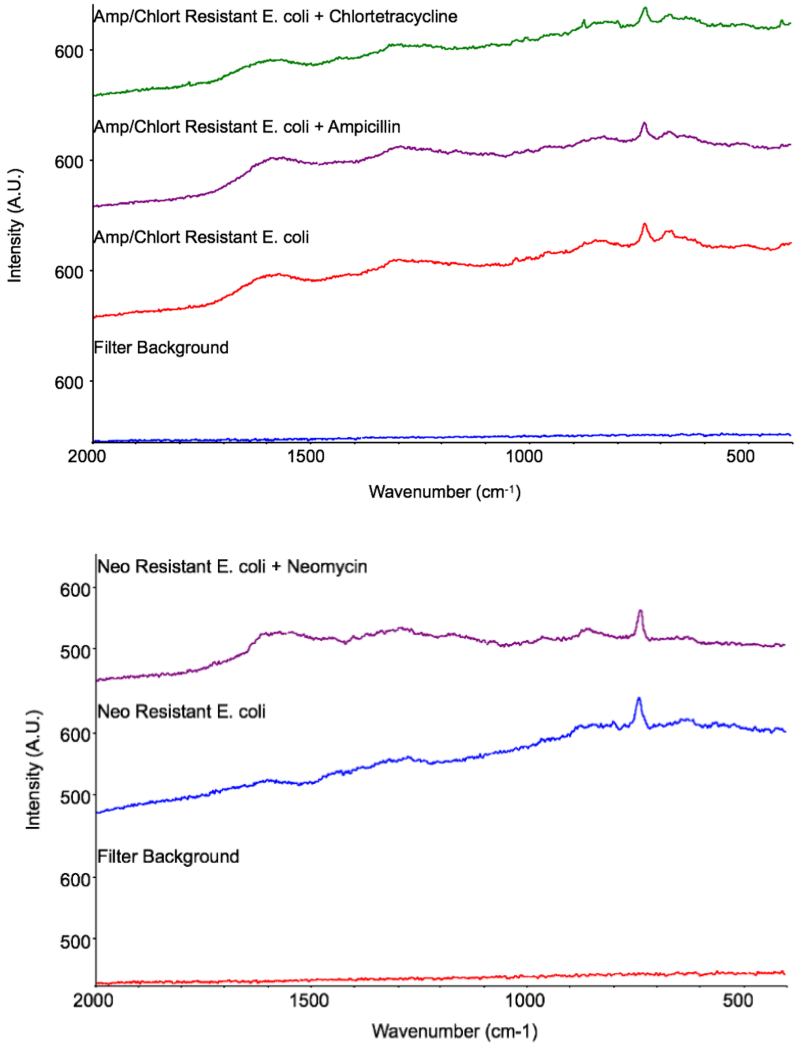

Figure 11. Portable SERS spectra of the antibiotic resistant *E. coli* samples combined with ampicillin, neomycin, and chlortetracycline.

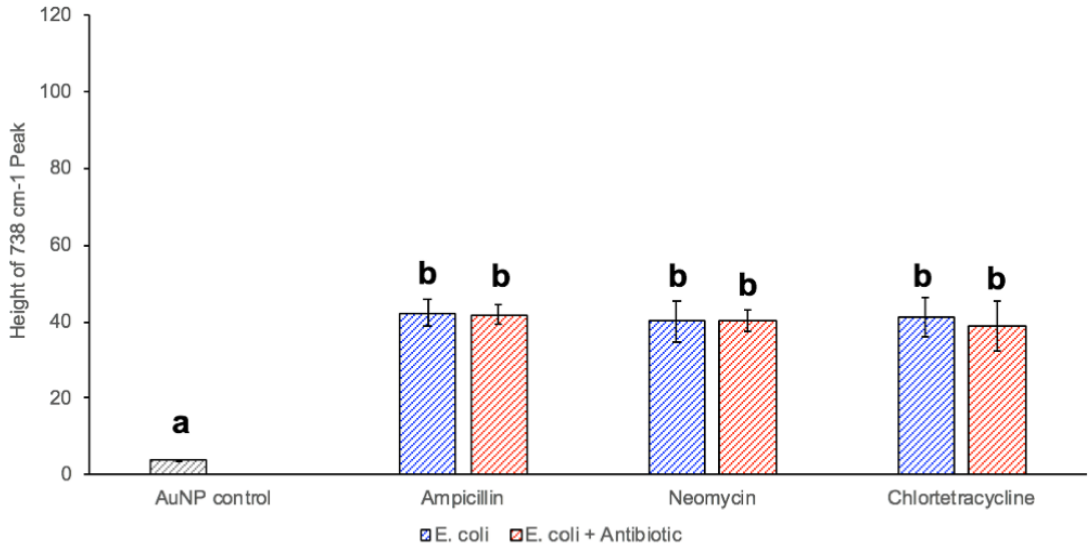

Figure 12. Height of the 738 cm^{-1} SERS peak in the antibiotic resistant *E. coli* samples combined with ampicillin, neomycin, and chlortetracycline. Error bars represent the standard deviation of the peak heights from the Raman spectra obtained from each sample.

4.3.2. Testing of Bacillus cereus and Salmonella enterica

Following the testing of the antibiotic sensitive and resistant *E. coli,* we obtained spectra using our portable SERS method from *B. cereus* and *S. enterica* combined with each of the three antibiotics. The average spectra obtained from *B. cereus* are shown in Figure 13. A comparison of the 738 cm^{-1} peak intensity from the *B. cereus* spectra is shown in Figure 14.

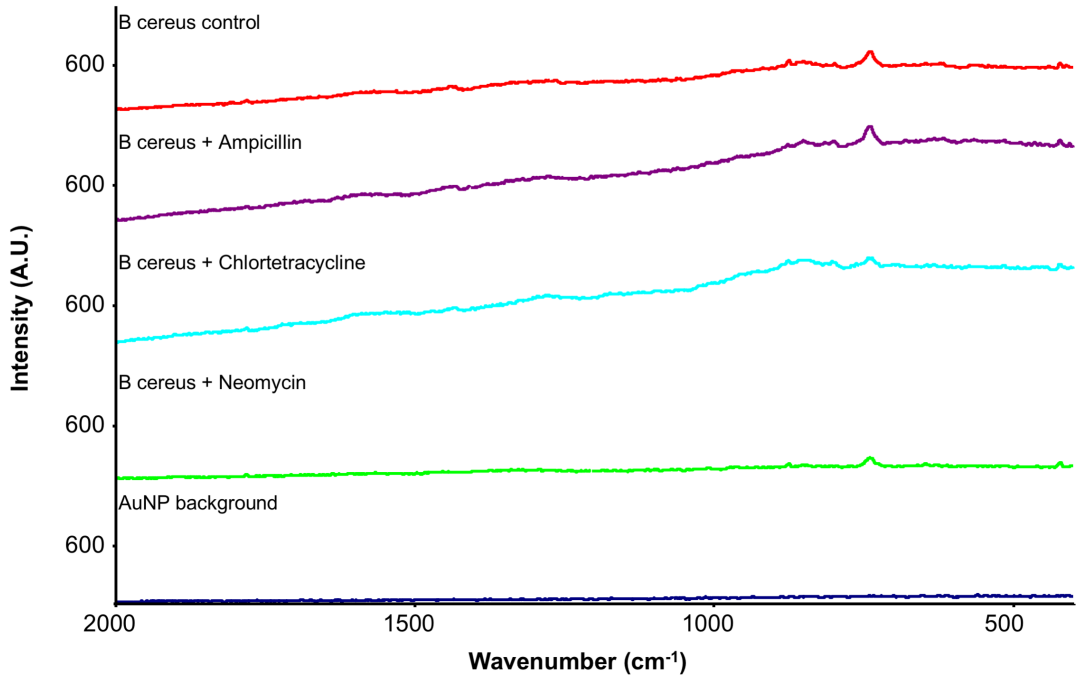

Figure 13. Average portable SERS spectra of *B. cereus* combined with ampicillin, neomycin, and chlortetracycline.

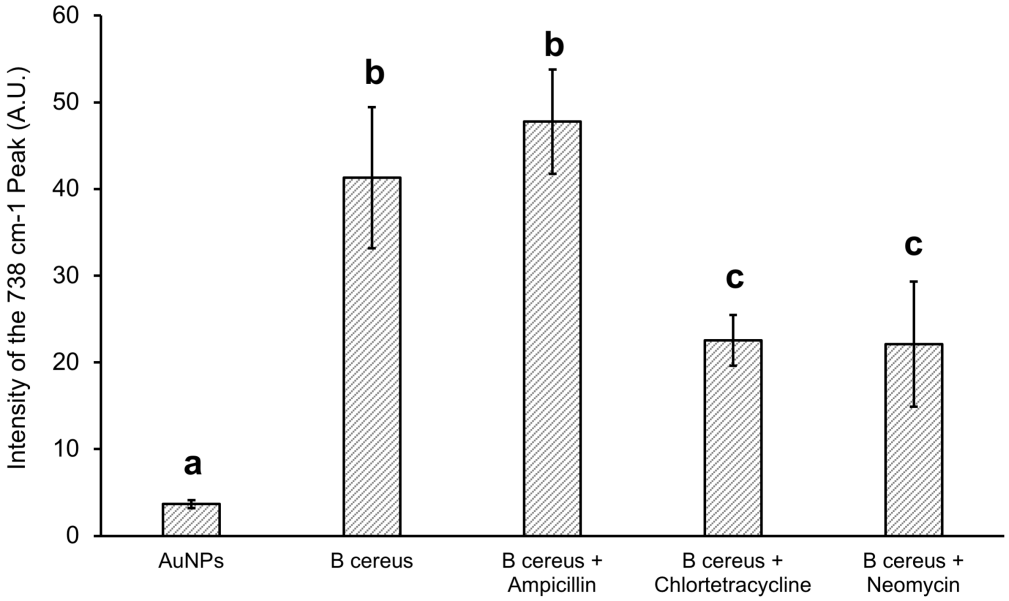

Figure 14. Average height of the 738 cm^{-1} peak in spectra from *B. cereus* samples combined with ampicillin, neomycin, and chlortetracycline. Error bars represent the standard deviation of the peak heights from the Raman spectra obtained from each sample.

As observed in Figure 13, the profile of the spectra obtained from *B. cereus* were largely identical were largely identical to the spectra previously obtained from the *E. coli*- the spectra were dominated by the 738 cm^{-1} peak. In response to antibiotic exposure, some differences, which can be seen in Figure 14, were found in comparison to the results previously obtained from antibiotic sensitive *E. coli*. While a decrease in intensity was observed after the *B. cereus* was exposed to chlortetracycline and neomycin, no significant increase in intensity was observed in response to ampicillin exposure. The corresponding data from the testing of *S. enterica* is shown below in Figures 15 and 16.

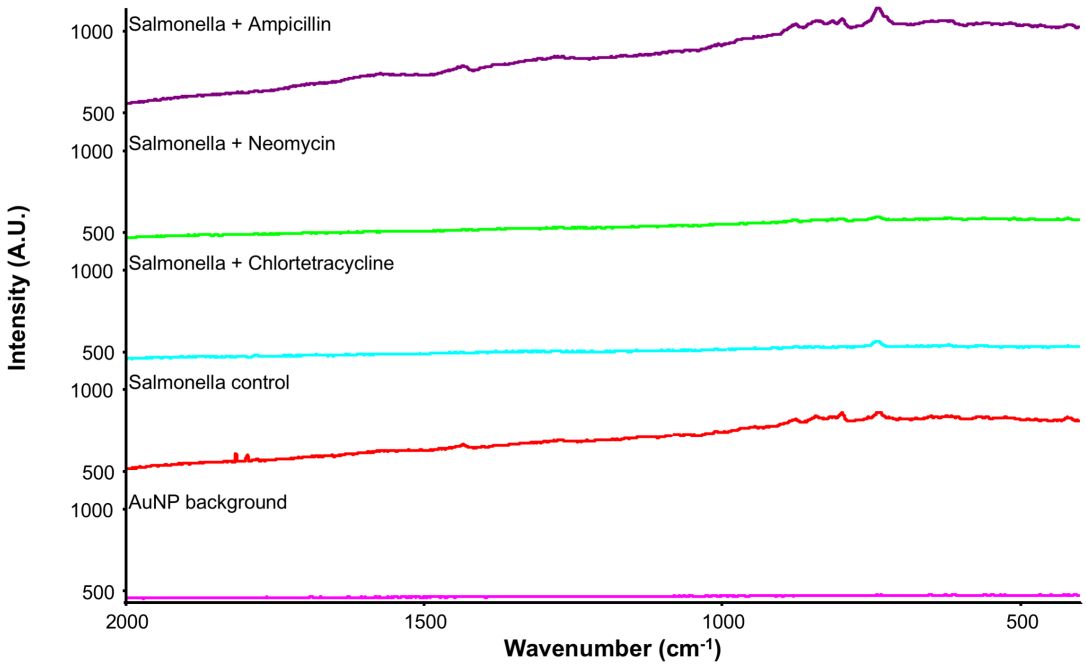

Figure 15. Average portable SERS spectra of *Salmonella enterica* combined with ampicillin, neomycin, and chlortetracycline.

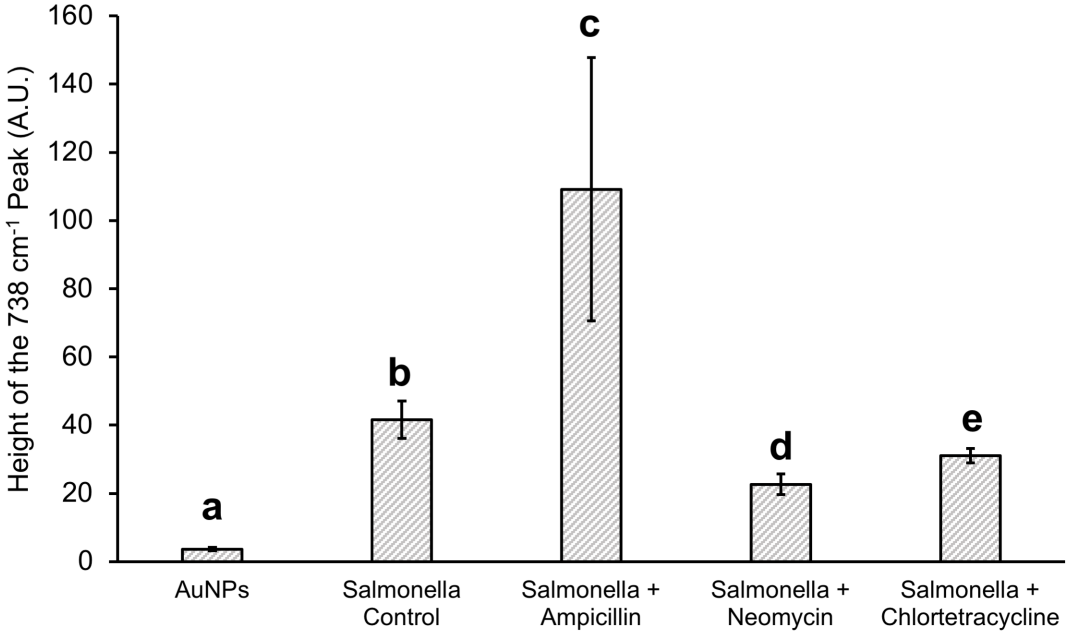

Figure 16. Average height of the 738 cm^{-1} peak in spectra from *Salmonella enterica* samples combined with ampicillin, neomycin, and chlortetracycline. Error bars represent the standard deviation of the peak heights from the Raman spectra obtained from each sample.

Once again, the spectra from *S. enterica* contained a peak at 738 cm^{-1}, and the height of this peak changed in response to exposure to antibiotics. Unlike the *B. cereus,* the patterns observed in the *S. enterica* samples were largely identical to the antibiotic sensitive *E. coli*- there was a significant increase in the peak height when the bacteria were exposed to ampicillin, and a significant decrease instead when they were exposed to neomycin and chlortetracycline. There was also a statistically significant difference between the average peak height of the bacteria exposed to neomycin and chlortetracycline, which was not observed in the other samples.

4.3.3. Testing of Bacteria Extracted from Ground Beef

Coliform colonies were successfully isolated from the supermarket ground beef- an image of the plate is shown in Figure 13, with the purple colonies identified as coliforms. We also found from the conventional antibiotic sensitivity tests that the bacteria we isolated were sensitive to all three antibiotics- ampicillin, neomycin, and chlortetracycline. The results from these tests are shown in Figure 14. It was found that the characteristic signal of the ground beef isolate bacteria was nearly identical to the previously tested *E. coli* strains. The most significant peak observed in the spectrum, like the *E. coli*, was around 738 cm^{-1}. To obtain a clear picture of the bacterial signal, the liquid filtered from the bacterial control sample during the sample preparation procedure was reserved, combined with AuNPs, and analyzed using our DXR Raman microscope. The resulting spectrum, and a more detailed description of the analytical procedure, are shown in Figure 15. The 738 cm^{-1} peak can be clearly observed in the bacterial spectrum, and it is not present in the background control spectrum of the AuNPs.

Figure 17. Image of a VRBA plate used to extract coliforms from supermarket ground beef.

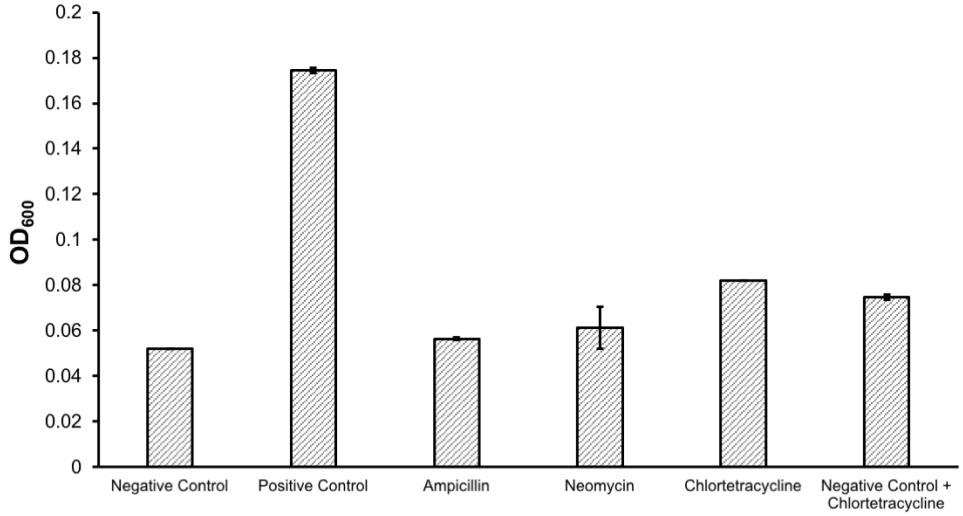

Figure 18. Antibiotic sensitivity testing of coliforms extracted from ground beef. To prepare these samples, the isolated coliform culture was grown to an optical density of 0.1 at 600 nm. 2 µL of the culture was added to 498 µL of either TSB or TSB containing 0.1% ampicillin. A blank sample was also prepared using 498 µL of TSB and 2 µL of water. The samples were incubated overnight and checked the next day for growth, and the optical density readings at 600 nm are shown. Three independent samples were prepared and averaged to obtain the results. The optical density readings were obtained using a SpectraMax M2 UV-vis spectrophotometer (Molecular Devices, San Jose, CA).

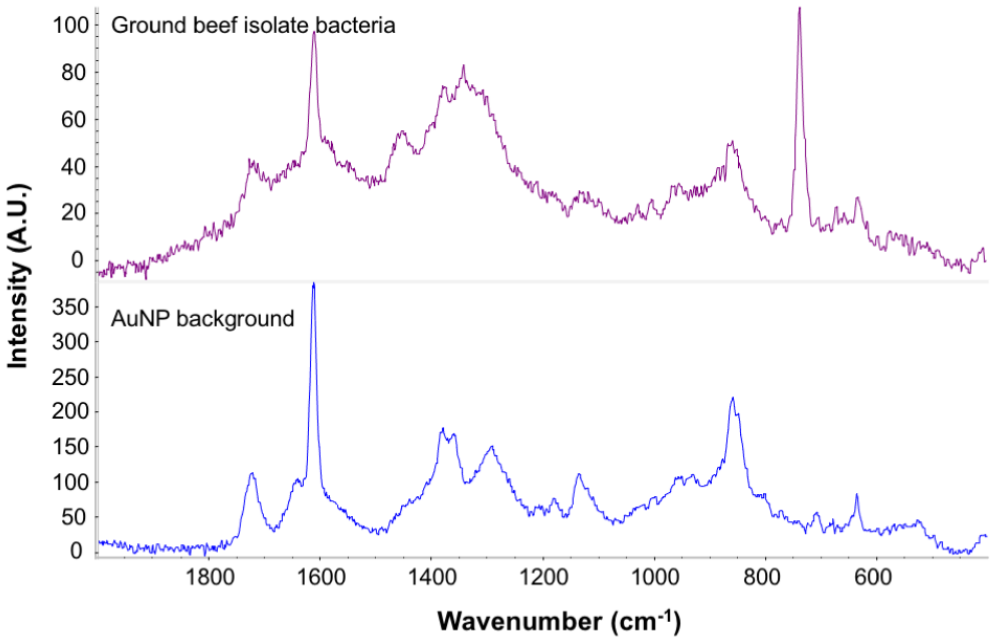

Figure 19. Extracellular matrix liquid spectrum from the ground beef isolate bacteria and the AuNP background signal. The liquid from the bacteria was combined in a 1:4 ratio with the AuNPs, dropped on a slide, and dried, along with a sample AuNPs to show the background signal. The resulting coffee rings were then tested using a DXR Raman microscope.

The results from the portable SERS antibiotic sensitivity tests are shown in Figure 16. Once again, the clearest peak observed in the control sample was the 738 cm^{-1} peak, and the same patterns observed previously in the antibiotic sensitive *E. coli* were observed in the ground beef isolate bacteria in response to antibiotic exposure. When ampicillin was added, it was found that the intensity of the 738 cm^{-1} peak increased, and the intensity decreased when neomycin and chlortetracycline were added. A clearer graphical depiction of the intensity of this peak in the different samples is shown in Figure 17. The results from the SERS testing indicate that the ground beef isolate bacteria are sensitive to each of these

three antibiotics, and this is consistent with the conventional antibiotic sensitivity tests of these samples.

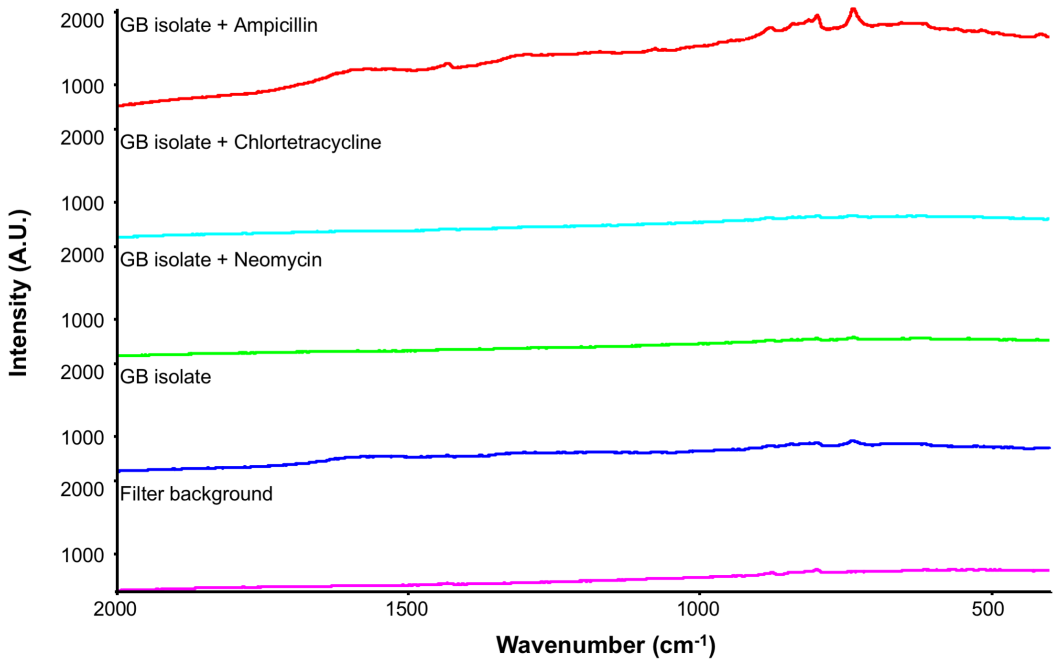

Figure 20. Portable SERS spectra of the ground beef isolate bacteria combined with ampicillin, neomycin, and chlortetracycline.

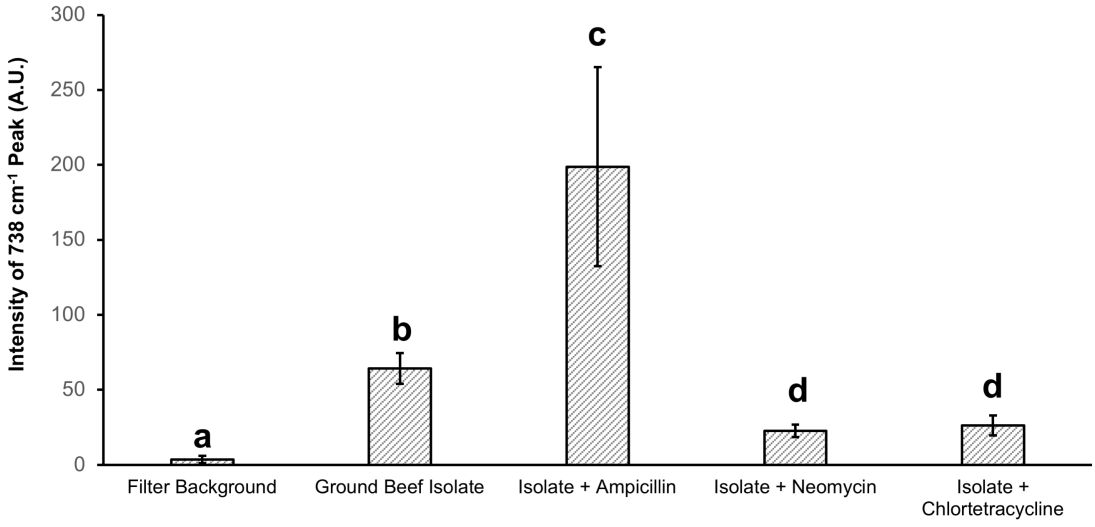

Figure 21. Intensity of the 738 cm^{-1} SERS peak in the ground beef isolate bacteria samples combined with ampicillin, neomycin, and chlortetracycline.

4.3.4. Discussion

The results obtained from testing the *E. coli* strains and the ground beef isolate bacteria demonstrate that our portable SERS procedure can be effectively used to determine the antibiotic sensitivity of bacterial samples. All of these samples showed a characteristic peak at 738 cm^{-1}, and were able to observe distinct patterns in the intensity change of this peak in response to antibiotic exposure when sensitive bacteria were exposed to ampicillin, neomycin, and chlortetracycline. These patterns appeared to correspond to biochemical changes occurring in the samples when the antibiotic was added. For ampicillin, an increase in the intensity of this peak was observed, which is consistent with the results obtained previously with our benchtop Raman microscope-based method in Chapter 3. Ampicillin, which is a beta-lactam antibiotic, disrupts the formation of peptidoglycan in the bacterial cell wall, which leads to ruptures in the cell wall which can be observed with electron microscopy (Wang et al., 2016b). This allows for substances and components from the interior of the cell to leach out into the surrounding liquid, which ultimately leads to the death of the cells. It also allows for more compounds from within the cell to interact with the AuNPs, which would lead to a more intense SERS signal. The bacterial SERS peaks have been previously attributed to biological compounds such as nucleotides and related metabolic byproducts (Premasiri et al., 2016), and the intensity increase of the 738 cm^{-1} peak in response to ampicillin exposure can be attributed to a higher concentration of these compounds escaping the cells and interacting with the substrate.

The other two antibiotics we tested, neomycin and chlortetracycline, have different mechanisms of action than ampicillin. Instead of affecting the cell wall, they target the

synthesis of proteins by ribosomes within the cell- both of them interact with the 30S subunit to inhibit its normal functioning (Kapoor et al., 2017). Unlike ampicillin, they do not cause the same level of disruption to the cell wall and a corresponding release of intracellular compounds was not observed, since the intensity of the peak in the SERS spectrum did not increase. A likely explanation for the decrease in intensity seen in these samples is that cells killed by these antibiotics were no longer producing adenine-related compounds such as ATP which are responsible for the 738 cm^{-1} peak, which resulted in its intensity decreasing. Since the cells did not lyse to the extent of the ampicillin-exposed samples, these compounds remained more contained within the cells so only a decrease in intensity was observed. These results show how the SERS spectra can be used to not only observe the antibiotic sensitivity of the bacteria, but also study the biochemical changes occurring in the samples. More research should be done in the future to confirm the biochemical mechanisms responsible for these SERS patterns, and additional classes of antibiotics can be studied as well to learn more about how different antibiotic mechanisms impact the characteristic peaks in the SERS spectra.

Another significant finding from these results is that these patterns appear to be consistent between different species of bacteria, including *E. coli*, *B. cereus*, *S. enterica*, and the ground beef isolate bacteria. All of the spectra we obtained from the bacteria samples using our portable SERS procedure contained the distinct 738 cm^{-1} peak, and the changes in peak height in response to antibiotic exposure appeared to be largely consistent between the different species. This is particularly notable due to the fact that there are significant biological differences between some of the bacterial species we tested- for instance, *E. coli* is a Gram-negative coliform, *B. cereus* is a Gram-positive spore former,

and *S. enterica* is a Gram-negative non-spore former, although it is not a coliform. Despite these differences, the SERS spectra observed from these samples were all very similar- this suggests that this type of SERS method is more non-selective, and could potentially be used with a wide range of potential bacteria species. This is potentially useful for future applications in which the sole function of this sort of test would be to identify whether any antibiotic sensitive or resistant bacteria are present in a sample, though there could be limitations for this sort of SERS method in uses where multiple types of bacteria need to be differentiated, since the spectra from different species could be very similar.

There were also some slight differences observed in the patterns in response to antibiotic exposure when comparing the various bacterial species. The most notable difference observed was that the *B. cereus* sample we tested did not exhibit a statistically significant increase in 738 cm^{-1} peak intensity, unlike the *S. enterica*. Compared to the antibiotic sensitive and resistant *E. coli*, this pattern suggests that the *B. cereus* is resistant to ampicillin, since it did not display a significant response to antibiotic exposure. This finding is indeed consistent with the established antibiotic sensitivity profile of this *B. cereus* strain- previously, this strain has been found to be resistant to beta-lactams (such as ampicillin), but sensitive to aminoglycosides and tetracyclines (Environment Canada, 2013). This shows that our portable SERS method is able to accurately assess the antibiotic sensitivity of biologically different species of bacteria, such as *B. cereus.* One additional observation of note is that there was a statistically significant difference between the 738 cm^{-1} peak intensity in the *S. enterica* samples combined with neomycin and chlortetracycline, which was not the case in any of the other bacteria samples. It is possible that some species of bacteria might exhibit observable differences in their responses to

neomycin and chlortetracycline, and more work can be done in the future to assess the potential differences in other kinds of bacterial samples, such as strains with differing sensitivity to aminoglycosides and tetracyclines.

The results obtained in this study and the SERS patterns observed in the samples are largely consistent with the previously obtained results from the benchtop Raman microscope-based methods in Chapter 3. This suggests that portable SERS methods can be just an effective in this application as the microscope-based laboratory methods, which is a useful finding- a portable SERS technique would be more accessible to wider audience and more useful for practical applications, since portable Raman devices are less expensive and can be transported much more easily. The spectrometer that we used can fit into a small briefcase, so it could be brought to different locations quite easily for potential on-site testing. As found in our study, our method could be used effectively with bacteria extracted from real food samples as well. Our method is well-suited for potential use in the food industry for studying the presence of antibiotic resistant organisms in food, agricultural, and environmental samples. More work should be done in the future to use this portable SERS method for analyzing different kinds of food samples and determine the capabilities and potential limitations of the method. Other potential areas of future study include using different substrates to try to further increase the intensity of the SERS signal, and testing samples containing a mix of bacteria types instead of just one isolated strain.

4.4. Conclusions

We have developed an effective portable SERS-based method for assessing the antibiotic sensitivity of bacterial samples. Our procedure was effective for testing *E. coli*

strains both sensitive and resistant to ampicillin, neomycin and chlortetracycline, and the overall SERS patterns were consistent with data previously obtained from benchtop Raman microscope-based methods. It was found that neomycin and chlortetracycline had different impacts on the characteristic bacterial peaks of the SERS spectra than ampicillin, and these patterns can be used to observe biochemical changes occurring in the bacteria in response to antibiotic exposure and determine whether or sample is resistant to the antibiotics. We were also able to successfully use our portable SERS procedure with other bacterial species, including *Bacillus cereus* and *Salmonella enterica*. Additionally, we were able to successfully isolate coliform samples from ground beef and accurately test their sensitivity to these three antibiotics as well. These results could be compared to traditional growth-based antibiotic sensitivity tests, which were performed separately. Overall, these results show that our portable SERS-based method can be effective for assessing the antibiotic sensitivity of a number of different types of bacterial samples, and more work should be done in the future to further test the capabilities of this method.

CHAPTER 5

OPTIMIZATION OF A PORTABLE SERS-BASED METHOD FOR TESTING ANTIBIOTIC SENSITIVITY USING BACTERIAL EXTRACELLULAR MATRIX LIQUID

5.1. Introduction

Antibiotic resistant organisms pose a major threat to global public health, and the growing usage of antibiotics around the world has accelerated the natural development of antimicrobial resistance in the environment (Davies and Davies, 2010; Ventola, 2015). In order to help prevent the further development and spread of antibiotic resistant organisms, it is essential to have effective methods capable of quickly and accurately testing the antibiotic sensitivity of bacterial samples, which can be used to identify resistant organisms. A number of different techniques already exist for testing antibiotic sensitivity, including growth-based methods, such as agar and broth dilution, as well as more rapid procedures such as PCR-based techniques and immunoassays (Pulido et al., 2013). However, these existing methods tend to have a number of disadvantages as well- for instance, growth-based methods are quite time-consuming and can take several days to obtain results, while PCR and immunological methods require prior knowledge of specific gene sequences or cellular proteins associated with antibiotic resistance. As a result, there is still more work to be done to develop improved detection methods that are able to rapidly test the antibiotic sensitivity of a wide range of bacterial samples.

A potential alternative to more traditional methods of testing antibiotic sensitivity is Surface-enhanced Raman Spectroscopy, or SERS. SERS makes use of Raman spectroscopy, an analytical technique that uses inelastic light scattering to create a spectrum

that provides information about the chemical composition of a sample. Raman signals typically tend to fairly weak, so SERS makes use of metallic substrates which can enhance the intensity of the Raman scattering by factors of up to 10^8 or even larger (Langer et al., 2020). Our goal is to develop an efficient and accurate SERS-based method for testing the antibiotic sensitivity of bacterial samples.

In previous chapters, we have successfully developed a number of SERS-based protocols for testing antibiotic sensitivity by analyzing the response patterns that show up in the SERS spectra following bacterial incubation with different antibiotics. In Chapter 3, we tested several different Raman microscope-based approaches, including testing the bacterial cells directly, analyzing the filtered extracellular matrix liquid, and SERS mapping. Of these three methods, testing the bacterial extracellular liquid, as opposed to testing the cells themselves, resulted in the strongest SERS signal and the clearest differentiation between antibiotic sensitive and resistant bacterial samples. There are a number of reasons by testing the bacterial liquid is a preferred approach compared to more traditional SERS methods of testing the bacteria directly- for instance, testing the liquid provides a much more uniform and consistent sample than testing a culture of bacteria, which contains millions of cells that may be in different stages of their life cycles or may be responding to the antibiotic in different ways. As a result, there is much more potential for variation when randomly selecting areas to test in a bacteria sample, compared to a liquid sample in which the bacteria have been removed. Another advantage of liquid analysis is that the sample preparation can be further streamlined- in a protocol such as the one used in Chapter 4, in which the bacteria are filtered prior to analysis, it could be possible to avoid the filtration step if the liquid is tested instead of the bacterial cells. It

could be possible to simply centrifuge the washed bacteria samples and pipette out the supernatant liquid to reserve for subsequent analysis, which would allow us to skip the filtration step of the protocol. This would be a significant improvement, because the filtration step is the most labor-intensive and time consuming part of the sample preparation process- the samples need to be filtered one at a time, and it would save a considerable amount of time to instead simply centrifuge all of the samples at once. It would also allow us to avoid the use of syringes for filtration, which can be a potential safety hazard when handling pathogenic bacteria samples.

As discussed in Chapter 4, using a portable spectrometer-based SERS method is much more accessible than a microscope-based SERS method for a wider audience, since portable devices are less expensive, require less specialized training, and can be easily transported for analysis in different locations. For an optimized SERS method for testing antibiotic sensitivity that is easily accessible, simple to perform, and provides clear results, a portable SERS method that analyzes the extracellular bacterial liquid would be ideal. However, there are some obstacles for testing the liquid using a portable spectrometer. The liquid samples were tested with SERS by selecting spots in the "coffee ring" of concentrated nanoparticles that forms as a sample droplet dries on a slide, which has the most intense Raman signal. This is easy to perform with a microscope which can zoom in on the sample, allowing micro-scale spots on the coffee ring to be accurately selected. With a portable spectrometer, there is much less accuracy for testing such a small area, and it can be difficult to get a clear signal from the coffee ring. It is necessary to further optimize the sample preparation to ensure that clear SERS spectra can be obtained from the liquid samples using the portable Raman spectrometer. This could potentially be achieved by

increasing the concentration of the AuNP substrate used in this procedure, which would provide a stronger SERS signal and could allow the target peaks to be detected more clearly with the portable Raman spectrometer.

In addition to overcoming the logistical challenges of testing the sample with the portable device, there are additional tests that can be performed to further optimize the detection method and obtain more information about the origin of the antibiotic response patterns observed in the SERS spectra, as well as to better understand the experimental conditions required to clearly observe these patterns. In Section 3.3.4, we discussed that a potential explanation for the signals observed in the extracellular liquid is the stress responses of the bacteria to being washed with distilled water. The ~730 cm^{-1} peak has been attributed purine compounds, such as adenine, which are released by bacteria as part of these stress responses (Premasiri et al., 2016). As observed previously in Chapters 3 and 4, the intensity of this peak changes in response to antibiotic exposure, indicating the antibiotic sensitivity of the bacteria. One question that remains unanswered is whether or not the washing step itself is necessary for seeing these antibiotic response patterns- it could potentially be possible to test the media used to incubate the bacteria with the antibiotics directly to observe these patterns, without washing cells. This would potentially allow us to further streamline the procedure by eliminating the washing step, and also provide more insight into whether the bacterial signals are indeed coming from the stress responses to washing. Additionally, more testing could be done to assess the impact of the type of liquid used for incubation with antibiotics on the resulting SERS patterns- incubating the cells directly in distilled water or a buffer may provide different SERS signals than simply adding the antibiotic to the broth used to grow the bacterial culture.

Our goal in this study is to optimize the conditions required for testing bacterial extracellular matrix liquid with a portable SERS-based method. First, we will experiment with different concentrations of AuNPs to assess the effect on the resulting SERS signal and determine what concentration of the substrate is necessary to clearly observe the antibiotic response patterns from the bacterial liquid samples using the portable spectrometer. We will also try testing the incubation liquid directly to determine if the washing step is needed to see the target SERS patterns, and also test the impact of replacing the broth with distilled water or phosphate buffered saline (PBS) prior to adding the antibiotics for incubation. These tests will allow us to determine the optimal conditions required for testing antibiotic sensitivity using SERS, and will also provide valuable insight into the origins of the SERS patterns observed in the bacterial samples. Once we have optimized the SERS detection method, we will test our procedure with antibiotic resistant bacteria isolated from real-world food samples.

5.2. Materials and Methods

5.2.1. Chemicals and Bacteria

Strains of *Escherichia coli* (ATCC® 43888™) and *Salmonella enterica* (ATCC® 51962™) were obtained from the ATCC. The bacteria samples were cultured overnight at 37°C for 16 hours in tryptic soy broth (TSB) to obtain a concentration of approximately 10^9 CFU/mL to be used in the subsequent experiments. Ampicillin, neomycin, and chlortetracycline were obtained from Sigma Aldrich (St. Louis, MO, USA) for the antibiotic sensitivity testing. 100 mg/mL stock solutions of the three antibiotics were prepared and diluted to the necessary concentrations for each experiment. TSB and PBS

were both obtained from Sigma Aldrich and used for incubating the bacteria with the antibiotics. Distilled water was obtained using a Thermo Fisher Scientific (Waltham, MA, USA) Barnstead MicroPure water purification system. Plate counts were performed using Petrifilm™ Aerobic Count Plates (3M, St. Paul, MN, USA).

5.2.2. Substrate Synthesis

AuNPs were synthesized based on the procedure used previously by Qu and He (2020) and in the prior studies in Chapters 3 and 4. In a clean and dry Erlenmeyer flask, 2 mL of aqueous 1% chloroauric acid tetrahydrate ($HAuCl_4$) solution was mixed with 200 mL ultrapure water and heated at 310°C on a stir plate at 350 rpm. 1.4 mL of 1% aqueous sodium citrate solution was added to the flask once the $HAuCl_4$ solution reached a boil. The contents of the flask were then heated and continually mixed until they turned a deep red color, which took approximately 30 minutes. The AuNPs in the resulting solution had an average diameter of 55 nm, and were diluted to 200 mL to obtain a final concentration of 0.1 mg/mL. The AuNP solution was then cooled and stored in a refrigerator until used in subsequent experiments. The chemicals used in this protocol were obtained from Sigma Aldrich (St. Louis, MO, USA). To prepare different concentrations for testing their effects on the subsequent SERS analysis, samples of the AuNPs were spun in a centrifuge at 10,000x g for 5 minutes to concentrate the AuNPs at the bottom of the tube. The supernatant liquid was then removed to obtain the desired final concentrations, and the AuNPs were resuspended in the remaining liquid. Three AuNP concentrations were tested in this study- 0.1 mg/mL (1x concentrated), 0.2 mg/mL (2x concentrated), and 1 mg/mL (10x concentrated).

5.2.3. Bacterial Sample Preparation

To obtain the extracellular matrix liquid for SERS analysis, 1 mL volumes of the bacterial cultures were first combined with 0.1% concentrations of each antibiotic. A control sample containing no antibiotics was also prepared by combining 1 mL of the bacterial culture with water, adjusting the volume of water so that the total volume is identical to the samples combined with the antibiotics. The samples were then incubated in a shaking incubator at 37°C for 90 minutes at 100 rpm. After the incubation period, the samples were centrifuged at 3000x g for 1 minute and the supernatant liquid was removed. To wash the cells, the pellets were each resuspended in 1 mL of water and centrifuged again at 3000x g for 1 minute. The supernatants were again removed and the pellets were resuspended in 250 mL of water. The samples were held at room temperature for 15 minutes, and then they were centrifuged once more at 3000x g for 1 minute. The resulting supernatant water was removed and reserved for subsequent SERS analysis. For each sample, 20 µL of the reserved water was combined with 80 µL of the AuNPs. A control sample containing 20 µL of distilled water and 80 µL of the AuNPs was also prepared for comparison. The mixtures were incubated at room temperature for 30 minutes, and then 5 µL drops of each sample were placed aluminum foil-wrapped glass slide and dried prior to SERS analysis. A diagram of this procedure can be seen in Figure 22.

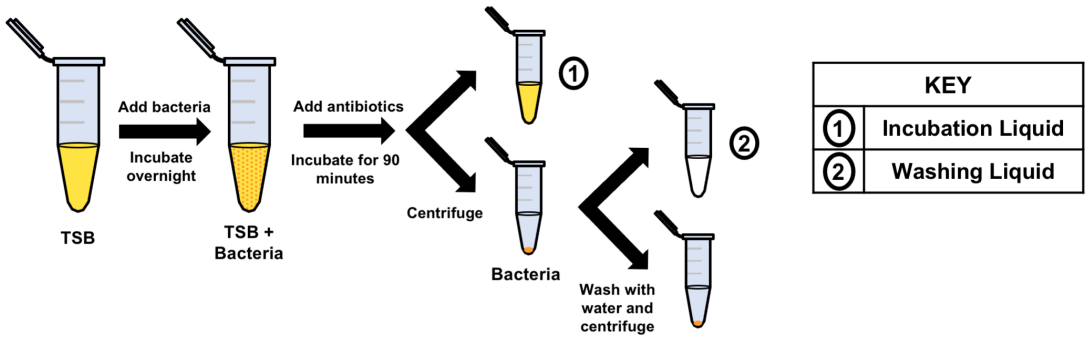

Figure 22. Procedure used to obtain liquid samples from bacteria for testing antibiotic sensitivity using our SERS method.

Several alternate versions of this protocol were also performed to assess the effect of different experimental designs on the SERS results. In the first variation, the post-incubation washing step was omitted, and the supernatant liquid from the first centrifugation was directly combined with the AuNPs and tested. This was intended to see whether the TSB media used to grow the bacteria and incubate with the antibiotics could be tested directly after the incubation, without the need for subsequent washing. The second variation of the protocol was identical to the original procedure, except the bacteria samples were centrifuged at 3000x g and the TSB was removed and replaced with distilled water prior to combining with antibiotics and incubating the bacterial samples. The subsequent incubation and washing steps were then conducted as previously described. The third variation of the protocol was similar to the second variation, except the bacteria samples were centrifuged at 3000x g and the TSB was removed and replaced with PBS (instead of water) prior to combining with antibiotics and incubating the bacterial samples. These two procedures were intended to assess the effect of the incubation medium on the resulting

SERS signal, and determine whether TSB, PBS, or distilled water was the most effective medium for incubating the cells with the antibiotics and obtaining the clearest SERS signal for determining the antibiotic susceptibility. A diagram showing the modified protocol with the additional step of replacing the TSB with PBS or distilled water is shown below in Figure 23.

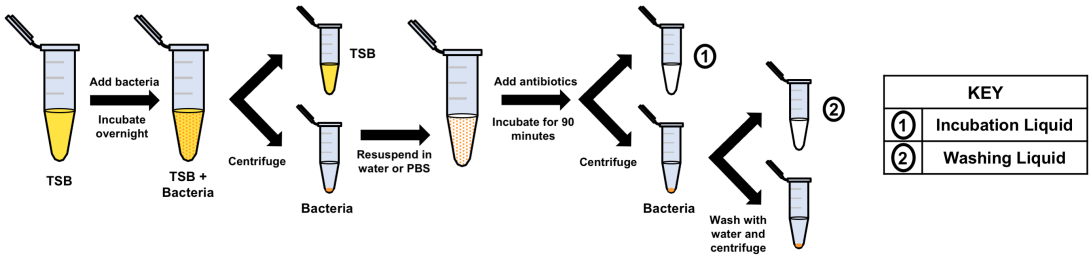

Figure 23. Modified procedure for obtaining liquid samples from bacteria for antibiotic sensitivity testing, used to test the effect of replacing the TSB with distilled water or PBS.

Additionally, plate counts were also performed on some of the samples to assess the effects of the antibiotics on the concentration of viable bacterial cells. The plate counts were performed by first incubating the bacteria with the antibiotics and washing with distilled water as described above. Serial dilutions of the bacterial samples were prepared, and 1 mL of each dilution was plated on Petrifilm^{TM} Aerobic Count Plates. The samples were incubated at 37°C for 48 hours, and then the number of colonies observed from each sample was recorded.

5.2.4. Bacteria Extraction from Ground Chicken

To obtain antibiotic resistant bacteria samples for SERS testing, ground chicken was first purchased from the local supermarket. 25 g of ground chicken was placed into a bag

with 225 mL of peptone water and placed in a Stomacher for two minutes. A series of tenfold serial dilutions were then prepared in peptone water using the stomached liquid, down to 10^{-5} g/mL. 1 mL of each dilution were plated on separate tryptic soy agar plates each containing 50 µg/mL of one of the antibiotics (ampicillin, neomycin, and chlortetracycline) to select for bacteria resistant to each antibiotic. The plates were incubated at 37°C for 48 hours, and afterwards colonies were randomly selected from each antibiotic plate and used to grow separate overnight cultures of bacteria resistant to each of the three antibiotics in TSB. Once the overnight cultures were prepared, the bacteria from each culture were tested for sensitivity to the corresponding antibiotic using the SERS protocol described previously in Section 5.2.3.

5.2.5. Data Acquisition and Analysis

For the portable SERS analysis, the spectra were obtained using a TSI ChemLogix portable Raman spectrometer (Shoreview, MN, USA). The laser was focused on the sample, and spectra were obtained from three different randomly selected areas in the concentrated "coffee ring" of AuNPs in each sample. For these experiments, a power setting two-thirds of maximum was utilized. The spectra were then saved and transferred onto a separate computer and analyzed using OMNIC software to characterize the samples and assess the intensity of the SERS peaks.

Some samples in this study, as indicated in Section 5.3, were also analyzed using a DXR Raman Microscope and DXRxi Raman Imaging Microscope (both manufactured by Thermo Fisher Scientific). For the DXR microscope analysis, a 20x magnifying objective was used to obtain the SERS spectra from each sample. A laser power of 4 mW and a grating of 400 lines/mm were used, with an excitation wavelength of 780 nm and an

exposure time of 1 second. Spots from the coffee ring of concentrated AuNPs in the dried samples were chosen for analysis, and spectra from 15 spots on each sample were obtained using a spectral range of 2000 to 400 cm^{-1}. For the analysis with the DXRxi microscope, 250 μm^2 SERS maps were created from sections of the coffee ring from each sample, using a laser power of 4 mW, an excitation wavelength of 780 nm, an exposure time of 0.002 seconds, a step size of 2 µm, and a 20x magnifying objective. OMNICxi Raman imaging software was subsequently used to analyze and prepare the maps.

5.3. Results and Discussion

5.3.1. Comparison of Different AuNP Concentrations

The average spectra obtained from portable SERS testing bacterial liquid samples with different AuNP concentrations are shown below in Figure 24. SERS maps of the different AuNP concentrations, obtained using the DXRxi Imaging Microscope, are shown in Figure 25.

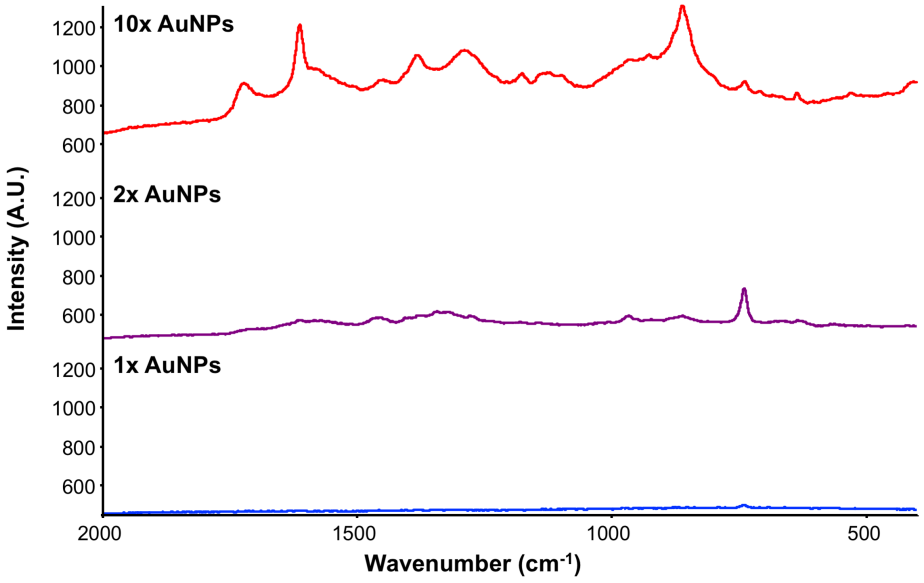

Figure 24. SERS spectra of different AuNP concentrations combined with washing liquid from a control *S. enterica* sample.

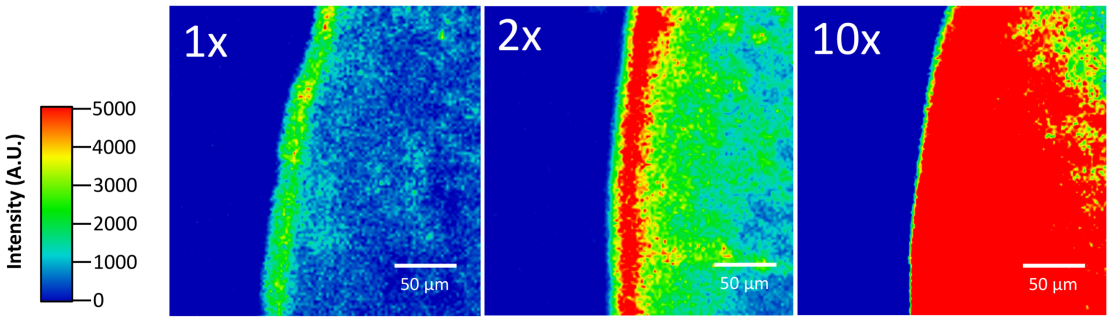

Figure 25. SERS maps obtained from coffee ring sections of each AuNP concentration, showing the intensity of the 1615 cm^{-1} peak from the AuNP spectrum across the surface of each sample.

Differences between the samples, in both the SERS spectra and the SERS maps, can be readily observed. As seen in Figure 24, the 1x concentration of AuNPs is insufficient for clearly observing the Raman peaks with the portable SERS method, while the 10x concentration of AuNPs results in a very strong signal from the nanoparticles. In the 10x concentrated AuNP sample, a comparatively weaker target 738 cm^{-1} peak from bacteria is also observed, which is difficult to distinguish clearly due to the intensity of the background AuNP peaks. The spectra from 2x concentrated AuNPs, by comparison, clearly show a distinctly enhanced 738 cm^{-1} peak as well as background peaks from the AuNPs which do not overwhelm the target bacterial signal. These differences can also be observed in the SERS maps of the AuNPs in Figure 25. As seen in this Figure, the intensity of the signal from the coffee ring of dried AuNPs clearly increases as the concentration of AuNPs

increases, and the results are consistent with the observations from the portable SERS results- the 1x concentration of AuNPs results in a noticeably weaker signal, while the 10x concentration results in a more intense signal that spans a larger area.

An additional finding regarding the effectiveness of more concentrated AuNPs can be observed in Figures 26 and 27, which show the 738 cm^{-1} peak in the SERS spectra from the 10x concentrated AuNPs combined with liquid from bacteria exposed to different antibiotics.

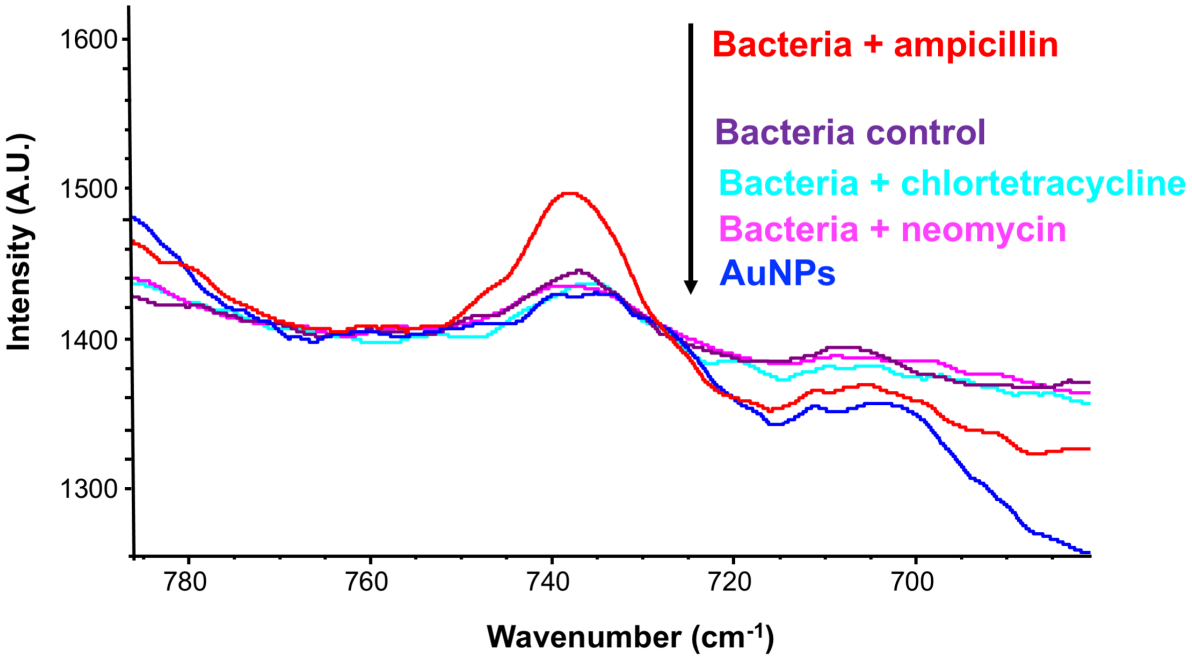

Figure 26. SERS spectra comparison of washing liquid from *S. enterica* exposed to different antibiotics, combined with 10x concentrated AuNPs.

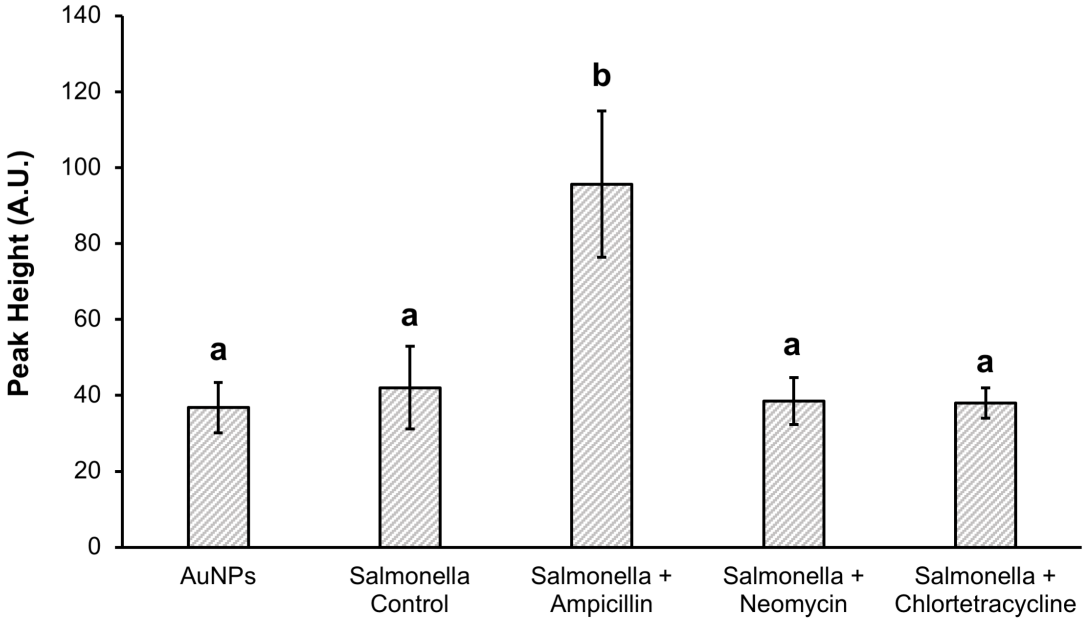

Figure 27. Comparison of the average 738 cm^{-1} peak heights from the spectra shown in Figure 26.

In Figure 26, it can be seen that the 738 cm^{-1} peak has similar intensity in all of the samples except for the bacteria combined with ampicillin. The peak height from these spectra is quantified in Figure 27, which shows that the AuNPs, the bacterial control, and the bacteria combined with neomycin and chlortetracycline cannot be clearly distinguished from one another. Only the bacteria combined with ampicillin, which causes an increase of the 738 cm^{-1} peak intensity as previously observed in Chapters 3 and 4, can be accurately distinguished from the other samples. This demonstrates a potential limitation of using too high of a concentration of AuNPs in this application.

5.3.2. Testing of Bacterial Washing Liquid

Using the 2x concentrated AuNPs, *E. coli* and *S. enterica* were then tested for their sensitivity to ampicillin, neomycin, and chlortetracycline using the portable SERS method. In these tests, the reserved water used to wash the bacteria following the incubation with antibiotics was analyzed. The SERS spectra obtained from the testing of *E. coli* can be seen below in Figure 28, and Figure 29 shows a comparison of the 738 cm^{-1} peak height from the different *E. coli* spectra.

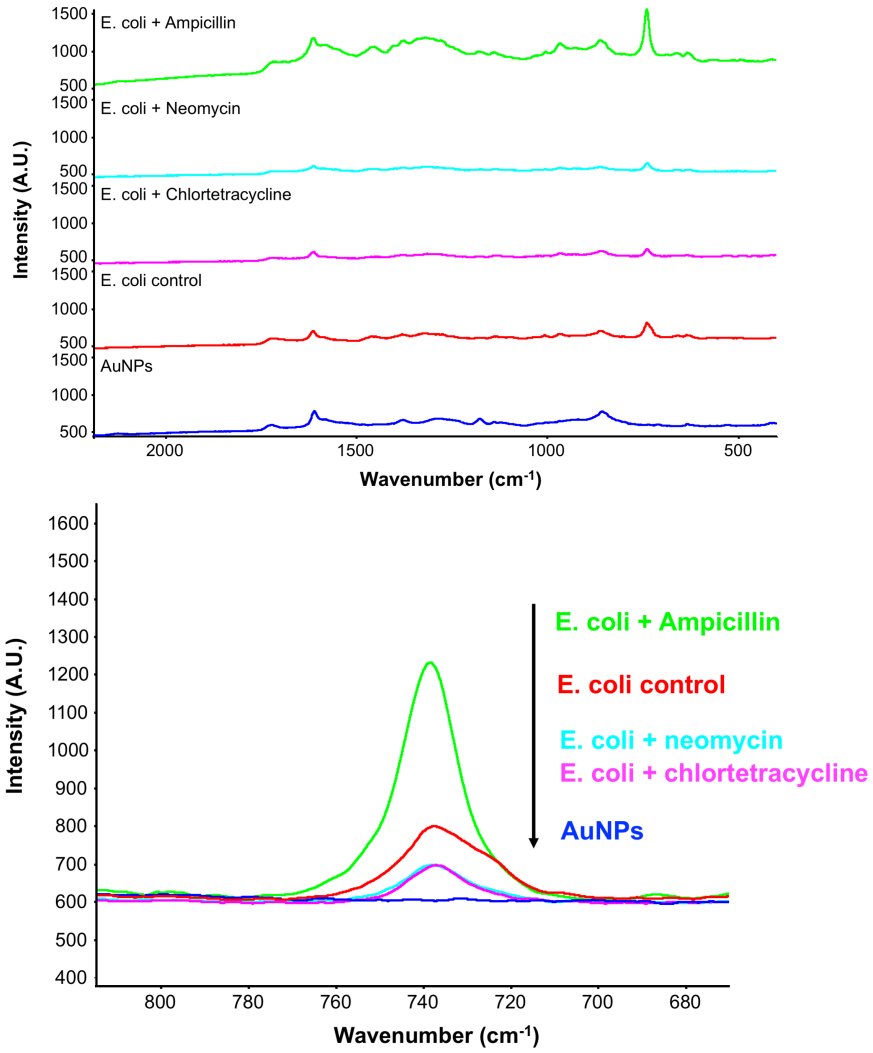

Figure 28. SERS spectra obtained from the washing liquid of *E. coli* samples exposed to different antibiotics, combined with 2x concentrated AuNPs. The top graphic shows the entire spectra, while the bottom shows a comparison of the 738 cm^{-1} peak.

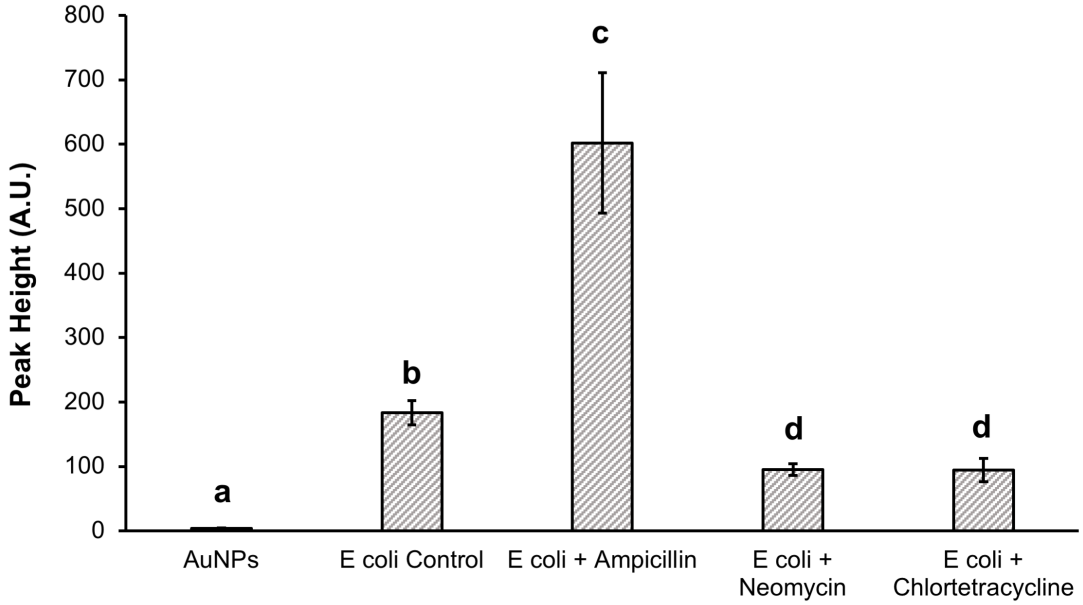

Figure 29. Comparison of the heights of the 738 cm^{-1} peaks from the *E. coli* washing liquid spectra shown in Figure 28.

As observed in Figures 28 and 29, there were significant differences in the spectra between the different *E. coli* samples. Compared to the control *E. coli* sample with no antibiotics, the height of the 738 cm^{-1} peak increased in response to ampicillin exposure, and decreased in response to neomycin and chlortetracycline exposure. These patterns are consistent with the previously observed responses to these antibiotics in Chapter 4. The changes in the 738 cm^{-1} peak were also seen in the *S. enterica* samples, shown below in Figures 30 and 31.

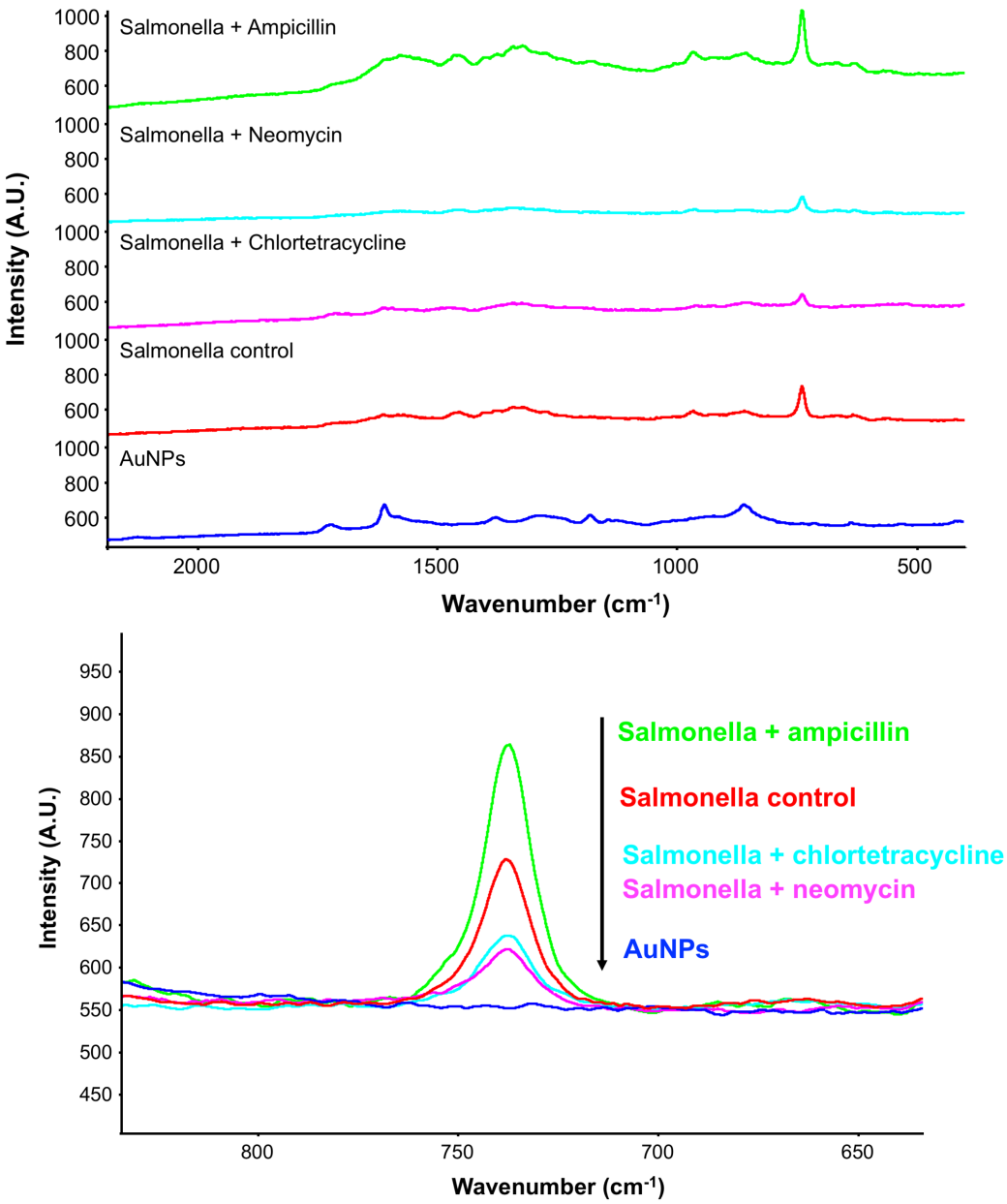

Figure 30. SERS spectra obtained from the washing liquid of *S. enterica* samples exposed to different antibiotics, combined with 2x concentrated AuNPs. The top graphic shows the entire spectra, while the bottom shows a comparison of the 738 cm^{-1} peak.

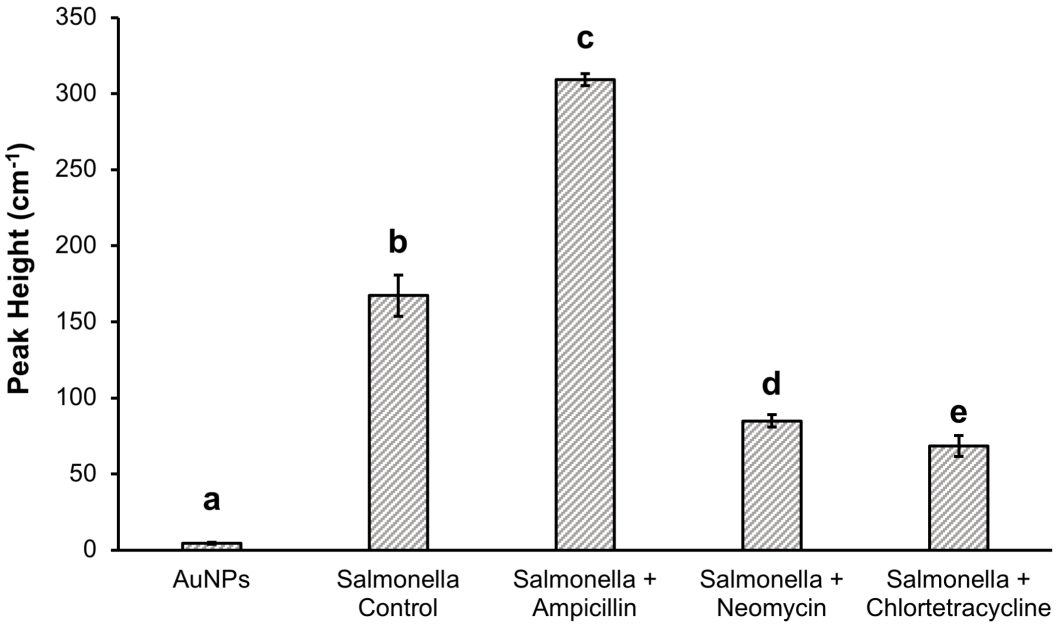

Figure 31. Comparison of the heights of the 738 cm^{-1} peaks from the *S. enterica* washing liquid spectra shown in Figure 30.

The patterns in response to antibiotic exposure were largely identical in both *E. coli* and *S. enterica*, and the portable SERS method appeared to be effective for testing the bacterial washing liquid to assess these patterns. Some relatively minor differences between the species can be observed, such as the variability in how clearly the 1615 cm^{-1} peak from the AuNP background shows up in the spectra- this peak is more clearly distinct in the *E. coli* spectra than the *S. enterica* spectra. There was also a more significant difference in the 738 cm^{-1} peak height between the *S. enterica* samples exposed to neomycin and

chlortetracycline, while the corresponding spectra were not clearly distinguishable in the *E. coli* samples.

5.3.3. Direct SERS Testing of Growth Media

The next set of experiments involved testing the growth media used to incubate the bacteria and antibiotics directly, to assess whether or not the washing step was necessary to see the target SERS patterns. First, a comparison was done to identify the background signal from the TSB media and assess how it changes after incubation with bacteria, as well as how it compares to the previously observed signal from the washing liquid. Figure 32 shows a comparison of the SERS spectra obtained using the DXR microscope of several different types of samples. Included are the spectra from the TSB used to incubate a control *E. coli* sample without any antibiotics, the washing liquid obtained from the same control *E. coli* sample, a sample of TSB that had not been used to incubate bacteria, and the background signal from the AuNPs.

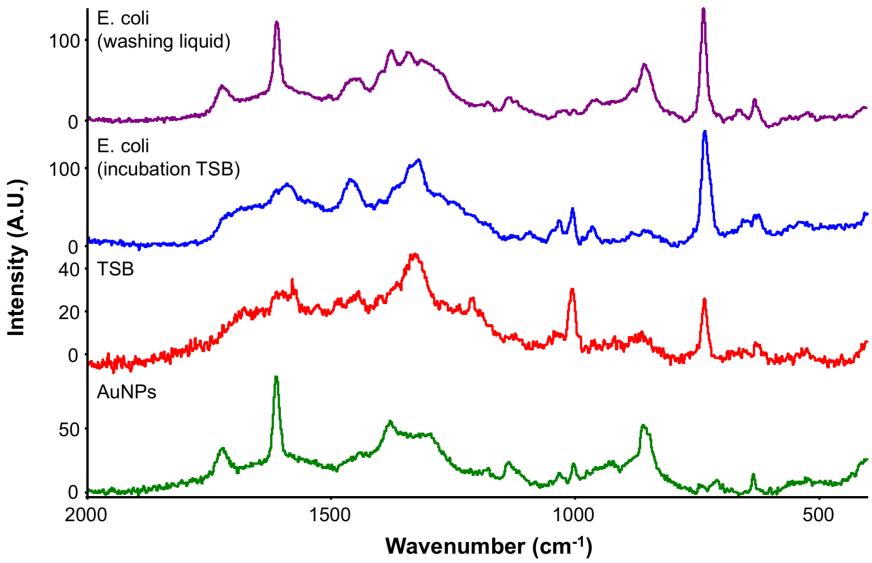

Figure 32. Comparison of SERS spectra obtained from the AuNP background, a TSB control sample, a sample of TSB used to incubate *E. coli*, and the liquid subsequently obtained from washing the bacteria.

Some clear differences between the samples can be observed- notably, the 738 cm^{-1} peak previously seen in the bacteria samples was also observed in the TSB control sample. An additional distinct peak around 1010 cm^{-1} was also observed in the spectrum of the TSB control. Changes in the spectral profile can be seen when comparing the TSB control to the media used to incubate the *E. coli*- most noticeably, the relative intensity of the 738 cm^{-1} peak increased substantially compared to the 1010 cm^{-1} peak after the TSB had been used to incubate the bacteria. In the washing liquid from the *E. coli*, the 1010 cm^{-1} peak largely disappeared compared to the TSB samples, while the 738 cm^{-1} peak remained as intense as it had been in the spectrum from the TSB that had been used to incubate the bacteria. The background peaks from the AuNPs could also be seen more clearly in the washing liquid spectrum, compared to the TSB samples.

Figure 33 shows the results of testing done with the portable Raman spectrometer on the media used to incubate *E. coli* with different antibiotics. As seen in the Figure, the spectra were all relatively similar, particularly in the intensity of the 738 cm^{-1} peak. Overall, these spectra did not appear to show clear changes in the intensity of the 738 cm^{-1} peak in response to antibiotic exposure.

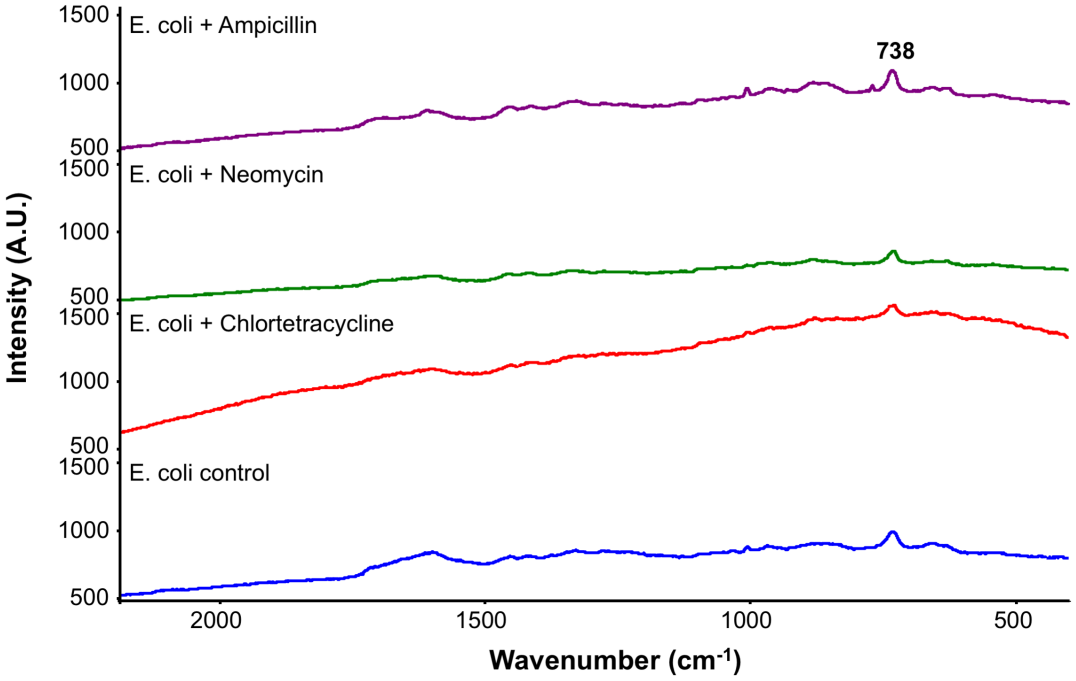

Figure 33. SERS spectra obtained from the incubation liquid of *E. coli* samples incubated in TSB with different antibiotics, obtained using the portable SERS method with 2x concentrated AuNPs.

5.3.4. SERS Analysis of Bacteria Incubated in Distilled Water and PBS

Next, *E. coli* samples were tested using our portable SERS method in which the TSB growth media was replaced with either distilled water or PBS prior to adding the antibiotics and incubating the bacteria. The purpose of these tests was to assess the effect of the incubation medium on the resulting SERS patterns observed in the bacteria in response to antibiotic exposure. For both of these sets of experiments, the incubation media and the resulting washing liquid were both tested. First, the results from *E. coli* incubated in distilled water are shown below. Figure 34 shows the spectra obtained from the water used to incubate the bacteria with the antibiotics, and Figure 35 shows a comparison of the 738 cm^{-1} peak height in these samples.

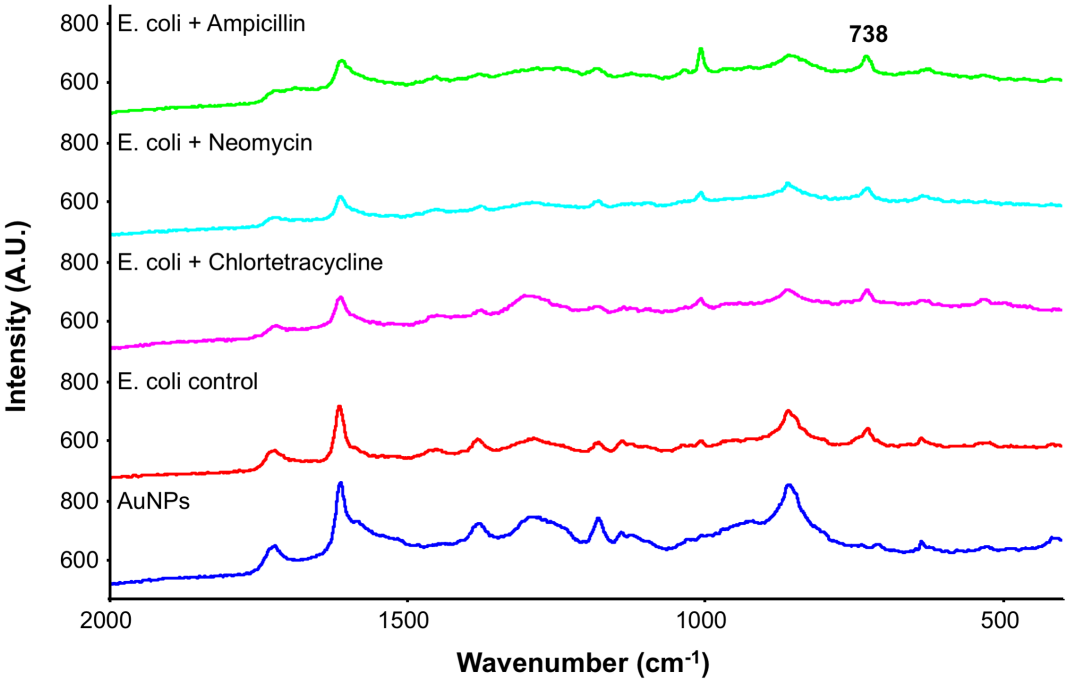

Figure 34. SERS spectra obtained from the incubation liquid of *E. coli* samples incubated in water with different antibiotics, obtained using the portable SERS method with 2x concentrated AuNPs.

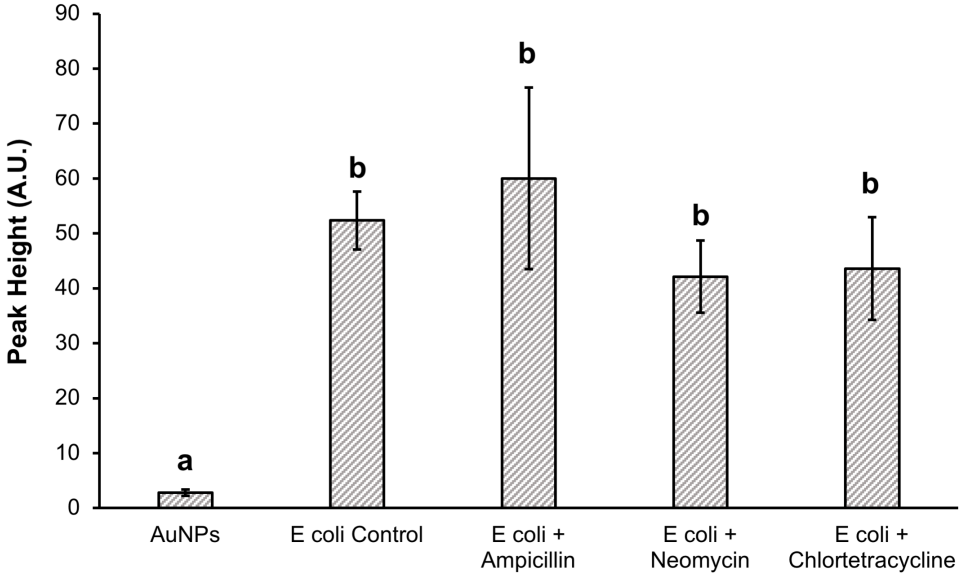

Figure 35. Comparison of the heights of the 738 cm^{-1} peaks from the SERS spectra of the incubation liquid from *E. coli* samples incubated in water shown in Figure 34.

As observed in these Figures, the 738 cm^{-1} peak can be observed in all of the bacteria samples, but there was no significant difference in the peak height between the samples exposed to different antibiotics. The spectra obtained from the washing liquid subsequently obtained from these samples are shown below in Figure 36.

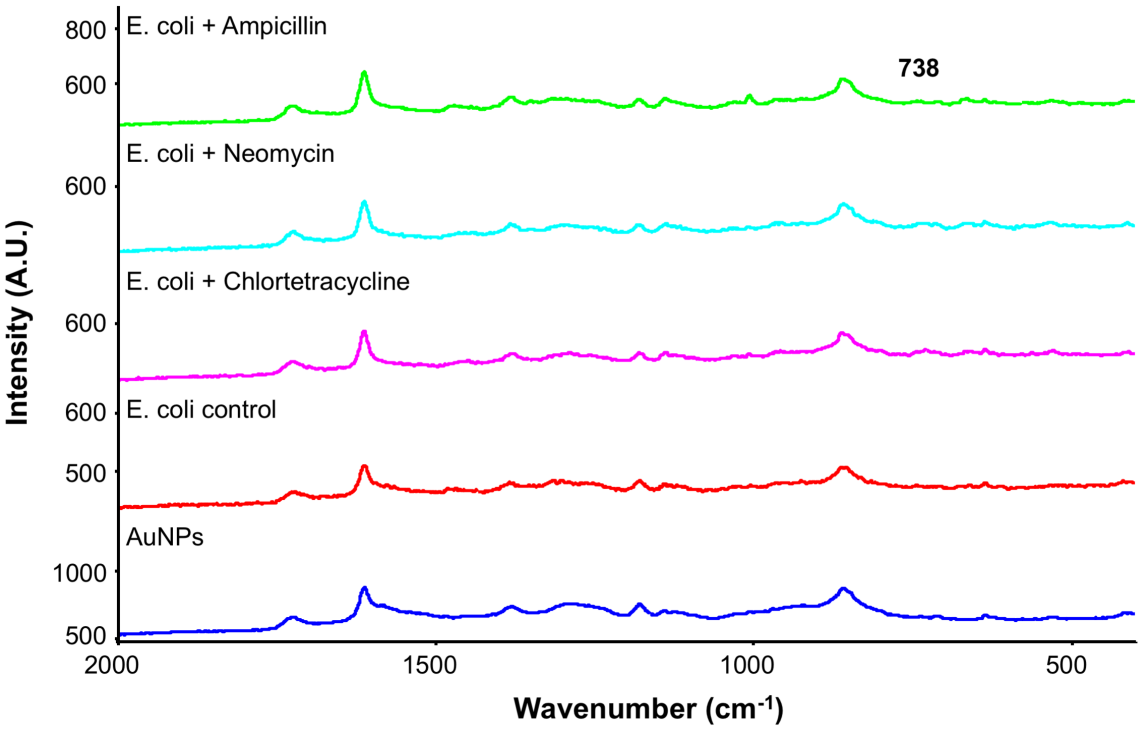

Figure 36. SERS spectra obtained from the washing liquid of *E. coli* samples incubated in water with different antibiotics, obtained using the portable SERS method with 2x concentrated AuNPs.

Notably, a major decrease in the intensity of the 738 cm^{-1} peak was observed in the washing liquid in all of the bacterial samples, and the peak was difficult to distinguish at all. These results indicate that distilled water is not effective as an incubation medium for this procedure, and the target SERS patterns in response to antibiotic exposure were not observed.

E. coli samples with the growth media replaced with PBS were also tested to see the effect of the resulting SERS spectra. The spectra obtained from the PBS used to incubate the bacteria with the antibiotics are shown below in Figure 37.

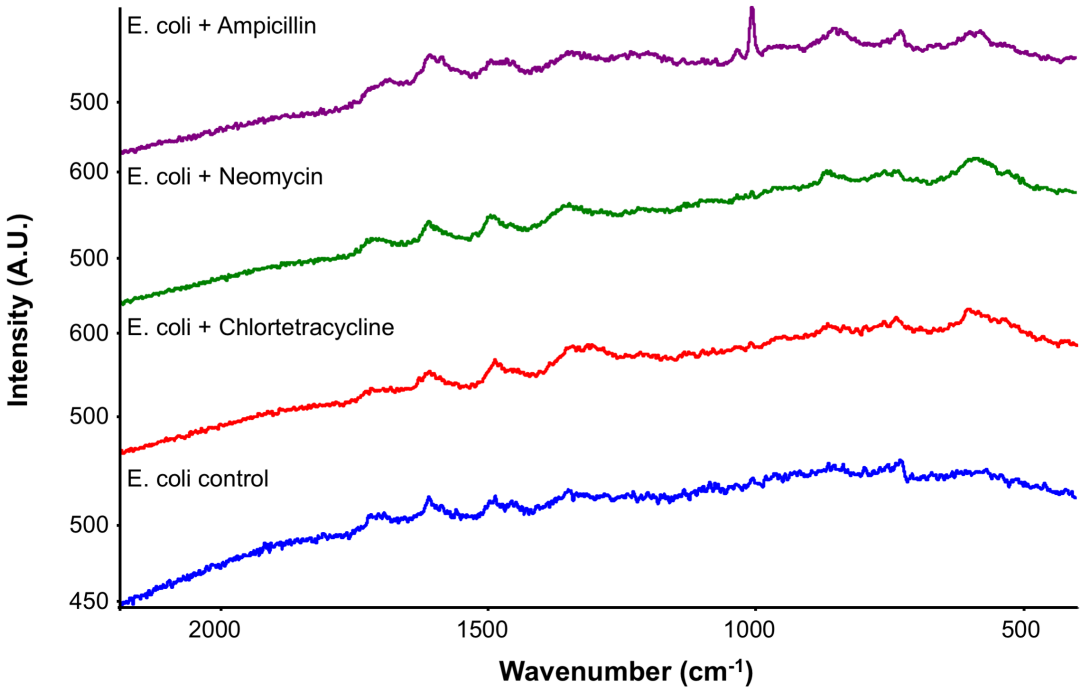

Figure 37. SERS spectra obtained from the incubation liquid of *E. coli* samples incubated in PBS with different antibiotics, obtained using the portable SERS method with 2x concentrated AuNPs.

Compared to the spectra from other bacterial samples that were previously tested, the effect of the PBS on the spectra can be clearly observed. The 738 cm^{-1} peak was much less distinct than in the previously tested bacterial samples, and there was a noticeable pattern between the range of 1200 cm^{-1} and 1700 cm^{-1} that was not observed in the non-PBS samples. The spectra from the washing liquid subsequently obtained from these samples are shown below in Figure 38.

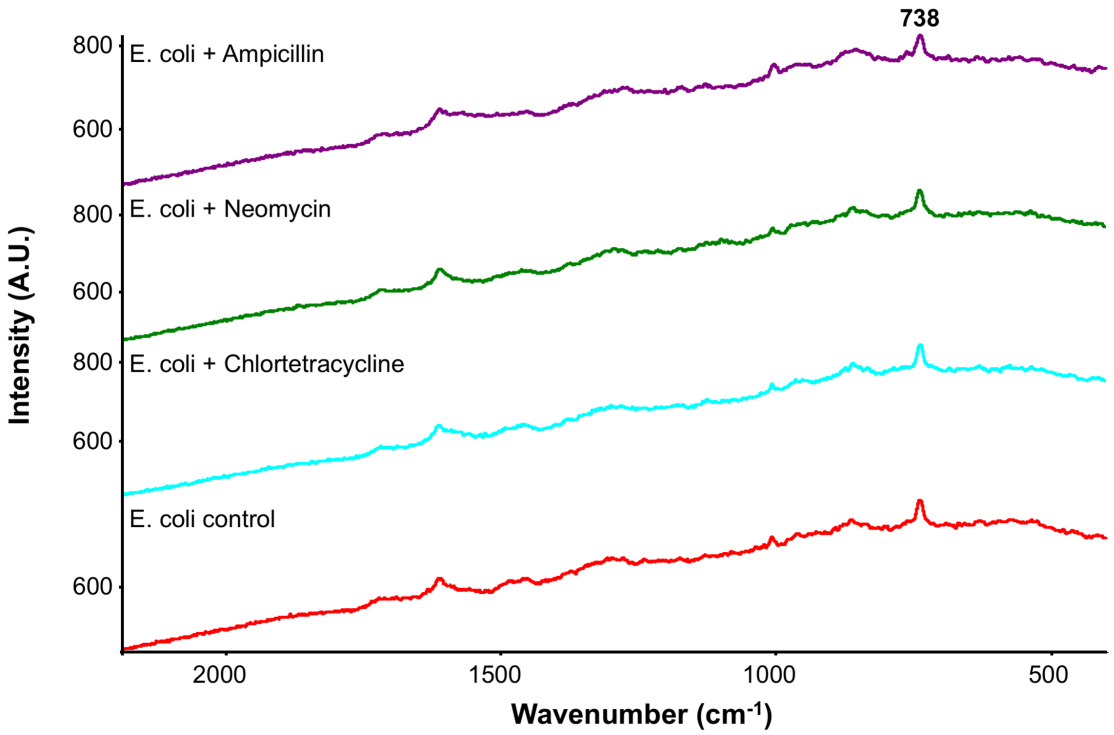

Figure 38. SERS spectra obtained from the washing liquid of *E. coli* samples incubated in PBS with different antibiotics, obtained using the portable SERS method with 2x concentrated AuNPs.

These spectra, from samples in which the PBS had been removed, did show a more distinct 738 cm^{-1} peak than the spectra from the incubation PBS. However, there was not any clearly observable difference in the intensity of the 738 cm^{-1} peak between the samples that had been exposed to different antibiotics. These results indicate that PBS is also not ideal as an incubation medium for testing antibiotic susceptibility with our portable SERS method, and it does not result in the desired patterns showing up in the SERS spectra that indicate antibiotic sensitivity. Comparing the results from the different incubation media, only incubating the bacteria with TSB resulted in clear changes in the SERS spectra that showed the bacterial responses to the antibiotics.

5.3.5. Plate Counts of Bacteria Exposed to Antibiotics

To compare the effects of the incubation medium on the number of surviving viable cells following antibiotic exposure, plate counts were performed on *E. coli* samples exposed to ampicillin, neomycin, and chlortetracycline. Two groups of *E. coli* samples were compared- samples incubated with the antibiotics directly in TSB, or samples in which the TSB was replaced with PBS prior to incubation with the antibiotics. The results of these plate counts can be found below in Figure 39.

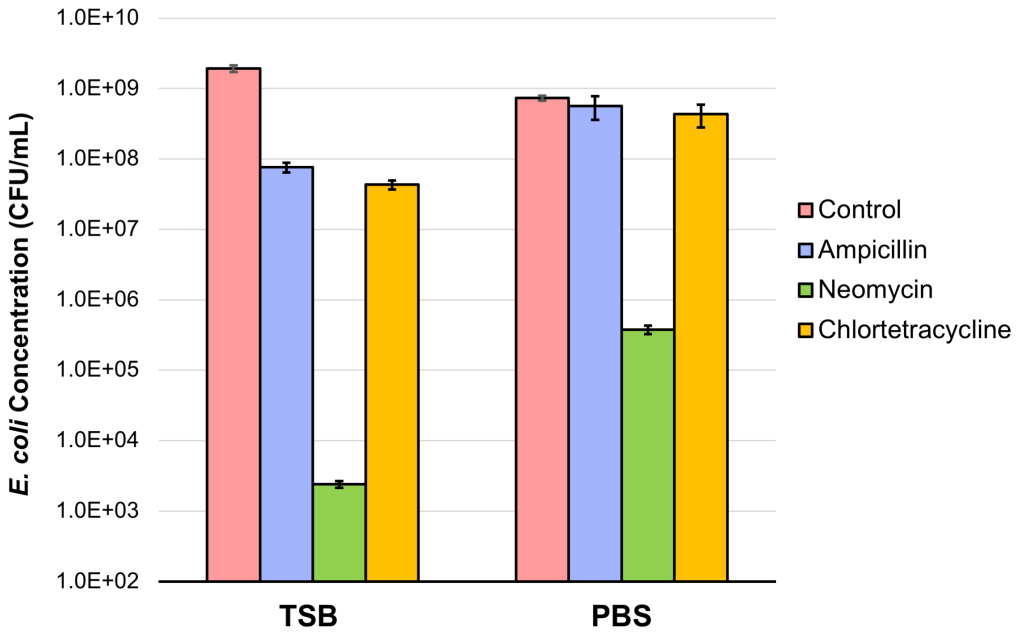

Figure 39. Average bacterial CFU concentrations obtained from the samples of *E. coli* incubated with antibiotics in TSB and PBS, calculated from the results of the plate counts.

As seen in the Figure, the antibiotics have a variable effect on the number of colony-forming units (CFUs) remaining in the cultures following antibiotic exposure- in both TSB and PBS, neomycin was found to have a greater effect than ampicillin and

chlortetracycline. Comparing the two types of incubation media, the antibiotics consistently caused a greater decrease in the number of CFUs compared to the control samples in TSB, compared to PBS.

5.3.6. Antibiotic Sensitivity Testing of Foodborne Bacteria

In addition to testing different experimental conditions, we also tried using the portable SERS method to test samples of antibiotic resistant bacteria obtained from supermarket ground chicken. Colonies of bacteria resistant to each antibiotic were successfully isolated from the ground chicken, and overnight cultures were grown in TSB using one randomly selected colony resistant to each antibiotic. The samples were then analyzed using our portable SERS method, and the spectra obtained from the tests are shown below in Figure 40. A comparison of the 738 cm^{-1} peak heights from these spectra is shown in Figure 41.

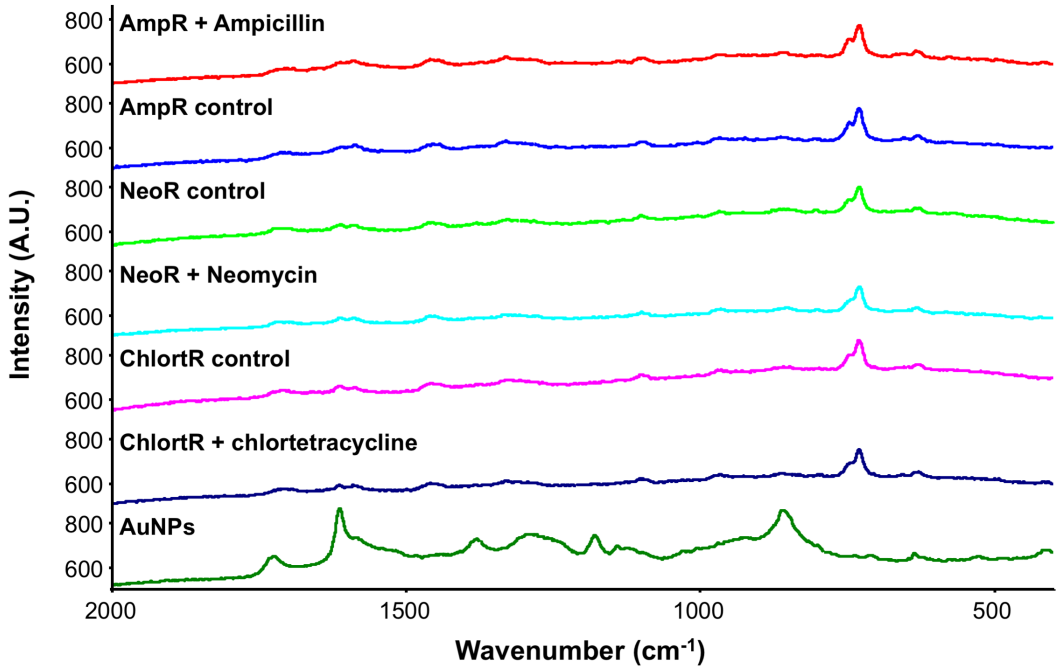

Figure 40. SERS spectra obtained from the washing liquid of the antibiotic resistant ground chicken isolate bacteria strains, following incubation with the corresponding antibiotic for each strain.

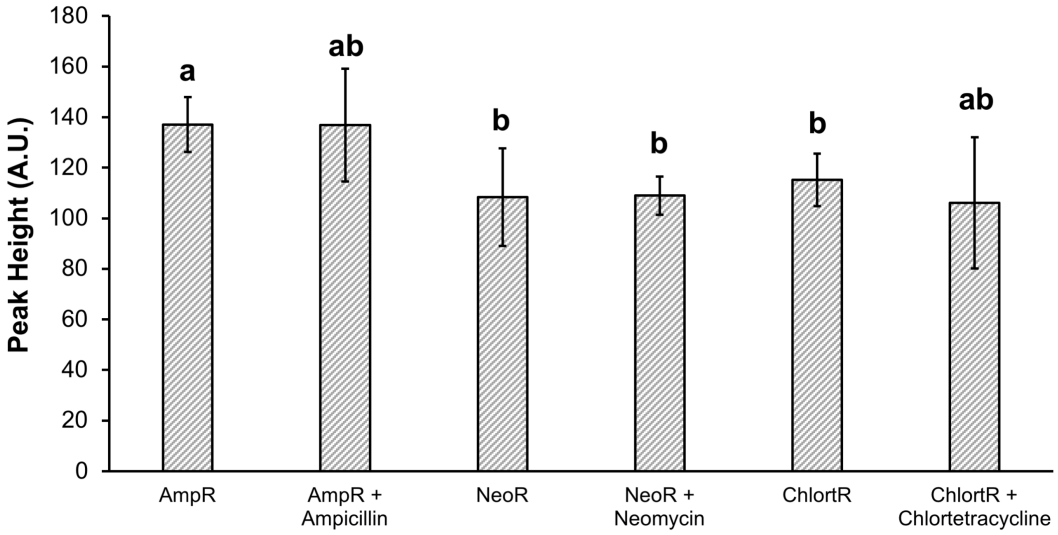

Figure 41. Comparison of the heights of the 738 cm^{-1} peaks from the SERS spectra of the washing liquid from the ground chicken isolate bacteria shown in Figure 40.

5.3.7. Discussion

Comparing the results obtained from the various experiments in this study provides some clear findings about the optimal conditions for testing bacterial matrix liquid using our portable SERS method. It is apparent from the results that the SERS signals and antibiotic response patterns observed in the bacterial spectra can vary quite significantly depending on a variety of factors, such as the method used to prepare the samples and the concentration of the substrate. In order to clearly identify the antibiotic sensitivity of bacterial samples using a portable SERS technique, it is necessary to properly optimize the various experimental parameters so that the desired patterns can be observed in the spectra of the extracellular matrix liquid.

One factor that played a major role in the clarity of the SERS spectra in our tests was the concentration of AuNPs combined with the bacterial liquid prior to the SERS analysis. As the results in Figures 24-27 show, the substrate concentration needs to be optimized sufficiently for a portable SERS method to be successful- if the concentration is too low then it will be difficult to clearly identify the desired signals, and if the concentration is too high then the background signals can overwhelm the target peaks and make it impossible to properly assess patterns such as changes in peak intensity. The results indicate that any substrate that is intended for a portable SERS method needs to be properly optimized for its specific application. This is particularly important for a portable spectrometer-based SERS method, compared to a microscope-based method- unlike a microscope, a portable spectrometer has much less capability to zoom in on a specific area of the coffee ring with the desired intensity. As a result, it is essential for SERS methods that do not use a microscope that the substrate concentration is high enough to ensure a sufficiently intense SERS signal in the larger area that is scanned with the Raman laser.

Other aspects of the experimental design, such as what specific sample of liquid from the bacteria was analyzed, also played a major role in the quality of the final SERS results. The two types of liquid samples we tested, the incubation liquid and the washing liquid, both resulted in very different SERS spectra- the antibiotic response patterns could be clearly identified in the spectra from the washing liquid, but not the spectra from the incubation liquid. This is particularly apparent when comparing the results from Figures 28 and 33, which were obtained from *E. coli* samples prepared in an identical fashion- the only difference being whether the incubation liquid or washing liquid was tested. The fact that the antibiotic response patterns can only be clearly observed in the washing liquid

suggests that the washing step itself plays a role in the patterns that appear in the resulting SERS spectra. This finding is consistent with the results obtained previously by Premasiri et al. (2016), who concluded that bacterial SERS signals can be attributed to purine metabolites that result from the starvation response of the bacteria. Washing the bacteria with water places them in a nutrient-free environment, resulting in the release of these purine compounds as part of their response to these conditions. These compounds are subsequently reflected in the spectra obtained from SERS analysis of the washing liquid. The differences between the spectra obtained from samples exposed to different antibiotics in Figures 28 and 30 suggest that the antibiotic-bacteria interactions during the incubation step also play a role in the determining the concentration of these compounds in the washing liquid, as reflected in the final spectra- however, these differences cannot be observed without performing the washing step, as shown in Figure 33. Therefore, this set of results shows that both the incubation step and the washing step are necessary to observe the effects of the antibiotics on the bacteria. During the incubation step, the antibiotics impact the bacteria in various ways, such as causing damage to the cell wall or inhibiting the synthesis of proteins, depending on the specific antibiotic mechanism. Based on the SERS results, these changes then impact the concentration of purine compounds released by the bacteria in the subsequent washing step, which are then reflected in the SERS spectra of the washing liquid by changes in the intensity of the 738 cm^{-1} peak. A summary of this proposed mechanism is shown in the schematic below in Figure 42.

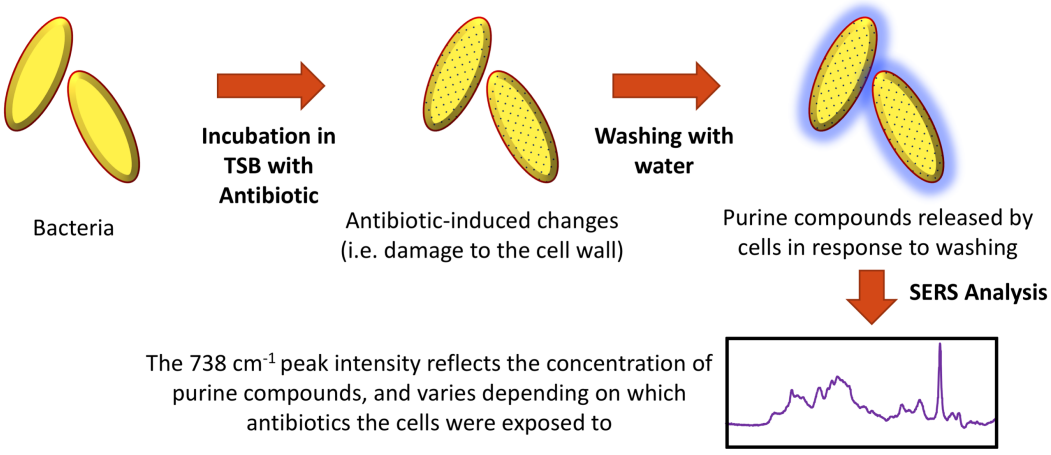

Figure 42. Schematic of the proposed mechanism for the antibiotic response patterns observed in our experiments, and the experimental steps necessary to observe them in the SERS spectra.

An additional result that underlies that the washing step is necessary for properly assessing the bacterial SERS signals is the comparison of the bacterial liquid to pure TSB in Figure 32. TSB naturally contains a peak at 738 cm^{-1}, which makes sense considering that TSB is made from soy extracts that contain the purine compounds that are responsible for this peak. The fact that this peak is naturally present in the growth medium can make it difficult to distinguish whether the peak observed in the spectra from bacterial samples should be attributed to the TSB or the bacteria themselves. It is necessary to wash the bacteria and remove the growth media in order to be sure that the signals observed are not coming from the TSB. Indeed, in the washing liquid spectrum it can be observed that other peaks from the TSB spectrum, such as the 1010 cm^{-1} peak, are largely eliminated, but the 738 cm^{-1} peak is still present and is considerably more intense than it was originally in the

TSB spectrum. This indicates that the 738 cm^{-1} peak seen in the washing liquid spectrum is originating from the bacteria, and not from the growth media.

The tests in which the TSB was replaced with distilled water or PBS prior to incubation with the antibiotics also provide useful information about the optimal conditions for assessing the bacterial responses to antibiotic exposure using SERS. As shown in Figures 34-38, the type of liquid used to incubate the cells with the antibiotics has a significant impact on what peaks and patterns show up in the resulting SERS spectra, both in the spectra from the incubation liquid and the subsequent washing liquid. In Figures 34 and 35, we can see that when distilled water is used to incubate the bacteria with the antibiotics, we can see the 738 cm^{-1} peak shows up in all of the spectra, but there is no significant difference in the peak height between the various samples. In the liquid used to wash these samples after incubation, seen in Figure 36, it can be observed that the 738 cm^{-1} peak does not appear to show up in the spectra at all. These results indicate that the cells are exhibiting a stress response to the initial suspension in the water, since the 738 cm^{-1} peak shows up in the incubation liquid spectra- however, the lack of antibiotic response patterns also suggest that the bacteria are not interacting with the antibiotics prior to these stress responses occurring, and any subsequent antibiotic interactions are not reflected in the SERS spectra. The lack of any 738 cm^{-1} peak in the washing liquid from these samples indicates that the compounds released by the cells from the initial stress response are removed by the washing step, and the cells subsequently do not release enough of these compounds during the washing process to be reflected in the SERS spectra of the washing liquid.

The testing with PBS used as the incubation liquid, shown in Figures 37 and 38, also provides useful insight into the origins of these SERS patterns. As seen in Figure 37, the 738 cm^{-1} peak is noticeably indistinct in the spectra from the PBS used as the incubation liquid. It then becomes more clear and defined in the spectra from the water used to subsequently wash the bacteria, seen in Figure 38. This is consistent with the proposed mechanism that these peaks arise from the bacterial stress responses to the washing step, as PBS provides a more stable environment for the bacteria than pure distilled water and these peaks should be less apparent in PBS than distilled water if this was the case. However, there were also no clear antibiotic response patterns observed in the washing liquid, suggesting that the bacteria and antibiotics do not interact in the same manner in PBS as they do in TSB, similar to the observations from the bacteria suspended in distilled water. All together, these results show that TSB is a more suitable medium for incubation than distilled water or PBS, as the antibiotic response patterns do not show up clearly in the SERS spectra obtained from the latter samples. This could potentially be attributed to decreased antibiotic effects in nutrient-poor environments such as distilled water or PBS, which have previously been shown to impact the effectiveness of antibiotics- for instance, direct correlations can be found between the growth rate of bacteria and the efficacy of certain antibiotics (Lee et al., 2018). The nutrient-poor environments could also potentially be inducing antibiotic persistence, allowing the bacteria to survive exposure to antibiotics and reducing their effectiveness (Cabral et al., 2018).

The results of plate counts comparing the effects of TSB and PBS on the number of viable bacteria remaining following antibiotic exposure, shown in Figure 39, provide further insight into the impact the incubation medium has on the effects of the antibiotics.

As shown in the Figure, the number of CFUs observed in the plate counts are consistently lower following antibiotic exposure in TSB, compared to PBS. This suggests that the TSB is indeed more suited for this application, as the observed efficacies of the antibiotics are higher in TSB than they are in PBS. There is also another interesting finding from these plate counts- there is considerable variation in the final concentration of the viable bacteria when comparing the three antibiotics, regardless of the incubation medium that is used. For instance, there is a significant difference in the number of CFUs observed following neomycin and chlortetracycline exposure in TSB. Despite this finding, the SERS spectra obtained from comparable samples (as seen in Figure 28) look largely identical. This suggests that the observed SERS patterns are not necessarily correlated with the number of viable bacteria remaining in the culture following antibiotic exposure. A likely explanation for this is that the patterns found in the SERS spectra are originating from the short-term post-exposure effects of the antibiotics on the cells, which do not necessarily result in the death of the bacteria. Previously, it has been found that short term antibiotic exposure can result in significant impacts on the rates of cellular processes, such as RNA, DNA, and protein synthesis (Stubbings et al., 2006). However, after several hours had elapsed since antibiotic exposure, these processes were observed to be restored back to their usual rates. In our results, the SERS spectra are showing the effects of the antibiotics immediately following exposure. As time passes and the cells recover following the removal of the antibiotic, the bacteria may recover to varying degrees, resulting in different numbers of CFUs showing up in the plate counts from samples exposed to different antibiotics despite the similarities in the SERS spectra obtained directly after the exposure.

Finally, the results from the ground chicken isolate bacteria testing in Figures 40 and 41 indicate that our method can be used to successfully assess the antibiotic sensitivity of antibiotic resistant bacteria isolated from food samples- while there was some variation in the average height of the 738 cm^{-1} peak between the different samples, there were no clear antibiotic response patterns were observed in any of the samples. This indicates that they are indeed resistant to the antibiotics, and that our SERS method can correctly show the absence of these patterns in antibiotic resistant bacteria samples. One notable finding was that the spectra from the three isolates were all largely identical, and yet slightly different from the spectra previously found from *E. coli* and *Salmonella*- most notably, a smaller peak around 750 cm^{-1} was observed in the samples, adjacent to the 738 cm^{-1} peak. The similarity of the three bacteria samples suggests they could all be the same species, potentially a multi-drug resistant strain. The differences from the *E. coli* and *Salmonella* also show that some bacteria species tested in real-world application may exhibit different peaks and patterns, and further study is needed to assess more kinds of these samples and see if the differences impact the effectiveness of this kind of SERS-based antibiotic sensitivity detection method. However, regardless of the specific properties of the bacteria tested here, our results show that our optimized SERS method can be used to accurately assess the antibiotic sensitivity of real-world foodborne bacteria samples using their extracellular matrix liquid.

5.4. Conclusions

Overall, we have developed an effective portable SERS method for testing antibiotic sensitivity using the extracellular matrix liquid from bacterial samples. We have optimized the concentration of AuNPs required to see the antibiotic response patterns in the liquid

samples, and these patterns could now be clearly observed with the portable SERS method. In addition, we have obtained valuable information about the sample preparation steps required to observe the antibiotic patterns- based on our results, both the incubation step and the washing step are required to see these patterns in the resulting SERS spectra. The type of media used for incubation also has a significant impact- using a nutrient-rich medium such as TSB allows us to clearly observe the antibiotic response patterns in the resulting spectra, but these patterns did not appear when distilled water or PBS was substituted for the TSB. Therefore, the preferred experimental procedure we found for testing antibiotic sensitivity was first incubating the bacteria with the antibiotics in TSB, followed by washing with distilled water. The antibiotic response patterns could then be observed in the SERS spectra obtained from the liquid used to wash the bacteria. Finally, we were able to successfully test the antibiotic sensitivity of resistant bacteria isolated from real food samples using our optimized method. Our SERS procedure, as optimized in this study for the portable Raman spectrometer, is a significant improvement on the previously tested protocols and allows bacterial samples to be tested much more rapidly and more effectively. This type of approach for testing antibiotic sensitivity could be of great use, and it has great potential to be further explored and tested in more real-world applications.

CHAPTER 6

OVERALL CONCLUSIONS AND FUTURE WORK

6.1. Overall Conclusions

The results obtained in this project show that SERS can be used to effectively test the antibiotic sensitivity of a variety of different bacterial samples. The SERS protocols we have developed have a number of advantages compared to existing detection methods- they are faster than the traditional growth-based procedures and can be used non-selectively with different kinds of bacteria, unlike methods such as PCR and immunoassays which require prior knowledge of specific targets in the cells. Compared to other Raman and SERS-based procedures that have been previously developed, our optimized protocol requires less sample preparation and makes use of more practical portable Raman technology. It is also safer to perform with more pathogenic bacteria samples than comparable methods that make use of syringes for filtration. We have also obtained valuable knowledge of the mechanisms that are responsible for the bacterial SERS responses to antibiotic exposure, and our results indicate that the type of media used to incubate the bacteria with the antibiotics as well as the subsequent washing step both play a significant role in determining what patterns show up in the resulting SERS spectra. To our knowledge, this is the most thoroughly optimized SERS method that has been developed for this application.

While our experiments show that SERS can be used effectively in this application, there are still a variety of questions which still remain to be answered before these types of methods can be as widely used as growth-based methods or PCR. Our results showed that

the experimental design, including the types of SERS substrate and liquid media that were used, plays a major role in determining what patterns appear in the SERS spectra of the bacteria. In order for this type of method to be used successfully in real-world applications, it is important the SERS patterns are consistent and can be accurately used to identify antibiotic sensitive and resistant bacterial samples. While the patterns were largely consistent in our experiments, there is still more work that can be done to assess whether these patterns can be clearly observed in different environments. Future studies can continue to explore the SERS responses to antibiotics using different types of substrates, bacteria and antibiotic concentrations, and with more types of food samples. This will help us to gain a better understanding of whether these SERS methods can be used effectively in more kinds of real-world applications, and will assist with further optimization of the procedure by testing the effects of more experimental conditions.

6.2. Potential Future Work

There are two main areas that can be further explored with our SERS procedures- additional optimization by testing more kinds of experimental conditions and samples, and adapting our SERS methods for other types of applications beyond simply determining the antibiotic sensitivity of a single type of bacteria. In this section, we will discuss a number of potential areas of study that could further improve our SERS procedures.

6.2.1. Testing Alternative Experimental Conditions

In Chapter 5, we explored a number of variations of the experimental conditions in our SERS method, including testing different AuNP concentrations for the substrate and

different types of media for incubating the bacteria with the antibiotics. The results showed that these conditions have a significant impact on the results, and more changes to the experimental design can be tested to assess their impacts on the patterns in the bacterial SERS spectra. Examples of potential variations to the substrate include using silver nanoparticles instead of AuNPs, or using other types of substrates such as dendrites or a concentrated nanoparticle mirror instead of simply using gold or silver nanoparticles alone. Other types of nutrient-rich media could also potentially be used as an alternative to TSB for the incubation media, and the effect of the media on the resulting SERS patterns could be further assessed.

Additionally, another topic which could have a significant effect on the results is the concentration of bacteria and antibiotics used in the experiments. In our procedures, we intentionally grew the bacteria to a high concentration and used antibiotics at a much higher level than the minimum inhibitory concentration so that we could clearly see the signal from the bacteria and the effects of the antibiotics on the SERS spectrum. However, one area which we have not yet explored in detail is whether these effects can be observed with lower bacteria or antibiotic concentrations. Previous studies have shown that the antibiotic concentration used to incubate bacteria does have an impact on the resulting SERS patterns (Wang et al., 2016b). Testing lower concentrations of the antibiotics and bacteria would provide more information on whether particular concentrations are required to see the SERS patterns clearly, and could potentially help to speed up the procedure by reducing the time required to grow the bacteria.

6.2.2. Other Types of Bacteria and Antibiotics

While we have tested a number of types of bacteria, including *Escherichia coli*, *Salmonella enterica*, and *Bacillus cereus*, there are certainly more types of bacteria that can be tested to further explore whether our method is effective. In addition to simply testing more the effects of antibiotics on more species of bacteria, we could also try to isolate more kinds of foodborne bacteria to be tested with our method. Potential food samples we could test include fish, leafy vegetables, and other kinds of meat products. These experiments could potentially be of significant interest- as shown in the chicken isolate bacteria testing in Chapter 5, some types of bacteria may exhibit different peaks and patterns beyond simply the changes we previously observed in the ~730 cm^{-1} peak. It is important to gain a greater understanding of the SERS peaks that may occur in these samples, so that they can be recognized in future testing and be used to identify the antibiotic susceptibility of the bacteria in the sample.

In addition to testing new types of bacteria, we also want to test more antibiotics. We have previously tested examples of three classes of antibiotics- ampicillin (a beta lactam antibiotic), chlortetracycline (a tetracycline antibiotic), and neomycin (an aminoglycoside antibiotic). These antibiotics have different mechanisms and impacts on the cell- beta lactams inhibit the formation of the cell wall, while tetracyclines and aminoglycosides impact the ribosomes and protein synthesis. While we could test more antibiotics in these classes and other classes which affect the same targets, we are particularly interested in testing classes of antibiotics which impact other parts of the cell and may have different impacts on the SERS spectra than the antibiotics we have tested previously. Examples include quinolones such as ciprofloxacin, which interfere with DNA replication, and trimethoprim, which inhibits the synthesis of folate. The biochemical

changes induced by these antibiotics could have very different SERS signatures than those we have tested previously, and it is important that we understand their effects on the bacterial SERS spectrum so that we can recognize them in future applications.

6.2.3. Testing Mixed Bacteria Populations

So far, we have focused on testing cultures grown from isolated bacteria colonies. These cultures would be expected to only contain one type of organism, and likely would have a relatively consistent SERS signal. However, in real-world food and environmental samples there may be a large number of different organisms that are simultaneously present. While it can be possible to isolate specific colonies from these samples for further analysis, it would be more efficient and less time-consuming if we could directly analyze mixed bacterial populations extracted from these samples without having to go through the isolation steps. Testing mixed bacteria populations present some distinct challenges compared to our previous research- there may be a wide range of bacteria types present with their own characteristic signals, and there would likely be a significant amount of variation in the results. As a result, testing randomly selected spots with our portable Raman spectrometer or DXR microscope may be less useful, as there could be excessive variation in different areas of the sample. For these types of samples, we would instead focus on SERS mapping with our DXRxi microscope, which could allow us to assess the variation within a larger area of the sample. Once we obtain a map, we could then adjust the parameters and look at the intensity of specific peaks, allowing us to potentially assess the antibiotic sensitivity of specific organisms in the sample.

To conduct these experiments, we could begin by simply mixing two known types of bacteria together and then testing them (such as *E. coli* and *Salmonella*). Once we have determined that our mapping procedure can be effective for these types of mixed samples, we could then try to test liquid samples extracted from foods prior to the isolation of specific organisms. It may be necessary to do some enrichment to increase the concentration of bacteria in the sample, and these types of food samples may present some unique challenges. Since we are looking directly at samples from foods, potentially without much additional preparation or processing, these samples may contain substances from the foods that could interfere with the bacterial SERS signals we are looking at, such as proteins or lipids. The bacterial signals may also not be sufficiently clear to determine the antibiotic sensitivity of specific organisms in the samples. Ultimately, we will have to test these samples and determine if any additional preparation is necessary. If these experiments are successful, it could considerably cut down the time required to conduct this analysis for bacteria from food samples and provide additional advantages for our SERS procedures compared to more conventional analytical techniques.

6.2.4. MIC Testing with SERS

Our previous experiments have focused on simply using a high concentration of antibiotics to determine whether or not the bacteria in a sample are sensitive or resistant. However, it would be useful at times to obtain more detailed information about the overall sensitivity of a sample to different antibiotics, particularly in potential clinical applications in which a specific antibiotic concentration may be necessary to effectively treat an infection. As a result, one useful way to further explore the applications of our SERS

methods is to use them to determine the minimum inhibitory concentration (MIC) of antibiotics for bacteria samples.

The procedures for this type of analysis would likely be quite similar to the experiments we have previously performed, except a range of different antibiotic concentrations will be tested instead of just one. We should be able to look for the same SERS patterns which we have studied previously- for instance, we could look for an increase in the intensity of the ~730 cm^{-1} peak in *E. coli* samples exposed to ampicillin. Lower concentrations of the antibiotic may have a reduced impact on the peaks in the SERS spectra, and we could assess the effect of different concentrations of specific target peaks. The goal of these experiments would be to identify the minimum concentration of the antibiotic which has a significant impact on the cells, based on the resulting SERS spectra. Conventional MIC testing, in which bacterial cultures are grown in the presence of different antibiotic concentrations to assess their inhibitory effect, would be separately conducted so that the results could be compared to the findings from the SERS analysis. Based on this data, we will be able to determine whether or not the patterns in the SERS spectra could be used to reliably identify the MIC of a bacterial sample. This would be a significant finding, and would make our SERS techniques even more useful in a variety of practical applications.

6.2.5. Assessing the Development of Antibiotic Resistance in a Sample

Another potential application of our SERS techniques is the analysis of how antibiotic resistance develops in a bacterial population over time. Our procedures are well-suited for this type of application, thanks to their ability to detect the biochemical changes

occurring in a sample. In order to conduct this analysis, we would grow a liquid culture of antibiotic sensitive bacteria and expose it to a low concentration of an antibiotic (preferably the highest concentration that is still below the MIC). Over a period of several days, we could grow successive cultures with increasing concentrations of the antibiotic present, each one inoculated with bacteria from the preceding culture. Ultimately, we will be able to see if antibiotic resistant bacteria develop by observing whether or not they are able to grow in the presence of higher antibiotic concentrations.

Each day of these experiments, samples of each culture will be removed and tested with our SERS procedures. To conduct this analysis, it may be preferable to use SERS mapping to assess the variation across a wider area of each sample. This would allow us to analyze individual cells or groups of cells which may have developed antibiotic resistance, and we could be able to visually distinguish resistant cells from sensitive cells by mapping the intensity of specific characteristic SERS peaks. We could use this data in a number of ways, such as estimating the number of resistant cells in each sample and taking a closer look at the biochemical changes that occur as antibiotic resistance develops.

These experiments are just one example of how our SERS techniques could be used as the basis for future research into antibiotic resistant organisms. Ultimately, these SERS-based methods could be used in many ways to make it easier to detect antibiotic resistance and study the biochemical characteristics and mechanisms occurring in these types of samples. The development of reliable and efficient SERS-based procedures for the analysis of antibiotic resistant organisms is a step forward in the battle against antibiotic resistance, and in the coming years these techniques will continue to be improved and optimized for

new kinds of technology and with applications for studying bacteria from food, agricultural, and environmental samples.

CHAPTER 7

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