Development of a SERS Sandwich Assay Platform for Rapid Detection of Bacteria

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DEVELOPMENT OF A SERS SANDWICH ASSAY PLATFORM FOR RAPID DETECTION OF BACTERIA

A Thesis Presented

By

BROOKE PEARSON

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

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Department of Food Science
DEVELOPMENT OF A SERS SANDWICH ASSAY PLATFORM FOR RAPID DETECTION OF BACTERIA

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By

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ACKNOWLEDGEMENTS

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The increased incidence of food pathogen outbreaks placed a new emphasis on the requirement of a rapid, sensitive, and reliable detection method for pathogens in food samples. Surface-enhanced Raman spectroscopy (SERS) is a technique that tremendously enhances the weak Raman scattering of an analyte by using a metallic nano-substrate. Herein, we developed an innovative SERS sandwich assay platform which is based on 3-mercaptophenylboronic acid (3-MPBA) or aptamer as a capturer, and 3-MPBA and silver nanoparticles (AgNPs) as the reporter for non-selective and selective detection of bacteria. Both optical and chemical (SERS mapping) imaging were used as mechanisms for bacterial detection and quantification. Using *Salmonella enterica* and *Listeria monocytogenes* as the model bacteria, we have identified a unique bacterial SERS signal upon the interaction between the captured bacteria, 3-MBPA and AgNPs, which was used as the base for reliable detection of bacteria using SERS mapping. The non-specific assay also possesses unique optical properties allowing for the enhanced visualization of bacteria at low microscope magnifications (10 and 20x objective lenses). Using 3-MBPA owe achieved sensitive detection and quantification of as low as $10^2$ CFU/mL and a capture efficiency of 92.1% for nonselective detection of *Salmonella*. The capability of the assay method to detect specific bacteria using an aptamer was also demonstrated. Besides the SERS applications of this assay, it
was discovered that the 3-MPBA coated gold chip developed for this assay enhances the visualization of bacteria under a light microscope allowing for facile and rapid detection and quantification. In anticipation for industrial applications, sample preparation methods and strategies were developed for simple and carbohydrate food matrices.
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LIST OF ABBREVIATIONS

AgNPs- silver nanoparticles
SERS- surface-enhanced Raman spectroscopy
3-MPBA- 3-mercaptophenylboronic acid
SE 1045- Salmonella enterica 1045
LM 21- Listeria monocytogenes 21
ATR-IR- attenuated total reflectance infrared spectroscopy
CHAPTER 1

INTRODUCTION

1.1 Justification

Foodborne illness presents a significant health hazard and economic burden on the United States. The USDA estimated in 2013 that 95% of known foodborne agents cost the United States economy $15.5 billion annually [1]. However, the CDC believes that unspecified agents cause the majority of foodborne illnesses (80%), hospitalizations (56%) and deaths (56%) [2]. As the USDA economic burden estimate only included known foodborne pathogens, the economic burden is probably much greater than this figure but isn’t currently being accounted for.

At the cornerstone of making the food system safer are powerful detection methods. In order to prevent a foodborne pathogen from causing illness, the pathogenic agent must be identified and an understanding of its microbial ecology is needed to create preventative measures to inhibit proliferation in the food source. Unfortunately, conventional culture methods can produce misleading results. An inherent weakness of these methods is that they depend on the assumption that the growth conditions (nutrients in media, temperature, atmosphere) the microbiologist chooses to use, are in fact those of the bacteria being targeted. Yet bacteria are incredibly diverse, and many grow in extreme conditions that aren’t routinely being tested for. The aerobic plate count is currently the gold standard test for total bacteria detection in food, yet only elucidates aerobic, mesophilic bacteria[3].

1.2 Conventional method for the detection of bacteria

The current culture protocol possesses weaknesses that cause microbiology testing to be under-utilized. The aerobic plate count for total bacteria detection requires a dedicated microbiology laboratory, the skill of a trained microbiologist, and a waiting time of 48 hours for preliminary results [3]. This is a significant investment and may be unrealistic for daily monitoring for many food producers [4]. The amount of time the test takes is particularly
problematic for all producers, no matter the size. The CDC has noted that perishable products including produce, meat and poultry, dairy and eggs, and fish and shellfish are the source of 95% of food borne illnesses [5]. A common trait in all these food items is their short shelf-life which probably makes meaningful microbiological tests difficult. To increase the safety of these products, a test should be developed that can provide same-day results.

1.3 Alternative bacteria rapid detection methods

Several other groups have proposed alternative methods to the Standard Plate Count for total bacteria detection, yet for industrial applications many barriers still exist. Probably the most well-known alternative is polymerase chain reaction (PCR) and its derivatives. This technology works by amplifying and detecting signature DNA sequences characteristic of the target bacteria. It is a highly precise technology that reduces detection time of bacteria to about 24 hours. It flounders from gaining wider adoption within the industry because it is expensive, requires highly trained personnel, is susceptible to environmental contamination, is difficult to quantify bacteria, and often requires a time-consuming sample preparation and enrichment step [6]. Hammons et al. found that an ATP luminescence technique to had non-significant differences in results compared to the Standard Plate Count [7]. While ATP luminescence is fast and inexpensive, it works by detecting the amount of ATP on a surface, which can be produced by all living things. This lack of specificity makes the technique better for preliminary testing instead of validating the food safety of the product. Other less commonly known techniques include flow cytometry [8] and fluorescent filter-based system [9].

1.4 Surface-enhanced Raman Spectroscopy (SERS)

Recently, surface-enhanced Raman spectroscopy (SERS) has emerged as a promising solution for rapid bacteria detection. SERS works by hitting a target molecule with a laser, producing vibrational energy that is measured. Molecules produce unique vibrations making it suitable for identification purposes [10]. Unfortunately, on their own, biomolecules give off a
weak Raman signal. However, a metallic nano-roughened surface can greatly enhance the Raman signal allowing for biomolecule identification [11]. Other potential advantages of using SERS include faster sample preparation and detection time, quantification of bacteria, and simultaneous bacteria detection. Using SERS assays, researchers have been able to detect and characterize an array of biomolecules including: bacteria [12–17], spores [18], viruses [19], yeasts [11] and biofilms [20].

1.5 Critical gaps in SERS detection of bacteria

In order for SERS to become widely adopted as a rapid detection method for bacteria, several key components of the assay must be refined. First, due to the robust signal and many species of bacteria in a sample, target bacteria of interest must be selectively captured and produce a robust, reliable, and unique spectrum. Next, a statistical method that can reliably distinguish between spectra is needed. Finally, specific clean-up methods should be found and tested to minimize the background of the food matrix while maximizing bacterial recovery[21].

1.5.1 Capture and Detection Techniques

Due to the high sensitivity of the Raman spectrometer, identifying and understanding the best way to capture target bacteria and enhance the signal they produce is essential for the success of this assay. Efrima and Zeiri have reported that the spectra of the same bacteria species are not always reproducible between different laboratory groups [21]. They speculate that this is because different components of the cell wall are targeted. Furthermore, elements like the wavelength of the laser, and the way the metal nanoparticles are mixed with the bacteria will significantly affect the resulting spectra. Despite these challenges, several research groups have reported various techniques that allow them to detect and differentiate between bacteria species and strains [13,15,22,23].

Antibodies are a commonly proposed solution because of their reputation for having a highly specific binding capacity. Najafí et al. developed a novel antibody sandwich structure.
This consisted of an antibody that was attached to a magnet, then the bacteria were added, and another antibody was attached to a SERS tag. They then used an external magnetic particle concentrator to collect the cells and used SERS to evaluate the presence of bacteria. Using this technique, they were able to detect *E. coli* in apple juice with a detection limit of $10^2$ CFU/mL [12]. Weidemaier et al. expanded on this technique by using three different types of pathogenic bacteria in more complex food matrices. Their detection limit was $10^5$ CFU/mL [13].

Aptamers may also possess good potential as SERS capturers. Aptamers are synthetically produced single-stranded nucleic acid ligands or peptides, that bind specifically to their target like antibodies [24]. While aptamers have never been used to detect bacteria using SERS they present a promising option for biosensor applications. Some of the advantages of aptamers over antibodies include easier production and stability in harsh conditions [24]. To further support their potential applications as a biosensor, aptamers have already been successfully used to detect multiple pesticides in apple juice using SERS [25].

Non-specific capturers like antibiotics and membrane disruptors have also shown some potential for SERS applications. Antibiotics work by non-selectively targeting specific features of bacterial cell walls. They have a high binding affinity to bacteria, and decrease the distance between the bacteria and the substrate therefore enhancing the Raman signal [26]. Wu et al. demonstrated its potential, identifying 6 different species and sub-species of bacteria in mung bean sprouts with a detection limit of $10^3$ CFU/mL [23]. Membrane disruptors work by targeting the degradation of the cell wall. This exposes proteins that produce unique spectra for different species of bacteria. Chen et al. reported a detection limit of $10^3$ CFU/mL in water of 6 species and sub species of bacteria using this method [27].

**1.5.2 Analysis of SERS spectra**

There are several ways to analyze detailed Raman spectra. The common means of determining significant bacterial difference for label-free bacteria spectra is Principal Component Analysis [13,23,27]. However, Chen et al. discussed that Principle Component Analysis was not
able to detect the difference between a *Pseudomonas* and *Listeria* bacteria [27]. While this may be due to a lack of specificity in the test, other approaches should be considered as more complex bacteria matrixes are analyzed. Xu et al. proposed a unique bar coding system in which they could identify unknown mixtures of 7 strains of *Vibrio parahaemolyticus* [28]. These statistical alternatives may give needed specificity to analyzing bacteria in samples.

Raman mapping is a powerful tool that can strengthen the reliability and usefulness of SERS data. Raman mapping is the collection of spectra from many discrete areas within a sample [29]. This approach strengthens SERS assays both qualitatively and quantitatively. From a qualitative sense, Raman mapping shows the distribution of the target within the sample. Larmour et al. successfully characterized the distribution of fat and sugars in a white chocolate sample using Raman mapping [30]. Perhaps more importantly, Chen et al. noted that because the laser hits such a small area of a sample, the probability of laser hitting a bacterium when the concentration is $10^5$ CFU/mL is only 40% [15]. This means that in order to accurately characterize the bacteria load in a sample, many data points must be taken. As many data points are taken, targets can be reliably quantified [29]. Incorporating Raman mapping into a SERS application, would strengthen its detection capabilities against rival technologies. Raman mapping may also simplify the spectra for technicians that do not have a strong chemistry background by creating presence-absence standards based on the presence of a few characteristic peaks.

### 1.5.3 Applications in a Food Matrix

Several research groups have demonstrated the feasibility of detecting bacteria in a food matrix [12,13,23]. Currently, most of the focus has been on better capturing techniques of bacteria. As these techniques become refined, the focus should shift to limiting background spectra from the sample in a cost-effective, time sensitive, reliable manner. Wu et al. used a two-step filtration process with a 74.6% recovery of target bacteria. Typically, it has been noted that the detection limit becomes higher when bacteria are isolated from a food matrix rather than
water [23]. This means that in order to detect bacteria in food at low levels, the limit of detection in water must be even lower.

1.6 Label-free vs. SERS labels

From this review it is clear that the detection limit of bacteria is a critical problem that must be solved. Label-free methods are appealing as they can give unique fingerprint spectral information about the molecule that was detected. The technique falters from gaining industrial acceptance due to the high spectral variation and low sensitivity when an enrichment step is not performed [14,15]. Many research groups have also explored the use of extrinsic SERS labeling [12,13]. SERS labels are bound to the target to increase its signal [10]. By doing this however, it limits the ability for accurate bacterial quantification at low concentrations, loses the intrinsic characteristic information about the cells, [27,31] increases the time and cost of the assay.
CHAPTER 2
DEVELOPMENT OF SERS SANDWICH ASSAY PLATFORM FOR NON-SPECIFIC DETECTION OF BACTERIA

2.1 Introduction

In Chapter 1, several major problems with conventional microbiological testing protocols were discussed. With the versatile detection capabilities of the Raman microscope, we wanted to develop an assay platform for the non-specific detection of bacteria using this system.

2.1.1 A marriage between two techniques: A SERS tag with an ability to distinguish

To overcome the weaknesses of both label and label-free SERS detection described in Section 1.6, we propose a marriage of the two techniques with a label that gives information about the molecule it binds to. The molecule we have identified to accomplish this goal is 3-mercaptophenylboronic acid (3-MPBA). This label has advantages over label-free techniques by being a very sensitive probe allowing for a low limit of detection and simplification of assay results due to the low spectral variation produced. In addition, due to the change is Raman signal it has added specificity over commonly used Raman reporters.

2.1.2 Boronic acids

Boronic acids have become popular choice as a capturers and indicator molecules for many assays[16,17,32,33]. They form a reversible, covalent bond primarily with 1,2 and 1,3 cis diols[34]. Bacteria cell walls are lined with glycoproteins that can bind to boronic acids making the molecule a good capturing and identification agent. The majority of assays tend to use 4-mercaptophenylboronic acid (4-MPBA)[17,32,33]. The reasons for this vary but are associated with increased stability and capturer affinity[32] as the molecular geometry of the molecule is optimal for binding to analytes. Tamer et. al[16] reported that 3-MPBA had increased binding affinity in an assay using bacteria. Based on this reporting, we first investigated the spectra produced when 3-MPBA binds to various diols.
2.1.3 Objectives of this study

The objectives of this study were to determine (1) develop a SERS assay platform for the non-specific detection of bacteria (2) evaluate its robustness against common food components that could interfere with the assay (3) determine the limit of detection of the assay.

2.2 Materials and methods

2.2.1 Sample preparation

2.2.1.1 Bacteria preparation

Salmonella enterica subsp enterica BAA1045 (SE1045) and Listeria monocytogenes FSL-J1-225M (Wiedmann Ithaca, NY) (LM 21) were used in this study. Frozen cultures of the SE1045 and LM 21 were revived in tryptic soy agar (Difco, Detroit, MI). Fresh bacteria culture was prepared by transferring a single colony into 9 mL of tryptic soy broth (Difco, Detroit, MI) and cultivated at 37 °C for approximately 16 hours.

Centrifugation at 6500×g for 3 minutes was used to collect the cells in a pellet. The supernatant was removed and replaced with 1 mL of double distilled autoclaved water. The cells were vortexed back into solution and centrifuged again. This was repeated two more times to clean the bacteria. After the third cleaning, the supernatant was removed and replaced with 1 mL of 50 mM ammonia bicarbonate (Fisher Scientific, Fair Lawn, NJ).

Autoclaved bacteria (~10⁹ CFU/mL) underwent the same procedure, but autoclaved double distilled water was added instead of ammonia bicarbonate. The bacteria sample was then autoclaved for 90 minutes at 250° F at 15 PSI. 100 μL of this solution was added to 900 μL of 50 mM ammonia bicarbonate.

Quantification of viable cells was conducted following the BAM Aerobic Plate Count method[3].

2.2.1.2 Simple sugar preparation
Simple sugar solutions were prepared by diluting 0.062 g/mL of fructose (Tate and Lyle London, UK) and 0.062 g/mL glucose (Sigma Aldrich, St Louis, MO) in 1 mL of 50 mM ammonia bicarbonate (approximately the concentration on of sugar in a soda).

2.2.2 3-MPBA diol in test tube reactions

For a preliminary understanding of the reaction, 3-MPBA was studied to see how it interacted with various diols. SE 1045 (~10⁸ CFU/mL), LM 21 (~10⁸ CFU/mL), fructose (0.062 g/mL) and glucose (0.062 g/mL) were suspended in 990 µL of 50 mM ammonia bicarbonate solution. 10 µL of 100 mM 3-MPBA (AstaTech Bristol, PA) ammonia bicarbonate solution was added to each analyte. The same concentration of 3-MPBA with just ammonia bicarbonate was also analysed as a control. The solution was vortexed and sat for 1 hour for the reaction to occur. The bacteria test tubes were then centrifuged at 6500 × g for 3 minutes. The supernatant was removed and replaced with 1 mL water for cleaning. The test tubes were vortexed and centrifuged again using the settings stated above. The supernatant was then removed and replaced with 1 mL 50 mM ammonia bicarbonate. Simple sugar samples were not washed, as no supernatant is formed during centrifugation. 50 µL of sample was pipetted in 100 µL of 60 nm citrated coated silver nanoparticles (nanoComposix, San Diego, CA) (AgNPs). Samples were gently mix and 10 µL of the solution was pipetted onto gold slides (Thermo Scientific Madison, WI) that had been cleaned with distilled water and allowed to air dry. Samples were air dried for approximately ten minutes.

2.2.3 Preparation of the 3-MPBA coated gold slide

The bacteria capturer chip protocol was adapted from a protocol by Bi et al.[32]. First, an Au slide (Thermo Scientific Madison, WI) was washed with deionized water and cut into rectangular pieces (approximately 2.5 by 3.5 mm) using a glass knife and cutter. The chips were then washed with ethanol (Pharmco-aaper Chicago, IL) for 30 seconds and placed into 4 mL of 1 mM 3-MPBA-ethanol (AstaTech Bristol, PA) solution in a sterile 5 mL test tube. The test tube was put onto a shaker (speed = 20 RPM) for approximately 17 hours. The next day, chips were
washed with ethanol for 20 seconds and put into a 96-well plate. 150 µL of ethanol was pipetted into each well to stabilize the chip until use[34] (approximately 1 hour).

Figure 1. Diagram of sandwich assay method

2.2.4 Sandwich assay protocol

The assay was conducted following an ELISA-like method (Fig. 1). When all the samples for the assay were prepared, the 96-well plate with chips was taken out. For each well that contained a chip, the ethanol was removed. Each well was then washed three times with 200 µL autoclaved double distilled water. Next, 100 µL of sample was pipetted into its respective well. The sample and chip incubated for 1 hour. After one hour, the supernatant was removed and washed three times with 50 mM ammonia bicarbonate. 150 µL 0.1 mM 3-MPBA-50 mM ammonia bicarbonate (fresh solution) was pipetted into each well. The supernatant was removed and washed three times with 200 µL of 50 mM ammonia bicarbonate. 100 µL of Ag NPs was pipetted into the wells and let to interact for 15 minutes. Then the supernatant was removed and washed three times with 200 µL 50 mM ammonia bicarbonate. Samples air dried for approximately 10 minutes.
2.2.5 Data collection

When chips were completely dry, the well plate was placed under a DXR Raman Spectro-microscope (Thermo Scientific, Madison, WI) for results. The microscope settings were a 20x microscope objective, 780 nm excitation wavelength, and 50 µm slit aperture for 1 s collection time. Laser power was 1 mW for studying the reaction and 4 mW for 3-MPBA sandwich assay. For SERS mapping, a 100 µm × 100 µm area was selected using a step size of 5 µm. SERS spectra and optical images were analyzed using Thermo Scientific OMNIC™ software (version 9.1) and TQ Analyst (version 8.0). Optical images were taken with the DXR Raman Spectro-microscope using the 20x microscope objective lens bright field setting.

To objectively analyse the optical video images, the OMNIC™ software Image Analysis tool was used. For each chemical spectral map that is taken, the software saves the video image as well. This video image is highlighted and then the software will analyze the pixel intensity of the selected area. The software does this by converting the image to greyscale and then creating a histogram of pixel intensity from low to high. The threshold of the lowest and highest pixel intensity is manually changeable. For this study, the lower threshold was always zero. Unfortunately, the threshold for each area map varies greatly (range 386 to 807). This is most likely caused by differences in original optical lighting of the image as well as the number of ‘black dots’ present on the slide. To overcome this, a guideline was set that the threshold was to be the point just before red flecks begin to appear in one corner or side of the optical image.

2.3 Results and discussion

2.3.1 Specificity of 3-MPBA

In order to increase the consistency and robustness of our results, the conditions of the reaction were first optimized. It is widely known that the boronic acid-diol reaction is more favorable at higher pH values[34,35]. Because of this, a 50 mM ammonia bicarbonate solution was used as a basic medium. Through our experiments it was found that ammonia bicarbonate
serves many critical roles in the success of this assay. These include enhancing the $K_{eq}$ of the reaction because of its increased pH (8.4)\cite{35}, protecting 3-MPBA from degrading due to its partial solubility in aqueous mediums\cite{34}, protecting bacteria from lysing due to an increased osmotic pressure effect, and enhancing the Raman signal due to its role as a salt. Ammonia bicarbonate may also cause boronic acid to be positively charged thus increasing binding affinity to negatively charged bacteria\cite{36} because MPBA has a pKa value of approximately 9.2\cite{37}. To ensure that the ammonia bicarbonate-3MPBA was not killing the bacteria we conducted a microbiology experiment comparing viable bacteria cells that were emerged in various iterations of these environmental conditions (Fig. 2). The results reinforce the benefits that were stated previously.

![Figure 2. Salmonella enterica under different environmental conditions.](image)

3-MPBA was first studied in test tube reactions to understand how it reacts with various diol-containing molecules. When 3-MPBA was diluted in ammonia bicarbonate, four sharp and strong characteristic peaks were emitted at 419, 997, 1073, and 1571 cm$^{-1}$ (Fig. 3A). The peak at 419 cm$^{-1}$ is attributed to the sulfur-gold bond\cite{38}, was used in this study as the indicator peak that
3-MPBA is bound to AgNPs. The peaks at 997 and 1073 cm$^{-1}$ are caused by the benzene-thiol ring in 3-MPBA[33]. The peak at 1571 cm$^{-1}$ is considered the marker of acidity for boronic acids[37] When only one peak is present, the boronic acid is in a pH below its pKa, in the trigonal form. When the pH is at the pKa a double peak (1571 and 1607 cm$^{-1}$) appears and the boronic acid is in the tetrahedral form[35]. Ammonia bicarbonate as our basic medium is a good choice for SERS experiments as the chemical gives off no Raman peaks (Fig. 3B).

Both SE 1045 and LM 21 give off similar SERS signals in both the control (Fig. 2B) and when combined with 3-MPBA (Fig. 3A). The reason this occurs is because the ammonia bicarbonate and silver nanoparticles create a non-favourable environment for bacteria to grow. This promotes metabolites of purine degradation to be elucidated in the bacteria SERS spectra[39]. Two characteristic purine peaks are present at 733 cm$^{-1}$ and 659 cm$^{-1}$. To ensure that 3-MPBA could indeed bind to both bacteria species, the bacteria-3-MPBA solution was washed once in the centrifuge. As the 3-MPBA and bacteria spectra is both distinctly present (Fig. 3A), we are confident that 3-MPBA can effectively bind to the bacteria cell wall. Because the spectra of SE 1045 and LM 21 in Figure 3A is very similar, there is evidence that 3-MPBA can bind to many different bacteria, as Gram negative (SE 1045) and positive bacteria (LM 21) have very

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Figure 3. Representative spectra of 3-MPBA in 50 mM ammonia bicarbonate bound to various molecules and controls. Concentration of analytes were: glucose (0.062 g/mL), fructose (0.062 g/mL), *Salmonella enterica* $10^8$ CFU/mL, *Listeria monocytogenes* $10^8$ CFU/mL.

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Figure 3A: Representative spectra of 3-MPBA in 50 mM ammonia bicarbonate bound to various molecules and controls. Concentration of analytes were: glucose (0.062 g/mL), fructose (0.062 g/mL), *Salmonella enterica* $10^8$ CFU/mL, *Listeria monocytogenes* $10^8$ CFU/mL.
Figure 3B: Representative spectra of 3-MPBA in 50 mM ammonia bicarbonate bound to various molecules and controls. Concentration of analytes were: glucose (0.062 g/mL), fructose (0.062 g/mL), *Salmonella enterica* $10^8$ CFU/mL, *Listeria monocytogenes* $10^8$ CFU/mL.
different cell envelope structures[40] in which 3-MPBA is binding to. One noticeable difference between the control 3-MPBA spectra and the bacteria-3-MPBA spectra is a new peak present at 1023 cm$^{-1}$. The exact assignment of this peak is unclear. Other references[16,33] and our bacteria spectra (Fig. 3A) have reported a peak at 1023 cm$^{-1}$. This indicates that the peak is probably inherent to the bound 3-MPBA molecule due to its change of orientation [37].

Simple sugars containing diols were reported to interact with MBPA as well. Therefore, two simple sugars that are commonly found in food were also tested to confirm whether the new peak was specific to bacteria, or merely an indicator that boronic acid was bound to a diol. Not only did the sugars tested not produce a peak at 1023 cm$^{-1}$, but fructose produced a different indicator peak at 880 cm$^{-1}$ (Fig. 3A). While this new peak was not further investigated, other groups have successfully created colorimetric assays that can differentiate between sugars based on differences in the molecular geometry of the diol-boronic acid complex[41][42]. Potentially, the experimental conditions may cause a similar differentiation of sugars to occur. The absence of the peak at 1023 cm$^{-1}$ is promising for future work involving more complex matrices. Fructose also had better interaction with 3-MPBA over glucose in terms of peak intensity. It is unclear whether this has to do with the inherent Raman signal of glucose when bound to 3-MPBA or is an indicator for binding affinity. In previous studies, it has been reported that fructose has a higher affinity towards boronic acids than glucose[35].

The presence of new peaks presents an advantage over other assays. Because there is an indication of what boronic acid has bound to, there is less need for the assay to be perfect. For example, if the signal of 3-MPBA did not change when bound to an analyte and there was some residual 3-MPBA left on the slide during washing, a false positive signal would occur. The specificity of 3-MPBA allows for increased confidence in the results because there is decreased potential that the signal could be coming from a different molecule. Therefore, the 3-MPBA has unique specificity making it an ideal indicator for SERS applications on bacteria.
2.3.2 3-MPBA Sandwich Assay
A sandwich assay was created using a 3-MPBA modified surface for non-specific capture of bacteria. Then 3-MBPA and AgNPs were used as secondary capturer and SERS tags for reporting the captured bacteria. Representative SERS spectra of various diols and bacteria using this sandwich assay are shown in Figure 4. The most noticeable difference between the spectra obtained in the sandwich assay and those from the test tube, is the decrease of spectral intensity. The reason for this is that in Figure 3, excess AgNPs and ammonia bicarbonate which amplify the signal are not washed away. There is also a new wide peak present between 1280 and 1400 cm\(^{-1}\).

In Fig. 5, the spectra of a plain gold slide this peak is also present, meaning it comes from the gold slide. The 3-MPBA coated gold slide with AgNPs dropped on the surface was also tested to ensure that the 1023 cm\(^{-1}\) was not present before bacteria were introduced to interact with 3-MBPA (Fig. 5).

![SERS spectra](image)

Figure 4. Representative spectra of non-specific 3-MPBA sandwich assay with different target molecules and diagram of sandwich assay structure. The concentration of the analytes were: glucose (0.062 g/mL), fructose (0.062 g/mL), *Salmonella enterica* \(10^7\) CFU/mL, *Listeria monocytogenes* \(10^7\) CFU/mL, and autoclaved *Salmonella enterica* \(10^7\) CFU/mL.
For the 3-MPBA sandwich assay (Fig. 4), the viable bacteria results were fairly consistent with the test tube results (Fig. 3). Both SE 1045 and LM 21 appeared to have similar binding affinities towards the slide (Fig. 6). It is also interesting to see that autoclaved bacteria produced negative spectra which demonstrate the assay has potential to discriminate between viable and non-viable bacteria. However, autoclaving a sample represents an extreme way of killing bacteria due to the combination of pressure and heat. Potentially, bacteria killed in less extreme ways such as compaction of the cells during centrifugation[43] may produce the 3-MPBA bacteria peak. This needs to be investigated in future study.

Figure 5. Controls for 3-MPBA sandwich assay.

Figure 6. Raman mapping of representative Gram positive and negative bacteria. Presence/absence criteria was determined by examining all the 1023 cm\(^{-1}\) peak, and selecting a cutoff of 3 times the standard deviation plus mean of the negative control (ammonia bicarbonate).
It is also interesting that the simple sugars produced negative spectra, different from the test tube study where a new peak at 880 cm\(^{-1}\) was clearly shown when fructose had bound to 3-MPBA. The reason for this is the molecular geometry of the diols. Boronic acids only forms bonds with 1,2 and 1,3-cis diol groups. Because there are not two sets of groups to bind to on the fructose molecule, it is not sandwiched. This is consistent with the results found by Bi et al\[32\]. Due to the presence of weak peaks consistent with 3-MPBA, fructose probably did bind to the slide surface, but its signal was unable to be amplified because it was not sandwiched. However, in his research glucose was able to be sandwiched by 4-MBPA due to its ability to form dicyclic boronates. In this assay, the boronic acid group is to the side (3- vs. 4-MPBA) and thus created a molecular positioning that was unfavorable to be sandwiched. Li et al\[37\] also found that high pH values hindered the ability of D-glucose to bind to 4-MPBA due to the esterification of the sugar with boronate. Because of this effect, glucose binds to the layer of MPBA but does not disrupt the Raman spectra produced by the capturing molecule. This may explain the depression effect observed in our 3-MPBA-glucose data (Fig. 3A).

2.3.3 Dual Sensing Mechanism: Optical and Chemical (SERS) Imaging

2.3.3.1 Optical Imaging

![Optical video images](image)

Figure 7. Optical video images (20x bright field objective) of a plain gold slide (left) and Salmonella enterica 1045 10\(^8\) CFU/mL suspended in water and let to dry on a plain gold slide.
A unique feature of our assay is the fact that there is a visual indication that bacteria has bound to the slide. This is significant because bacteria placed under a light microscope are very difficult to see due to their transparency under white light[44]. In Figure 7 there is a picture of what SE 1045 looks like when suspended in water and let to air dry on a gold slide using the same microscope that was used to take the optical images in Figure 9A. To overcome this, microbiologists typically employ staining techniques or use a phase contrast microscope to visualize bacteria cells[44]. As shown in Figure 9A, when high concentrations are bound to the sandwich assay that was described previously, the slide is dark and ‘black dots’ are present. In the negative control and low levels of bacteria concentrations, the slide is simply a grey color. The visual indication of bacteria is a tremendous strength of this assay. When making this assay, the visual indication helped with troubleshooting problems with the assay in terms of if bacteria were present or not. Similarly, we can get an initial idea that bacteria has bound to chip and then use SERS mapping to verify this observation. SE 1045 was used as the model bacterium, but this effect was also observed with regards to Listeria (Fig. 8). Future work will include an investigation into the cause of the black dots and understanding the mechanism that causes them to form.
Figure 8. Optical video images of *Listeria monocytogenes* and *Salmonella enterica* (10^6 CFU/mL, left, and 10^7 CFU/mL, right) under bright field 10x microscope objective.

In order to use the optical imaging for preliminary quantification, pixel intensity analysis was conducted. In Fig. 9B, one can see the areas that the software deemed to be darker than the background of the slide. For additional information on how the analysis was conducted, refer to Section 2.2.5.
Figure 9. (A) Optical video images of a 100 µm × 100 µm area of *Salmonella enterica* on 3-MPBA sandwich chip at log concentrations (a): ammonia bicarbonate negative control (b) 3.5×10⁰ CFU/mL (c) 3.5×10¹ CFU/mL (d) 3.5×10² CFU/mL (e) 3.5×10³ CFU/mL (f) 3.5×10⁴ (g) 3.5×10⁵ CFU/mL (h) 3.5×10⁶ CFU/mL (i) 3.5×10⁷ CFU/mL. (B) Optical analysis of adjacent video images. Refer to labelling stated previously for concentration (C) SERS chemical mapping corresponding to adjacent video images. Refer to labelling stated previously for concentration. (D) Relationship between *Salmonella enterica* concentration and percent of optical image positive for ‘black dots’ (E) Relationship between *Salmonella enterica* concentration and percent of chemical image positive for bacteria.
2.3.3.2 Chemical Imaging

From the Raman mapping, one can get a clear and reliable idea of the density of bacteria within the slide. Raman mapping is critical as it verifies that the spots selected are in fact bacteria, and not environmental contamination. This is determined by examining all the 1023 cm\(^{-1}\) peak, and selecting a cutoff of 3 times the standard deviation plus mean of the negative control (ammonia bicarbonate) to create a presence/absence standard. For potential error, each laser point was reviewed to ensure that all 5 peaks (and no more than 5) were present. If the spectra did not meet this standard, it was rejected as being negative. A 400 data point map was taken at each a series of log bacteria concentrations to understand the density of bacteria on the chip (Fig. 9).

While the Raman mapping (Fig. 9C) clearly demonstrates that the assay can detect bacteria at different concentrations, the actual spectrum itself was found to also give important quantification information. At higher bacteria concentrations (10\(^6\) and 10\(^7\) CFU mL\(^{-1}\)), the intensity of the Raman peaks are also higher (Fig. 10). This could be caused by the laser hitting multiple cells that are clustered together. In terms of quantification, this is an important factor to take into account.
Figure 10. Representative spectra of *Salmonella enterica* at various concentrations from Raman mapping in Fig. 9C

There are limitations to the quantity of bacteria that can be detected. Around a bacteria concentration of $10^8$ CFU/mL both the indicator 3-MPBA as well as the bacteria itself can be repelled from the slide. The large biomass of bacteria may decrease the pH and cause 3-MPBA to be negatively charged and thus repel the negatively charged bacteria. This hypothesis is supported by the Raman spectra produced (Fig. 10). At most bacteria concentrations, a double peak is observed at 1573 cm$^{-1}$ showing that boronic acid is in the basic form with an extra OH$^-$ in the p orbital of boron (Fig. 3 and 4). However, at $10^6$ CFU/mL and $10^7$ CFU/mL the pH is below the pKa value and the p orbital is empty keeping boronic acid in its acidic state. This effect has been explained by two research groups and is referred to as the marker bands for acidity of MPBA[33,37]. For practical applications, if a high bacterial load is suspected, the analyst should merely test multiple dilutions of the same sample as is done in conventional microbiology plating. Even with this stipulation, the bacteria load (expressed in logarithmic base) that our assay can...
detect presents a huge advantage over the conventional method where only 2 logs can be used for detection[3]. This could allow for efficiency and saving of resources.

2.3.4 Select Point Method
In addition to the (Fig. 9C) where a random area is selected and data points are systematically taken, an alternative method, named the select points method, was also developed leveraging the advantage of optical imaging. The goal of using this method was to validate that there was indeed a correlation between black dots and bacteria. In the select point method any black dot approximately 2 µm by 2 µm is selected. As can be seen in Figure 11, the number of positive signals at both the 1023 and 419 cm\textsuperscript{-1} peak were analyzed. The reason for this was to demonstrate the specificity of our assay. When a data point is positive for the 419 cm\textsuperscript{-1} peak but not the 1023 cm\textsuperscript{-1} peak it indicates that environmental interference has been sandwiched and/or captured. While not all black dots give off a bacteria signal, it does indicate that the area will produce 3-MPBA peaks. The added specificity of our assay demonstrates an advantage that our assay has over SERS tag assays that have already been proposed.
Figure 11. Percent of positive signals for bacteria (1023 cm$^{-1}$) and indicator (419 cm$^{-1}$) at different bacteria concentrations.

The limit of detection of our assay is very low with bacteria cells being consistently detected at $10^3$ CFU mL$^{-1}$. There are many reasons why this could be. First of all, our indicator is robust, because 3-MPBA produces such a strong, unique, consistent signal, it is easy to get a signal from just one molecule that it has bound to. While autoclaved bacteria were tested to determine if a signal existed, this represents an extreme heat treatment. We hypothesize that potentially bacteria that have been killed or injured in ways that may make them unviable to grow in culture media, may still be elucidated through our mapping technique. Thus the actual quantity of bacteria that was physically present was higher than our aerobic plate count growth results show. This is supported by a control we did with the growth culture media before centrifugation. The process of centrifugation and washing tended to cause a loss of approximately 1 log of viable bacteria cells (Fig. S8). This could lead to some interesting advantages over the growth culture method. One could detect all the bacteria that were potentially killed during processing in order to validate a kill step. Another reason why the detection limit is low is that it is based on the physical presence of the bacteria. Growth culture media relies on viable cells to grow[3], however
if the growth conditions aren’t correct for that specific bacteria, growth will not occur, and bacteria will not be detected.

Figure 12. Comparison of viable *Salmonella enterica* cells before and after washing.

While both Raman mapping and the select point method can detect bacteria at low levels, the mapping method is more reliable for limit of detection work. As one can see from the select points method, the variation is high. Furthermore, the spectra produced are more likely to contain alternate peaks that are inconsistent with the bacteria spectra that was studied and discussed in earlier figures. This makes sense, because in the select points method one had a higher chance to target at interfering background rather than cells. For detection work, the Raman mapping method is the preferred method for quantification, but the select points method can be useful for preliminary information as data collection takes less time.
2.4 Conclusion

A versatile and sensitive sandwich assay platform with dual sensing mechanisms, optical and chemical imaging, has been developed for rapid detection of bacteria. 3-MPBA and AgNPs were used to probe both highly sensitive SERS tag signal as well as characteristic bacterial signal for robust SERS detection. The visual observation of bacteria cells added one benefit for rapid screening. We can detect as low as 100 CFU mL⁻¹ using the developed assay, and the total analytical time for optical detection is 1.25 hours and for SERS imaging is 3 hours. Future work will include studying the cause of the black dots of the 3-MPBA chip surface, how different methods of inactivating bacteria and damaged cells respond to 3-MPBA and developing and testing the assay in food matrices. In addition, the sandwich assay can be easily modified by changing the capturer layer with a specific antibody or aptamer for selective detection of target bacteria.
CHAPTER 3
INVESTIGATION INTO THE ENHANCED VISUALIZATION EFFECT OF THE NON-SPECIFIC SERS SANDWICH ASSAY

3.1 Introduction
Optical sensing mechanisms have often been incorporated into bacteria detection platforms due to their robustness and ability to detect whole cells. Unfortunately, bacteria are not easy to see under the common light microscope due to their translucent optical properties[44]. Despite this hurdle, many research groups have developed methods to optically detect bacteria [45–48]. In Chapter 2, we described and demonstrated the potential to detect and quantify a visual effect that is correlated to bacteria concentration. Due to the many advantages that optical detection possesses, an investigation into the cause and understanding of the mechanism was conducted.

3.1.1 Bacteria microscopy
Ever since the discovery of bacteria under a microscope by Anthony van Leeuwenhock in the seventeenth century, microscopes have played a critical role in our understanding of the domain of bacteria [49]. Today, the most commonly used microscope in a microbiology lab is the compound light microscope. This microscope has a resolution power of 0.2 µm. To increase the resolving power of the microscope, an oil immersion lens is often used due to oil’s higher refractive index compared to water (1.52 vs. 1.33). The light microscope has four major subdivisions: bright field, dark field, fluorescence and phase contrast, two of which will be discussed further. Bright field is the most common use of the light microscope. It uses white light to illuminate objects. Because bacteria are translucent, they absorb too much light and on their own, are very difficult to under this instrument. For this reason, microbiologists have developed an array of staining techniques like methylene blue, the Gram stain and Congo red in order to clearly visualize cells and study their morphology. In 1932, Fritz Zernicke developed the first phase-contrast microscope allowing for the visualization of bacteria cells in their natural state without
the aid of a stain. This microscope works by amplifying small differences in the refractive indices and converting them to differences in light intensity[44]. While more powerful microscopes utilizing the power of electrons have been developed like SEM, these are highly expensive instruments that are unavailable to the general microbiologist. While there are clearly many good techniques for visualization of bacteria, we believe that the optical properties of the 3-MPBA coated chip that we developed could be an important advancement for on-site detection of bacteria. Some of the advantageous properties include visualization of bacteria at low objectives (10x) (Fig. 8), the use of a simple and widely available light microscope, and the ability to easily quantify the bacteria concentration with the aid of minimal chemicals.

3.1.2 Objectives of this study
The objectives of this study were to (1) identify what the optical effect was actually measuring (2) determine the principle components that cause the optical effect to occur (3) and to characterize and explain why this effect occurs.

3.2 Materials and methods
3.2.1 Sample Preparation
Refer to Section 2.2.1.1 for bacteria preparation.

3.2.2 Bacteria chip protocol
Refer to Section 2.2.3 for preparation of the 3-MPBA chip and Section 2.2.4 for the sandwich assay protocol. Refer to Section 2.2.5 for Raman microscope settings.

3.2.3 SEM sample preparation
A chemical drying procedure was used to prepare the 3-MPBA sandwich assay chips for the scanning electron microscope (SEM). First a 0.5 M HEPES solution was prepared (?). This solution was refrigerated at 4° C until use. The HEPES buffer was used as a diluent for a 2.5% glutaraldehyde solutions (1:10), also kept at 4° C. 150 µL of this solution was pipetted into each well plate that had a chip in it. The well plate with solution was placed in the refrigerator 4° C for 16 hours. Then, the solution was removed and washed 3 times with 200 µL autoclaved distilled
water. Next, 200 µL of a 25% ethanol solution was added to each well plate and let to interact for 5 min at room temperature. The solution was then removed and a higher concentration of ethanol was added. This was repeated with 50%, 75%, 90% and 100% (2x) ethanol solutions. Finally, a few drops of 100% acetone was dropped on each chip and air dried for 1 hour. Then samples were placed in a desiccator until use. Images were taken using a FEI Magellan 400 (FEI, OR) with an accelerating voltage of 10 kV under low vacuum conditions.

3.2.4 Visual spectrophotometer sample preparation
Bacteria were cleaned according to the protocol in Section 2.2.1.1. Then, 990 µL autoclaved double distilled water, 50 mM ammonia bicarbonate or 0.1 mM sodium hydroxide (Fisher Scientific, Fair Lawn, NJ) was added to each bacteria pellet. The bacteria and solution was vortexed until mixed. Then 10 µL of 100 mM 3-MPBA was added to each solution and vortexed. For controls, 1 mL of the above diluents were added to the bacteria pellet. The solutions were allowed to sit for an hour to allow the 3-MPBA to bind to the peptidoglycan on the bacterial cell envelope. After the hour, all the test tubes were centrifuged at 6,500 g for 3 minutes to concentrate the bacteria in a pellet. The supernatant was removed and 1 mL of autoclaved distilled water was added. The solution was vortexed into solution and centrifuged down to a pellet using the same settings stated previously. The supernatant was removed and replaced with 1 mL of autoclaved double distilled water and the bacteria pellet was vortexed until into solution. Next, 200 µL of each sample was pipetted into a well in a sterile 96 well plate. The well plate was placed in the UV-Vis spectrophotometer. Results were read at 600 nm on a SpectraMax M2 (Molecular Devices, Sunnyvale, CA).

3.2.5 ATR-IR sample preparation
Bacteria samples were cleaned using the protocol in Section 2.2.1.1. Then, 990 µL autoclaved double distilled water or 50 mM ammonia bicarbonate was added to each bacteria pellet. The bacteria and solution was vortexed into solution. Then 10 µL of 100 mM 3-MPBA was added to
each solution and vortexed. For controls, 990 μL autoclaved double distilled water or 50 mM ammonia bicarbonate was mixed with 10 μL of 100 mM 3-MPBA. All solutions were let to sit for 1 hour. Solutions were then re-vortexed, and 5 μL of the each solution was pipetted onto a clean gold slide. The droplet was air dried on the slide for approximately 15 minutes until the slide was visibly dry. The gold slides were then pressed against a zinc-selenide crystal using a high pressure clamp. Measurements were taken on an IRTracer-100 (Shimadzu, Columbia, MD) equipped with a Pike MIRacle ATR accessory.

3.3 Results and discussion

3.3.1 3-MPBA sandwich assay video image effect
When the sandwich assay was created, a unique visual effect, described previously in Section 2.3.3.1 was observed. The visual effect is very consistent, covering the entire slide. From general observation, it correlates with the number of bacteria introduced to the slide (Fig. 12). In addition, in Fig. 9D, it was demonstrated that when the visual effect is analyzed by pixel intensity, a standard curve can be generated. While this effect is highly advantageous and obvious at higher concentrations, below $10^5$ CFU/mL the visual effect should only be used as a preliminary screening tool to identify areas that could contain bacteria.

While the optical appearance seems to be correlated with bacteria concentration, there are many other things it could be associated with. The ammonia bicarbonate crystals or 3-MPBA could somehow be correlated with bacteria concentration. The AgNPs may aggregate in a way that black dots appear. Both of these scenarios are possible thus a microscope with higher magnification is needed to definitively understand what is occurring.
Figure 13. Representative microscope images of the following bacteria concentrations using a 20x bright field objective lens. (A) Ammonia bicarbonate negative control (B) $10^1$ CFU/mL (C) $10^2$ CFU/mL (D) $10^3$ CFU/mL (E) $10^4$ CFU/mL (F) $10^5$ CFU/mL (G) $10^6$ CFU/mL (H) $10^7$ CFU/mL (I) $10^8$ CFU/mL (J) $10^9$ CFU/mL.

Due to the unique advantages this assay presented, it is of interest to fully understand how and why this effect occurs. With a complete understanding of the effect, we will have more confidence in what we are measuring and observing, and may be able to leverage this effect for other applications.
3.3.2 SEM imaging to understand the cause of optical effect

In order to further understand what is exactly being measured, Scanning Electron Microscope images were taken. In Figure 13, there is a comparison of what SE 1045 $10^8$ CFU/mL on the 3-MPBA sandwich assay looks like under the Raman microscope (A) and under SEM (B). From this figure, it is clear that the black dots are primarily bacteria and not one of the chemicals that have been added in the sandwich assay. This bodes the question though that potentially one of the chemicals is coating the bacteria in a way that would optically enhance it.

![Image of SEM and Raman microscope images](image)

Figure 14. Comparison of \textit{Salmonella enterica} $10^8$ CFU/mL on 3-MPBA sandwich assay under (A) Raman microscope 20x bright field objective and (B) SEM.

Images of SE 1045 close up were taken to understand if there was something on the bacteria cell wall that would optically enhance it. In Figure 14A, one can see what SE 1045 looks like when suspended in water and air dried on a plain gold slide. The surface of the cell wall appears smooth. In counterattraction, in Figure 14B, one can see the appearance of SE 1045 in the 3-MPBA sandwich assay. The cell wall surface appears rough, with a coating of some material on top of it. Unfortunately, from this picture, it is unclear what exactly this coating is composed of. Due to the irregular shape of the coating, it is clear that AgNPs are not the primary source of the coating, yet they still could be an important contributor to the overall effect. For this reason, a series of experiments were conducted to deconstruct the 3-MPBA and discover the ultimate cause of the enhanced visualization of bacteria.
Figure 15. Comparison of *Salmonella enterica* under different conditions. (A) *Salmonella enterica* suspended in water and air dried on a gold slide. (B) *Salmonella enterica* in 3-MPBA sandwich assay

### 3.3.3 Deconstructing the assay to understand key components of the enhanced visualization effect

To first determine how the 3-MPBA sandwich assay enhances the visualization of bacteria, a component analysis was conducted to see how each chemical that is added to SE 1045 effects its appearance under the Raman microscope. All samples were dropped onto a gold slide and air dried. In Figure 16A one can see that SE 1045 is translucent and is only apparent due to the high concentration of bacteria pipetted onto the slide. Ammonia bicarbonate (Fig. 16B) appears to maintain a large crystal structure. However, when it interacts with SE 1045 (Fig.16E), the crystals appear to be smaller in size. While the ammonia bicarbonate appears to make the SE 1045 more black in appearance, it is most likely caused by the smaller crystal size than any change in the bacteria itself. 3-MPBA suspended in water (Fig. 16C), appears clear and is not easily seen under these microscope settings. However, when it interacts with SE 1045 (Fig. 16F), black dots are present. The black dots have a unique spatial distribution that is different from the other controls. Furthermore, the residual watery appearance seen in the bacteria control (Fig. 16A), is not present. This may mean that 3-MPBA may repel water from the bacteria surface. Finally, in Figure 16D, one can see the appearance of AgNPs, with small dark spots in the control. When the AgNPs interact with SE 1045, the black dot appearance is enhanced. Like the ammonia bicarbonate, the physical appearance seems to be modified by the high number of
bacteria cells present, yet does not change like the 3-MPBA did when it interacted with bacteria. Therefore, this experiment demonstrated that 3-MPBA may be the primary cause of the enhanced visualization, yet it is unclear how the other components in the assay may also be enhancing the effect.

Figure 16. Visual component analysis of 3-MPBA sandwich assay with SE 1045 $10^5$ CFU/mL (A) SE 1045 suspended in water. (B) 50 mM ammonia bicarbonate (C) 1 mM 3-MPBA suspended in water. (D) AgNPs (E) SE 1045 suspended in 50 mM ammonia bicarbonate (F) SE 1045 suspended in water and 1 mM 3-MPBA (G) SE 1045 suspended in water and AgNPs. All images were taken using the Raman microscope 20x objective bright field setting.

To further confirm the hypothesis that 3-MPBA was primarily responsible for the enhanced visualization of bacteria, optical images were taken at each stage of the 3-MPBA sandwich assay protocol. In Figure 17, one can see that the gold slide on its own (A), and when coated with 3-MPBA (B), the slides appear a plain grey color. However, in Figure 17C, when SE 1045 binds to the slide, the enhanced visualization effect clearly occurs. While more 3-MPBA (Fig. 17D) and AgNPs (Fig. 17E) are added later in the assay, it does not seem to effect or change the enhanced visualization effect.
Figure 17. Optical images of 3-MPBA sandwich assay at each step when exposed to *Salmonella enterica* $10^8$ CFU/mL (A) Plain gold slide (B) 3-MPBA coated gold slide (C) SE 1045 bound to 3-MPBA gold slide (D) 3-MPBA coated SE 1045 bound to 3-MPBA gold slide (E) Complete 3-MPBA sandwich assay (F) Comparison of bacteria coverage of chips using pixel intensity analysis at the steps listed in A-E. ANOVA analysis found a significant difference ($a=0.01$) when SE 1045 was bound to the chip.

The fact that the 3-MPBA coated chip can induce an enhanced visualization of bacteria when the bacteria is suspended in ammonia bicarbonate is highly advantageous and may become useful in other applications. For this assay, the fact that bacteria are optically apparent after just 1 hour of interaction with the chip means that the optical sensing mechanism can be analyzed after just 1 hour, for very rapid detection of bacteria, followed by chemical verification with the
Raman mapping after an additional 2 hours. Being able to see the bacteria represents an advantage of SERS over other rapid detection techniques like PCR or ATP testing where this is not possible. While this assay utilized the enhanced visualization effect to increase the probability of hitting bacteria with a laser, this discovery is not limited to SERS work. Currently, microbiologists use staining techniques in order to visualize bacteria. This protocol often involves washing steps, that actually wash away the very bacteria that the microbiologist is trying to visualize. Because the chip is coated with 3-MPBA, the bacteria stays on the chip after being washed with ammonia bicarbonate allowing quantification of the bacteria sample to be possible.

From Figure 17, it is clear that 3-MPBA and ammonia bicarbonate are the two components responsible for the enhanced visualization, but it is unclear which, or both of these components is the root cause. Therefore, an experiment was conducted utilizing the 3-MPBA coated chip to understand how critical ammonia bicarbonate is to the enhanced visualization of bacteria. In Figure 18A, SE 1045 was suspended in autoclaved double distilled water and let to interact with the 3-MPBA chip and then washed in water. It is clear from the image, that the 3-MPBA coated chip on its own can produce the ‘black dot’ effect. This is highly surprising, because only one side of the bacteria is interacting with the 3-MPBA and that is not the surface one can see from the microscope objective lenses. Because of this surprising finding, an investigation of the optical properties of the 3-MPBA coating was conducted and is detailed in Section 3.3.4.
Figure 18. *Salmonella enterica* $10^8$ CFU/mL suspended in different diluents and allowed to interact with 3-MPBA coated gold slide for 1 hour. (A) SE 1045 suspended in water (B) SE 1045 suspended in 0.1 mM sodium hydroxide (pH 8.4) (C) SE 1045 suspended in 50 mM ammonia bicarbonate (pH 8.4). (D) Percent of chip area positive for optical imaging using pixel intensity analysis conducted in triplicate. ANOVA analysis was conducted and a significant ($a=0.01$) difference was found between the ammonia bicarbonate treatment and both the water and sodium hydroxide treatments.

It is well known that in basic media, the boronic acid-diol reaction becomes more favorable. To understand the secondary effect that ammonia bicarbonate may be contributing, SE 1045 was suspended in both sodium hydroxide and ammonia bicarbonate with the concentration in both being adjusted so that the pH was 8.4. Because sodium hydroxide is a much more powerful, only a small amount is needed to achieve the target pH. Thus we can understand what role ammonia bicarbonate as a salt plays in the assay. When comparing, Figure 18A and 18B it is clear that there are more black dots in Fig. 18B. This is expected as the reaction is more favorable, and thus more bacteria stayed on the slide. There also appears to be a noticeable difference between Figure 18B and 18C when the pH of the dilutent was controlled. Thus ammonia bicarbonate plays a critical secondary enhancement of the chip. Based on the results in
Figure 14B, it did not appear that much excess ammonia bicarbonate crystals were sticking to the slide. This implies that ammonia bicarbonate somehow enhances bacteria’s attraction to the chip.

There is a large body of research that details ammonia-borane, a simple yet versatile complex that has many interesting chemical functionalities [50,51]. One of these properties is its ability to act as a Lewis base and acid complex. Furthermore, this complex can form metallic amidoboranes when a metallic cations (such as silver nanoparticles) replace a protic hydrogen [51]. Raman spectroscopy of isotopic derivatives of ammonia-borane reveal a medium intensity peak present between 708 and 785 cm$^{-1}$, depending on the isotope, coming from the nitrogen-boron bond [52]. However, when the SERS peaks of the 3-MPBA reaction with and without bacteria in the two different solutes, this peak is not present (Fig, 19). This is somewhat not surprising, because in order to generate the boron-nitrogen bond, a solvent such as ether or tetrahydrofuran is needed [50,51]. That being said, some sort of attraction may be occurring that creates the enhancement observed in Figure 18 which could be interesting to study by someone with a stronger chemistry background.

![Graph](attachment:image.png)
Figure 19. Comparison of 50 mM ammonia bicarbonate and sodium hydroxide solutes on 1 mM 3-MPBA with and without *Salmonella enterica* 10⁷ CFU/mL studied using the test tube method (Section 2.2.2).

**3.3.4 Characterization of the enhanced visualization of bacteria effect using optical spectroscopy**

In order to understand why the optical effect occurs, optical spectroscopy experiments were conducted to understand the absorption and reflectance properties of SE 1045 before and after it had bound to 3-MPBA.

Due to the black appearance of the bacteria under the microscope, we hypothesized that one of two things was occurring. When objects appear optically opaque, it is because either all light is reflected or absorbed [53]. To first understand, why the bacteria appear more optically opaque after binding to the 3-MPBA coated gold chip, a test tube a visual light spectrophotometer experiment was conducted to determine which of the two behaviors was occurring. For reference, bacteria is optically translucent, meaning that some light is reflected, some is absorbed and some goes through it [53]. From Figure 20, it was found that the absorbance of light at 600 nm is significantly decreased (a=0.01) when SE 1045 was allowed to bind to 3-MPBA in the presence of 50 mM ammonia bicarbonate. This means that the enhanced visualization effect is caused by light being reflected from the bacteria.
Figure 20. Absorbance at 600 nm of SE 1045 $10^{9}$ CFU/mL under different treatments and re-suspended in water. The 3-MPBA and ammonia bicarbonate treatment significantly ($a=0.01$) affected the absorbance of the bacteria.

Based on the finding that light is being reflected, we decided to investigate how light behaved in the mid-infrared range using the attenuated total reflectance (ATR) technique. This technique utilizes a prism that is in direct contact to a surface to generate evanescent waves to probe the surface of the sample [54]. The evanescent wave will probe the surface to a depth that is determined by the IR wavelength and refractive indices of both the prism and sample [55]. This is obvious in the equation,

$$d_p = \frac{\lambda}{2\pi n_{IRE} \sin^2 \theta - \left(\frac{n_{smp}}{n_{IRE}}\right)^2},$$

where $d_p$ is the depth of penetration, $\lambda$ is the wavelength, $n_{IRE}$ is the refractive index of the prism, $n_{smp}$ is the refractive index of the sample and $\theta$ is the critical angle[55]. We hypothesized that because the bacteria reflected more light under the 3-MPBA-ammonia bicarbonate treatment, there would be more peaks present in ATR spectra in the mid-infrared region due to a change in the refractive index.
Figure 21. ATR-IR spectroscopy results of SE 1045 $10^9$ CFU/mL exposed to different treatments and air dried on a gold slide.

From Figure 21, we can see that the ATR-IR spectra does indeed change when SE 1045 is exposed to different treatments. While the difference between SE 1045 in water and SE 1045 in 3-MPBA and ammonia bicarbonate seems to be drastic (Fig. 22), the intermediates may give insight into what is causing this effect. Surprisingly, the spectra of SE 1045 in water and SE 1045 in water and 3-MPBA (Fig. 21) appears to be the same. This may be caused by a combination of the reaction being less favorable at a pH of 7 and a less than ideal critical angle which would cause the spectra to be less detailed. Another interesting finding is the spectrum of SE 1045 immersed in ammonia bicarbonate and SE 1045 immersed in ammonia bicarbonate and 3-MPBA is very similar. This observation was further analysed in Figure 23.
Figure 22. Subtraction result of ATR-IR spectra *Salmonella enterica* $10^9$ CFU/mL treated with 1 mM 3-MPBA and 50 mM ammonia bicarbonate minus the spectra of *Salmonella enterica* $10^9$ CFU/mL treated with water. Figure 22 confirms that a change in refractive index has occurred due to the appearance of new peaks and a shift in peaks which is indicated by the S-shape (sharp decrease then increase) in the subtraction result. A more concrete explanation of the change in refractive index is described in the next section.

Figure 23. Subtraction result of ATR-IR spectra SE 1045 $10^9$ CFU/mL treated with 1 mM 3-MPBA and 50 mM ammonia bicarbonate minus the spectra of SE 1045 $10^9$ CFU/mL treated with just 50 mM ammonia bicarbonate.
While the spectra of SE 1045 immersed in ammonia bicarbonate with and without 3-MPBA are very similar, the subtraction results to demonstrate the presence of one new shift (Fig. 23). This spectrum demonstrates the important role ammonia bicarbonate plays in the enhanced visualization of bacteria. The coating of ammonia bicarbonate may make the surface of SE 1045 more optically smooth causing increased specular reflection. Initially, it had been hypothesized that 3-MPBA, which causes oxidation of the peptidoglycan[41] layer may increase the amount of free electrons on the surface of the bacteria cell wall. The refractive index of a material is directly related to the dielectric constant using Maxwell’s equation, \( n = \sqrt{\varepsilon_r} \) [56]. Therefore, it was of interest to understand if the electrons were at a higher energy state causing the presence of new peaks. A series of temperature experiments were conducted in the hopes of inducing ‘hot peaks’, but no new peaks appeared at elevated temperature [57].

3.3.4.1 Determining the Refractive Index of SE 1045 before and after the enhanced visualization effect

Due to the fact that the refractive index is so critical for increasing the resolution powers of microscopes, an investigation into determining the refractive index before and after treatment with 3-MPBA was conducted. The refractive index of a medium is commonly defined as the ratio of the velocity of light in a vacuum to its velocity in a specified medium. For the optics of solid materials, the refractive index is defined mathematically as: \( n(\lambda) = n_r(\lambda) + ik(\lambda) \) where \( n(\lambda) \) is the real refractive index and \( k(\lambda) \) is the imaginary refractive index [58]. The real refractive index is the ratio of the velocity of light in a vacuum to its velocity in a specified medium. The imaginary refractive index details the absorption behavior of the material and accounts for energy dissipation [59]. For transparent materials like glass, \( k(\lambda) \) is negligible and the refractive index can be assumed to equal \( n(\lambda)[56] \). However, for opaque materials both the real refractive index and its correction factor must be taken into account.
The imaginary refractive index \(k(\lambda)\) can be determined using the ATR data that was collected in Figure 21. The equation to transform the ATR data into the imaginary refractive index is given by the formula \(k(\lambda) = \frac{\alpha \lambda}{4\pi}\) where \(\alpha\) is absorbance and \(\lambda\) is wavelength [58,59]. The results of \(k(\lambda)\) analysis are summarized in Figure 24. Overall, the data supports the findings of the UV-Vis experiment in Figure 20. At many wavelengths, the imaginary refractive index is higher when SE 1045 is immersed in water than when it is treated with ammonia bicarbonate and 3MPBA meaning that the untreated bacteria is absorbing more infrared light than the treated bacteria. However, as discussed in Fig. 21-23, more peaks exist in the treated bacteria as \(k(\lambda)\) is a function of absorption.

![Graph showing imaginary refractive index of SE 1045 in ammonium bicarbonate and 3MPBA compared to SE 1045 in water](image)

**Figure 24.** The imaginary refractive index of *Salmonella enteria* \(10^9\) CFU/mL immersed in water and ammonium bicarbonate & 3-MPBA as a function of wavelength.

The real refractive index, \(n(\lambda)\), is difficult to determine for organic molecules. Only one research group has experimentally found the refractive index of bacteria [60]. In this study, they utilized the Kramers-Kronig equation and a critical angle optical spectroscopy to determine \(n(\lambda)\). Some research groups have proposed using the Sellmeier equation which is commonly employed
for determining the refractive index of glass[61]. An Abbe refractometer must be used to determine the coefficients and a series of linear equations can solve for the constants A, B, C etc. (depending on the complexity of the chosen equation).

Other research groups have found that $n(\lambda)$ is primarily determined by the water content in biological samples. It has been suggested by Jacques [59] that for biological samples $n(\lambda)$ can be measured using the merely the water content of the sample in the equation:

$$n(\lambda) = n_{dry} - (n_{dry} - n_{water})W$$

where $n_{dry}(\lambda)$ is 1.50, $n_{water}(\lambda)$ is 1.33 and W is the water content of the sample. This is actually a very interesting fact in light of the microscopy observation that was made in Figure 16. In that image, it appeared that 3-MPBA on its own seemed to repel water away from bacteria. 3-MPBA on its own is only partially soluble in water [34], so the chemical behavior of the molecule supports this observation. Potentially, part of the enhanced visualization effect occurs due to a dehydration of the bacterial cell causing an increase in refractive index.

3.4 Conclusion

An investigation into the optical properties of the enhanced visualization of bacteria effect observed in the 3-MPBA sandwich assay was conducted. It was discovered that the black dots that were observed and measured in Chapter 2 are bacteria. From SEM observation it is clear that there is a coating surrounding the bacteria, although it is unclear what the coating is composed of. The effect occurs after just 1 hour of allowing the bacteria to bind to the 3-MPBA chip surface allowing for even more rapid preliminary detection and quantification. While the enhanced visualization is primarily caused by the 3-MPBA coated gold chip, ammonia bicarbonate plays an essential secondary role. The optical properties of the visually enhanced bacteria were probed and it was found that these bacteria absorb less light in both the infrared and UV-visible light regions than untreated bacteria.
CHAPTER 4
DEVELOPMENT OF SERS SANDWICH ASSAY PLATFORM FOR SPECIFIC DETECTION OF BACTERIA

4.1 Introduction
To make the assay platform feasible for industrial application, detection of specific bacteria also must be developed. The design of the 3-MPBA sandwich assay described in Chapter 2, can be easily changed for the detection of specific bacteria by changing the capturer layer from 3-MPBA to an aptamer. For this study, a SE 1045 aptamer will be used for the detection of Salmonella, but using an aptamer specific to another bacterium could easily be used for detection of other desired bacteria.

4.1.1 Objectives of this study
The objectives of this study were to (1) develop a proof of concept that the general sandwich assay method described in Chapter 2 could be modified for the specific detection of bacteria.

4.2 Materials and Methods
4.2.1 Preparation of bacteria
Refer to Section 2.2.1.

4.2.2 Preparation of aptamer-coated gold slide
A SE 1045 specific chip was fabricated using an aptamer coated slide using a method adapted from Pang et. al[25]. First, a SE 1045 aptamer was diluted in 1x TE buffer (stock solution = 400 µM) (Eurofins, Huntsville, Al). This solution was vortexed and then stored in the freezer until just before use. The stock solution was placed on the bench top to defrost. Then, 20 µL of aptamer stock solution was heated for 3 minutes at 96°C and then cooled down to 4°C for 1 minute and then placed in an ice bath for an additional minute. Then, 60 µL of TCEP (Thermo Scientific, Madison, WI) was added to break the disulfide bonds and the solution was vortexed (stock solution = 100 µM). Next, 40 µL of the aptamer solution was added to 3960 µL of 50 mM ammonia bicarbonate. The solution was vortexed and gold slides fabricated in the method stated
earlier were placed in the solution overnight (~16 hours) on a shaker (speed = 20 RPM). Slides were washed for approximately 20 seconds with double distilled water and placed in a 96-well plate (Fisher Scientific, Fair Lawn, NJ). 150 µL of 50 mM ammonia bicarbonate was pipetted into each well-plate until use.

4.2.3 Sandwich assay protocol
Refer to Section 2.2.3-2.2.5 with the following amendment. The laser power of the Raman was changed from laser power 4 to laser power 10.

4.3 Results and Discussion

![Figure 25](image)

Figure 25. Representative spectra of the *Salmonella enterica* aptamer-3-MPBA sandwich assay and diagram of sandwich structure. The concentration of analytes were: *Listeria monocytogenes* $10^6$ CFU/mL *Salmonella enterica* $10^6$ CFU/mL. Refer to Figure A4 for controls.

When the bottom layer is switched to an aptamer, the assay can be used for detection of specific bacteria. For this study, a SE 1045 specific aptamer was used. We found that when SE 1045 was introduced to the slide, a positive signal was produced (Fig. 25). However, when a different species of bacteria was introduced, such as LM 21, no bacteria signal was generated. An important distinction to note between the 3-MPBA sandwich assay and the aptamer assay is that when something is bound to the capturer layer, the capturing molecule comes through in the final
spectra. The reason this is less apparent in the 3-MPBA sandwich assay is that the capturing and indicating molecules are the same, so the signal may be amplified by the bottom molecule, but the signal does not change. This effect can be useful as an additional control in the aptamer assay. If a non-specific bacterium is not washed away during the assay, and 3-MPBA binds to it, a signal from 3-MPBA will be produced, but the aptamer signature does not come through, and the spectra can be dismissed as a false positive (Fig. A5).

4.4 Conclusion

Through this preliminary data, it is clear that the 3-MPBA sandwich assay platform can easily be modified to accommodate the specific detection of bacteria utilizing an aptamer coated gold chip. Further optimization and limit of detection work will be conducted by Alexander Mills.
CHAPTER 5
USING THE NON-SPECIFIC SANDWICH ASSAY FOR DETECTION IN FOOD MATRICES

5.1 Introduction
In Chapter 2, I demonstrated the sensitivity of the 3-MPBA sandwich assay in a simple matrix. For industrial application though, it is important to understand if the assay has the capability of detecting bacteria in real food matrices.

5.1.1 Objectives of this study
The objectives of this study were (1) generate a standard curves for the detection of unknown quantities of bacteria (2) determine the capture efficiency (3) determine the assay’s ability to detect bacteria that were not cultured in a laboratory setting (4) develop sample preparation strategies for a variety of food commodities for detection of bacteria using the 3-MPBA sandwich assay.

5.2 Materials and Methods

5.2.1 Bacteria preparation
Refer to Section 2.2.1.1. SE 1045 incubation was modified by reducing the incubation time from 16 hours to 6 hours. Refer to Section 5.3.1 for justification.

5.2.2 3-MPBA sandwich assay protocol
Refer to Sections 2.2.3 – 2.2.5. In Figure 10, it was discussed that the indicator solution sometimes did not bind to the bacteria at high bacteria concentrations due to a decrease in pH. Therefore, for the 10^8 CFU/mL concentration, 23 µL of sodium hydroxide was used to increase consistency and reproducibility for this concentration. Adding this amount of sodium hydroxide to the solution did not affect the number of positive points at lower concentrations. In fact, it made positive signals lower, because the pH was too high for the assay to work properly. For additional information refer to Figure B5.
5.2.3 Optical density detection of bacteria

9 mL of sterile TSB was inoculated with 100 µL of SE 1045 stock solution. The solution was vortexed and 1 mL of the solution was placed in a plastic cuvette to take the optical density reading at 600 nm using a Shimadzu Biotech Biospec mini spectrophotometer. After the reading, the solution was placed back into the test tube, and the test tube was placed in the 37°C incubator on a shaker (speed = 120 rpm) for 1 hour. The test tube was then removed from the incubator and another optical density reading was taken. This process was repeated for 17 hours to achieve an optical density growth curve for SE 1045.

5.2.4 Pond water preparation

On the day of the experiment, approximately 15 mL of pond water was collected from the Campus pond at the University of Massachusetts Amherst in a sterile test tube from near the surface of the pond. The water was vortexed and approximately 5 mL of water was removed to achieve a volume of exactly 10 mL. Ammonia bicarbonate powder was added to the sample making the concentration of ammonia bicarbonate in the solution 50 mM. The sample was then vortexed and 100 µL of the sample was added to the 3-MPBA sandwich assay and the protocol described in Sections 2.2.3 -2.2.5 was followed with the following amendments. 18 µL of 0.02 M sodium hydroxide was added to the 3-MPBA indicator solution, making the solution additions: 972 µL 50 mM ammonia bicarbonate, 18 µL of 0.02 M sodium hydroxide and 10 µL 1 mM 3-MPBA. 3 100-point maps were taken to of each chip to evaluate the bacteria concentration on the chip.

5.2.5 Spinach leaf preparation

1 gram of spinach (2 leaves) (Whole Foods, organic baby spinach leaves pre-washed) were taken from the container and placed in a petri dish. For the sanitized spinach treatment, the EPA’s recommended protocol for fruit and vegetable washing was followed. In this method, the spinach was placed into 1 L of distilled water for 2 minutes keeping the leaves always immersed
in the basin using a scoopula. The spinach leaves were removed and placed in a second 1 L basin containing a freshly prepared 25 ppm chlorine solution (Clorox, Oakland, CA). Spinach were kept immersed within the basin for 2 minutes using a scoopula again. Sanitized leaves were then placed back into the petri dish and dried using an air blower until all water droplets were removed from surface of the spinach leaves. Both leaves then air dried in a hood for an additional hour.

After this time, the leaves were placed into sterile Whirl-Pak bags with 10 mL 50 mM ammonia bicarbonate and stomached for 5 min using an interscience BagMixer (Woburn, MA). Then 100 µL of the supernatant was removed and placed on the 3-MPBA sandwich assay chip for analysis using the protocol described in Section 2.2.3 with the following amendments. 18 µL of 0.02 M sodium hydroxide was added to the 3-MPBA indicator solution, making the solution additions: 972 µL 50 mM ammonia bicarbonate, 18 µL of 0.02 M sodium hydroxide and 10 µL 10 mM 3-MPBA. 3 100-point maps were taken of each chip to evaluate the bacteria concentration on the chip.

5.2.6 Detection of chemically inactivated bacteria

SE 1045 were washed according to the protocol in Section 2.2.1.1 using a 6-hour incubation time. Instead of suspending the bacteria in ammonia bicarbonate, the bacteria were suspended in 1 mL 25 ppm chlorine solution or 1 mL of 80 ppm peroxyacetic acid (Tsunami 100, Ecolab, St. Paul, MN) for 2 or 5 minutes. Then this solution was centrifuged for 3 min at 6500 g to form a pellet. The supernatant was then removed and 1 mL of autoclaved distilled water was added to the test tube and vortexed into solution. The solution was again centrifuged and the supernatant was removed. 1 mL of 50 mM ammonia bicarbonate was added to the test tube and the solution was diluted to make the final bacteria concentration 10⁸ CFU/mL.

5.2.7 Juice sample preparation

1 mL of 100% apple juice (Minute Maid, Atlanta, GA) was first pH adjusted using 100 µL 500 mM ammonia bicarbonate and 31 µL 2 M sodium hydroxide to achieve the same pH of
the solution used in Chapter 2. SE 1045 was then cleaned using the protocol described in Sections 2.2.1.1 and 5.2.1. The cleaned bacteria were suspended in the pH adjusted juice matrix and diluted to the desired bacteria concentration using more pH adjusted juice. Then 5 µL of 10 mM 3-MPBA was added to each bacteria juice sample and vortexed. After 5 min, samples were vortexed again and 100 µL of the solution was placed on a 3-MPBA coated chip and the protocol described in Sections 2.2.3 - 2.2.5 was followed with the following amendments. 8 µL of 0.02 M sodium hydroxide was added to the 3-MPBA indicator solution, making the solution additions: 982 µL 50 mM ammonia bicarbonate, 8 µL of 0.02 M sodium hydroxide and 10 µL 10 mM 3-MPBA. 3 100-point maps were taken to of each chip to evaluate the bacteria concentration on the chip.

5.3 Results and Discussion

5.3.1 Calibrating the microbiology culture method and 3-MPBA sandwich assay

Bacteria growth kinetics are broken into four basic life cycles: the lag phase, logarithmic phase, stationary phase and death phase. Long-term stationary phase is the life cycle that the majority of bacteria isolated in the environment are in [62]. Therefore, in this thesis, all bacteria tested (with the potential exclusion of environmental samples) were in the stationary phase. The stationary phase is induced in a bacteria population when the population exhausts an essential nutrient [63]. Many bacteria growth models thus assume that when bacteria have reached the stationary phase growth completely halts [63,64]. Recently, however, some researchers have suggested a more nuanced view of nutrient depletion, dependent on the limiting nutrient, where cells may continue to grow, although at a slower rate [65,66].

When initial tests of environmental samples were conducted on spinach leaves, there seemed to be a problem with the standard curve data that had been generated using a 16-hour incubation due to a slope that was too steep for environmental samples. It was hypothesized that potentially the bacteria were reaching stationary phase well before 16 hours, and additional
growth was occurring during the time the bacteria remained in stationary phase leading to an incongruence between the 3-MPBA sandwich assay and the culture results. To test this hypothesis, a standard curve using optical density was first generated (Fig. 26).

Figure 26. Optical density growth curve of *Salmonella enterica* 1045 at 600 nm.

After conducting an optical density-based growth curve, it appears that SE 1045 reaches stationary phase in just 6 hours (Fig. 26). Therefore, in the work conducted in Chapter 2, the bacteria were held in stationary phase for an additional 10 hours (16-hour total incubation time). The optical density readings seem to confer with our hypothesis that additional growth occurs once the cells reach stationary phase with an increase in optical density of 39.7% between hour 6 and hour 16. The only other reason why additional changes would be observed would be due to a Maillard reaction occurring between sugars and proteins in the TSB causing browning to occur.

To confirm that SE 1045 had indeed reached stationary phase after 6 hours, the bacteria procedure described in Chapter 2 was modified by shortening the incubation period of the bacteria to 6 hours. Then a comparison of cultures incubated for 6 or 16 hours was conducted to evaluate differences using both the 3-MPBA sandwich assay and the culture method (Fig. 27).
Figure 27. Differences in optical imaging and culture results between a 6-hour and 16-hour incubation of *Salmonella enterica* 1045. (A) *Salmonella enterica* 1045 $10^{1}$ dilution incubated for 6-hours and observed on the 3-MPBA coated chip (B) *Salmonella enterica* 1045 $10^{1}$ dilution incubated for 16-hours and observed on the 3-MPBA coated chip (C) Comparison of optical imaging results between a 6 and 16-hour incubation period when cells were placed on the 3-MPBA coated chip. The incubation periods resulted in the 16-hour incubation having a significantly ($a=0.01$) higher amount of cells on the chip using a t-test (D) Comparison of viable cell counts of bacteria that was incubated for 6 and 16 hours. Difference in culture results were not significantly different.

The results in Figure 27 show dramatic differences between both the optical imaging and culture results when SE 1045 is incubated for either 6 hours or 16 hours. From the optical imaging (Fig 27C), there is a significant ($a=0.01$) increase in cells between the 6 and 16-hour incubation time. This finding supports the hypothesis the growth continues to occur during the stationary phase (refer to Fig. A8 for additional controls). Alternatively, aspects of the chip could be causing this change including: changes in the cell wall during stationary phase leading to an enhanced binding affinity towards the chip [67], or variation in the chip surface area. In somewhat of a contradiction to this, the viable cell count (Fig. 27D) in the 6-hour incubation is
almost a log higher. The reason that the viable cell count is lower in the 16-hour incubation may be due to the fact that when cells form a pellet during centrifugation they are stickier and take much longer to re-vortex into suspension. The cells are also much older and may be more susceptible to death when placed in a non-favorable environment. It should be noted that not all stock cultures (changed monthly), produce the consistently higher optical imaging results. This may imply that a genetic mutation may cause the cells to continue to grow in stationary phase or have an increased binding affinity towards the chip [68]. Colony morphology of plated samples were consistent amongst replicates and days, making the possibility of contamination unlikely. Furthermore, due to the high number of viable cells observed in the 6-hour incubation, cell loss during the washing process appears to be low. Using the shorter incubation time also solves the problem discussed in Figure 12 where a 1 log reduction in viable cells after the washing and centrifugation process was observed.

The decision to switch to a 6-hour incubation time was primarily motivated by the higher plate count numbers, as well as a decrease in variation observed in optical imaging results. Because behavior of bacteria in the stationary phase was not a primary focus of this research, more controls would be needed to confirm the hypothesis that SE 1045 continues to grow during the stationary phase. That being said, the 3-MPBA chip that was developed in this study could be a powerful tool to test bacteria growth kinetic theories in the stationary phase due to its ability to detect intact, but non-viable cells that the culture method cannot, as well as the ease of quantification. Potential theories to test could include; growth advantage in stationary phase (GASP), glucose hunger, and phosphate starvation [62,65,66].
5.3.2 Standard curve and capture efficiency

![Standard Curve](image)

Figure 28. Standard curves for (A) optical and (B) chemical imaging of *Salmonella enterica* on the 3-MPBA sandwich assay

With the new 6-hour incubation time, a standard curve was generated taking 3-100 point maps of each concentration from 3 different cultures (Fig. 28). Both the optical and chemical standard curves have a good linear relationship with $R^2$ values 0.94719 and 0.98643 respectively. The optical imaging is slightly less precise than the Raman imaging, but results are more rapid and have less variability within a sample. In addition, the optical imaging is more sensitive at high bacteria concentrations to slight differences within a log as discussed in Figure 27. The major disadvantage of the optical imaging is that it lacks specificity and could potentially be more susceptible to a false positive result. Furthermore, at lower concentrations, quantification is more difficult and the optical imaging should be used as a tool to increase the likelihood of finding
bacteria by using the ‘Select Point’ method to rapidly screen potential points. However, it is essential at lower bacteria concentrations to follow this up with SERS mapping to verify the optical imaging result. The chemical imaging on the other hand has a higher specificity and precision, but more variation. Due to this variation, its ability to detect within log bacteria changes is limited. Together, this dual sensing mechanism presents a powerful tool for bacteria detection.

![Figure 29](image_url)

**Figure 29.** Capture efficiency of *Salmonella enterica* on 3-MPBA coated gold chip using $10^8$ CFU/mL concentration.

The capture efficiency of the 3-MPBA coated gold chip was conducted to better understand properties of the assay. The capture efficiency of using $10^8$ CFU/mL concentration resulted in a 91.7% capture of bacteria cells (approximately 1 log). To an extent, this demonstrates why the 3-MPBA sandwich assay can detect low concentrations of bacteria due to the high affinity of bacteria to the chip. This efficiency could probably be further improved by fabricating chips that fit exactly into the well-plate.
5.3.3 Adapting the 3-MPBA sandwich assay to detect bacteria in food matrices

Due to the chemically diverse nature of food matrices, sample preparation protocols and strategies need to be developed in order to make the matrix suitable for detection using the 3-MPBA sandwich assay that has been developed.

The pH of the matrix is very important as demonstrated in Figure 18. Due to the unique ability of ammonia bicarbonate to help capture bacteria, the chemical was added in the same molar concentration to all of the environmental samples. Without this addition, binding to the chip is noticeably decreased (Fig. A8) even when the pH is appropriately adjusted to that of the 50 mM ammonia bicarbonate solution. If the pH of the adjusted sample was too high, a noticeable decrease in bacteria binding affinity occurred, and if the pH was too low, fouling of the chip occurred. The juice matrix that was tested was a high acid food, with a pH of 3.44. Ammonia bicarbonate will not raise the pH of the juice matrix to 8.4. Therefore, a small amount of a highly concentrated sodium hydroxide solution can be used to adjust the pH of any sample to the target pH of 8.4.

Another crucial aspect of the assay that needs food-specific optimization is the pH of the indicator solution. In Figure 12, it was observed that the chemical imaging worked less well because the pH was too low. For all food samples tested, this effect also seemed to occur. By adding small amounts of concentrated sodium hydroxide (8-18 µL 0.02 M NaOH), this hurdle can be overcome. The quantity of sodium hydroxide needed is highly specific to the bacteria concentration/environmental sample, and too little or too much of the chemical can cause the chemical imaging to work sub-optimally (refer to Fig. A6 and A11). The reason why environmental samples depress this pH when in the assay is unclear. As discussed in Figure 18, some reaction may be occurring where more hydroxide molecules are needed. When similar amounts of sodium hydroxide were added to control bacteria solutions in ammonia bicarbonate, the chemical imaging did not work well. Figure 19 also confirms that when sodium hydroxide is used as the medium, the SERS peaks do not differ. For faster optimization of this parameter, the
1571 and/or 1607 cm\(^{-1}\) Raman peaks should be used at localized pH probes based on which peak is dominant.

### 5.3.4 Detection of bacteria in pond water

Figure 30. Comparison of detection of bacteria in pond water using the 3-MPBA sandwich assay and culture methods.

Pond water was selected as a matrix for facile detection of environmental bacteria. For sample preparation, the analyst must simply collect the sample and then add the appropriate amount of ammonia bicarbonate to make the molar concentration equal to 50 mM. Using this method, bacteria in pond water was detected in triplicate. The results of the pond water experiment are somewhat different than the microbiology results.

The reason the dissonance occurs is either due to the assay’s ability to detect more bacteria cells or from environmental contamination. Flow cytometry evaluation of drinking water found that flow cytometry consistently detected 1-2 logs more bacteria than the aerobic plate.
count method did [69]. Cho et al. reported that there are over 30 known bacteria species that can enter into a viable but non-culturable state when exposed to fresh water. They were able to identify $10^5$ CFU/mL of viable, but non-culturable *S. typhi* cells in pond water using green-fluorescent labeled cells [70]. In Figure 27 it was shown that the assay was capable of detecting non-viable cells. It has been reported that PCR can detect non-viable *E. coli* cells in pond water for three weeks after they have become inactivated. Potentially some of the bacteria detected had already died [71]. The bacteria in the pond water may also be ill-suited for the culture conditions used in this detection evaluation (TSA, 37° C, 48 hours) [72]. Finally, environmental interference such as dirt may be creating false positive results. Due to the general agreement of chemical and optical imaging in the middle of the quantification curve, this seems somewhat unlikely. Due to the robustness of this method, the 3-MPBA sandwich assay may be a good method to detect the presence of bacteria when bacterial contamination is suspected, but culture methods do not lead to positive results [2].

5.3.5 Detection of bacteria on spinach leaves

According to the CDC, the food commodity that causes the most foodborne illnesses are leafy greens [5]. For this reason, it was important to know if the assay we developed could detect bacteria on pre-washed organic spinach that one can pick up at a grocery store. To show that our assay is sensitive enough to detect differences in environmental samples, not only were spinach leaves simply tested, but the EPA protocol for fruit and vegetable washing was conducted in order to reduce the bacteria cell content.
Figure 31. Comparison of detection of bacteria on organic spinach leaves using the 3-MPBA sandwich assay and culture methods. The EPA protocol for sanitizing fruit and vegetables was conducted on a sub-set of spinach leaves to determine if the detection methods could detect a difference between treatment groups using 25 ppm chorine (Clorox) for 2 min. (A) Chemical and optical sample maps for control spinach leaves (B) Chemical and optical sample maps for sanitized spinach leaves (C) Comparison of 3-MPBA chemical & optical imaging with the microbiological culture method. Using a paired t-test, all three methods were able to detect a significant (a=0.05 for chemical and optical, a=0.01 for culture) difference in bacteria between spinach treatment groups.

When spinach leaves were tested (Fig. 31), all three methods were able to detect significant differences in bacteria concentration using a paired t-test (a=0.05 for 3-MPBA sandwich assay, a = 0.01 for culture method). In this case, the microbiology culture results are still lower than that of the 3-MPBA sandwich assay, but there is less of a log difference than see in the pond water environmental samples. Unsurprisingly, the microbiology results also detected a greater difference in control and sanitized spinach leaves. Therefore, the culture result is a more sensitive test for bacteria cell viability. Nevertheless, the 3-MPBA sandwich assay also is sensitive enough to detect differences in the un-treated and treated spinach leaves. Due to the fact
that there is agreement between the optical and chemical imaging result, it seems that less bacteria was captured in the sanitized spinach leaf samples. This would imply that bacteria are physically washed away during the sanitizing treatment. However, a more controlled experiment is necessary to make definitive conclusions about the 3-MPBA’s sensitivity to food-grade chemical antimicrobial treatments.

![Figure 32](image)

Figure 32. Understanding the 3-MPBA sandwich assay’s ability to detect chemically inactivated *Salmonella enterica* using food-grade approved sanitizing protocols (i) 80 ppm peroxyacetic acid (Tsuami 100) for 5 min (ii) 25 ppm chlorine (Clorox) for 2 min (iii) 25 ppm chlorine (Clorox) for 5 min. (A) Reduction in percent positive for optical imaging compared to control SE 10^8 CFU/mL (B) SERS spectra of control and chemically inactivated bacteria (i-iii).

To understand more fully, the result of the spinach leaf experiment, a known quantity of SE 1045 was treated with the sanitizing treatment used in the spinach leaf experiment, as well as
two other food-grade antimicrobial treatments for fruit and vegetable washing. Figure 32A shows that the chemically inactivated SE 1045 had a reduced ability to bind to the 3-MPBA coated gold slide. Interestingly, the type and time of chemical treatment had varying degrees of effectiveness in allowing the bacteria to bind to the chip. The SE 1045 treated with 25 ppm chlorine for 5 min had the lowest binding affinity for the chip, while the 80 ppm peroxyacetic acid treatment for 5 min had the highest affinity. While both of these antimicrobial solutions are oxidizing agents, the mechanism behind their ability to inactivate bacteria is very different [73]. The chlorine in bleach targets thermolabile proteins in bacteria cells, similar to the proteins that are targeted during heat shock. On the other hand, peroxide-based antimicrobials do not cause protein aggregation. This may explain why autoclaved bacteria (extreme heat shock) bacteria do not have the bacteria signal, while chemical treatments that do not target proteins effect the assay very little. The SERS chemical signal was also studied in Figure 32B. In the case of the chemical food-grade inactivating treatments, no change in the 3-MPBA spectra was observed. There was a decrease in the general intensity of the signal, but this may be due to a decrease in captured cells. It may be interesting to study sanitizing level chemical treatments (ex: 500 ppm chlorine) to see how a more extreme treatment may change these results.

The results obtained in Figure 32 agree with those found in environmental spinach leaf experiment. Sanitized bacteria cells were most likely physically washed away during the treatment, or less able to bind to the chip, but bacteria that was able to bind was detected by the 3-MPBA sandwich assay.

5.3.6 Detection of bacteria in apple juice

Theoretically, the food component that will create the most interference for the 3-MPBA sandwich assay should be carbohydrates. Carbohydrates could be problematic for this assay because boronic acid has a high affinity for diols and has been frequently used for carbohydrate detection [32,34,35]. While it was already demonstrated in Figure 4 that simple sugars are not
sandwiched under these assay conditions, they still may be able to bind to the 3-MPBA coated chip and block binding sites. Thus, it was of interest to develop a method to enhance the capture of bacteria in a high sugar matrix such as juice.

The method utilized to enhance bacteria capture and detection in a high sugar matrix takes advantage of simple sugars high binding affinity for boronic acid. Before bacteria spiked juice samples were introduced to the 3-MPBA coated chip, boronic acid was added directly to the juice matrix for 5 minutes. Because the simple sugars probably have a higher $K_{eq}$ than bacteria they will bind to the boronic acid introduced into the juice matrix first [35]. Furthermore, many simple sugars such as glucose and fructose have just one binding site for boronic acid. Therefore, the sugars that bind to boronic acid before being introduced to the 3-MPBA coated chip cannot block binding sites. Figure 33 demonstrates the effectiveness of this strategy, with an increase in optical imaging of over 2 logs.

![Figure 33](image_url)

Figure 33. Comparison of optical imaging result for apple juice spike with *Salmonella enterica* $10^8$ CFU/mL without and with 3-MPBA pre-treatment of bacteria spiked juice sample. Refer to Figures A8-A11 for optimization experiments.

Using this method, bacteria in juice samples were able to detected as low as $10^2$ CFU/mL (Fig. 34). At both medium and low concentrations, bacteria detection was not significantly
changed. At higher levels, such as $10^8$ CFU/mL there was approximately half a log of decreased binding affinity.

Figure 34. Limit of detection of *Salmonella enterica* spiked apple juice samples.

5.4 Conclusion

Testing the 3-MPBA sandwich assay in real environmental samples brought new insight into challenges, constraints and advantages this assay possesses. The 3-MPBA sandwich assay shows high sensitivity in the stationary phase and could be a powerful tool for quantifying bacteria that is at risk of being non-culturable due to its high capture efficiency and decreased sensitivity to viable cells. When detecting bacteria in environmental samples there was good agreement between optical and chemical sensing mechanisms, but the assay showed higher quantities of bacteria than the results of the culture method. Strategies including matrix pH adjustment and indicator pH adjustment were found to be critically important for getting consistent results. Finally, the success of a carbohydrate-specific strategy for detection in high sugar matrices demonstrated the capability of the assay to detect bacteria in a wide range of food samples.
CHAPTER 6

CONCLUSION

A versatile and sensitive SERS assay platform has been developed for the non-specific and specific detection of bacteria. The method leverages an ELISA-like sandwich assay for detection of bacteria using a dual optical and chemical sensing mechanisms. For added specificity, the assay utilizes a distinguishing label, 3-MPBA, that produces different spectra based on the molecule it binds to due to changes in polarity. The sandwich assay method demonstrates good selectivity towards bacteria as smaller molecules like simple sugars cannot be sandwiched. Furthermore, the assay shows some sensitivity to dead bacteria with a change in the SERS spectra observed for autoclaved bacteria and a decreased binding affinity observed in optical imaging of chemically inactivated bacteria. The assay is very sensitive with a limit of detection of $10^2$ CFU/mL and a capture efficiency of 91.7%. A unique aspect of this assay is its ability to optically detect and quantify bacteria. It was discovered that the optical effect is caused by the 3-MPBA coated gold chip due to a change in the refractive index of the bacteria. This chip may be a powerful tool for a variety of bacteria applications outside of SERS. Finally, it has been demonstrated that the assay is capable of detecting bacteria that are not cultured in a laboratory setting, showing its industrial applicability. While the 3-MPBA non-specific assay was the focus of this paper, it was demonstrated that the capturer layer can be switched to an aptamer for detection of specific bacteria using an aptamer. Further development of this assay will be continued due to the powerful detection capabilities this assay has demonstrated.
Figure A1. Optimization of 3-MPBA indicator interaction time 3-MPBA sandwich assay SE 1045 \( \sim 10^7 \text{ CFU mL}^{-1} \). Refer to Chapter 2.
Figure A2. Optimization of 3-MPBA capturer and indicator concentrations 3-MPBA sandwich assay SE 1045 $\sim 10^7$ CFU mL$^{-1}$. Refer to Chapter 2.

Figure A3. Optimization of 3-MPBA sandwich silver nanoparticle interaction time using SE 1045 $10^7$ CFU mL$^{-1}$. Refer to Chapter 2.
Figure A4. Controls for aptamer-3MPBA sandwich assay. Refer to Chapter 4.

Figure A5. Aptamer-3mpba sandwich assay demonstration of false positive signal. Refer to Chapter 4.
Figure A6. Optimization of incremental amounts of 0.02 M sodium hydroxide added to indicator solution to increase consistency of results (discussed in Figure 12) for SE 1045 $10^{-1}$ concentration.

Figure A7. Percent positive for optical imaging using the same starter culture (controlled experiment) with SE 1045 incubated for 6 and 16 hours respectively and bound to 3-MPBA coated gold chip done in duplicate.
Figure A8. Optimization of solute used to pH apple juice using the 3-MPBA pre-treatment and spiking sample with SE 1045 $10^8$ CFU/mL.

Figure A9. Optimization of 3-MPBA pre-treatment concentration to SE 1045 $10^8$ CFU/mL spiked juice sample
Figure A10. Optimization of 3-MPBA pre-treatment time to SE 1045 10^8 CFU/mL spiked juice sample

Figure A11. Optimization of the pH of the indicator solution in juice samples spiked with SE 1045 10^8 CFU/mL.
Figure B1. Schematic of Raman microscope detailing how the laser and light hit a sample in the machine. Critical components of a phase contrast microscope including the phase ring and condenser are not present. Schematic is provided courtesy of Thermo Fisher Scientific.
REFERENCES


