Detection of bacterial pathogens in low moisture foods with Dual Immunological Raman-Enabled Crosschecking Test (DIRECT) and Raman mapping

Cheng Pan
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Detection of bacterial pathogens in low moisture foods with Dual Immunological Raman-Enabled Crosschecking Test (DIRECT) and Raman mapping

by

Cheng Pan

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Agricultural and Biosystems Engineering

Program of Study Committee:
Chenxu Yu, Major Professor
Byron F. Brehm-Stecher
Jacek Koziel

The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this thesis. The Graduate College will ensure this thesis is globally accessible and will not permit alterations after a degree is conferred.

Iowa State University
Ames, Iowa
2020

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ABSTRACT

Food safety has always been an essential topic in our lives; about 30 foodborne disease outbreaks occurred each year for the last decade. People paid more attention and were more cautious about the food safety issue than ever before. Food manufacturers also aim to provide more healthy and safer food products to customers. In the past, low moisture foods (LMFs) have not drawn too much attention and concern as being with a high risk of contamination with foodborne pathogens. More and more cases of outbreaks related to LMFs changed that perception, and now LMFs are no longer assumed safe automatically.

Conventional detection methods such as ELISA (Enzyme-linked immunosorbent assay) and PCR (Polymerase chain reaction) used to test the foodborne disease pathogens nowadays often require a flow-based system relies on multiple washing steps to collect the pathogens from foods for analysis. These systems cannot provide a rapid and convenient test for LMFs, which, by definition, do not flow. In this thesis, A Dual Immunological Raman-Enabled Crosschecking Test (DIRECT) scheme was developed to provide direct and rapid detection of foodborne disease pathogens in LMFs. Surface-Enhanced Raman Scattering (SERS) technique was applied in this detection method to achieve a low limit of detection threshold (at $10^2$ CFU/g in model LMF systems). We demonstrated the feasibility of this approach with two MRAs (molecular recognition agents). With the help of a tape-based sampling and Raman mapping, the method has the potential to become a powerful tool for the detection of pathogens in LMFs to address this critical need for the food industry.
CHAPTER 1: GENERAL INTRODUCTION AND BACKGROUND

1.1 Introduction

Food safety is always a goal among human society, but foodborne disease outbreaks still occur from time to time worldwide. The impact of foodborne outbreak disease could lead to not only threats to public health but also significant economic loss. The first line of defense against foodborne pathogens is to detect their existence in food so that measures could be taken to avoid further impact. For the past decades, scientists have developed numerous technologies to detect the pathogens in food, including traditional culture-based methods, molecular recognition-based methods like enzyme-linked immunosorbent assays (ELISA), genetic recognition-based methods such as polymerase chain reaction (PCR), and various automatic biosensors for rapid readout and easy operation.

In this study, the goal is to develop a rapid method based on Dual Immunological Raman-Enabled Crosschecking Test (DIRECT) to detect bacterial pathogens in low moisture foods (LMFs). We will start by introducing the problems caused by pathogens in LMFs and the concerns they brought to food safety in general. Then we will summarize the current research on the detection of pathogens in low moisture food by discussing the advantages and disadvantages of various methods that have been explored. It will become clear that the DIRECT method that we developed in the context of this research offers an excellent solution to the detection of pathogens in LMFs, particularly in powder foods. It has the potential to become a powerful tool for the detection of pathogens in LMFs.
1.2 Food Safety Concerns related to pathogens in LMFs

1.2.1 Microbes in LMFs

Low moisture foods (LMF, or low water activity ($a_w$) foods) are foods with low moisture content and $a_w < 0.6$. These foods include dried vegetables and fruits, spices, chocolate, peanut butter and peanut-containing foods, raw nuts, children’s snack foods, hydrolyzed vegetable protein, and powdered infant formula. Traditionally, low moisture foods are believed to be of low microbial risk as the low $a_w$ in these foods are unable to support the growth of pathogenic and spoilage microorganisms. According to the United States Department of Agriculture (USDA), bacteria will not reproduce well under the conditions that temperature is lower 40 °F or higher 140 °F and water activity less than 0.85 [15]. Hence, for the food in the market, thermal processing and dehydration are the most common methods to prevent microbial proliferation and extend the food shelf life under room temperature. Although in LMFs, microbes are not expected to grow, under these conditions (temperature, water activity, etc.), they just stop reproducing; they can still survive. Once the bacteria are brought into a human body and pass the stomach, where the low pH acidic liquid may also kill them, those who survived entering the small intestine are being provided with an ideal living environment and can reproduce rapidly and cause illness [15]. According to recent studies [16], many microorganisms can survive the drying process in food production. Although the metabolism activities in these microbes are greatly reduced when the moisture level decrease, which could shut down the growth, vegetable cells and spores can still be viable for several months or even years.
In this case, they are often able to persist longer time than those microorganisms contained in high moisture food. Another concern with low moisture food safety is that processing for low moisture food usually does not utilize high-temperature treatment typical for high moisture or liquid food [17]. People tend to think low moisture food is more sterile, which may lead to less care being paid to food safety risks, which, ironically, could elevate the exact risk of foodborne diseases related to the LMFs.

1.2.2 Salmonella in Low moisture Food

By far, the most critical pathogens in LMFs are Salmonella. Salmonella can rapidly be destroyed by heat treatment in high moisture food; as the water activity decreases, the heat resistance of Salmonella increases [16]. Air-dried Salmonella cells, which have significantly lower water activity were more heat tolerant [17]. Many low moisture foods could be susceptible to Salmonella contamination, which may be due to improper operation during the dehydration process, contamination associate with poor sanitation practices or lack of using GMPs, contamination during the packaging process, etc. [17]. Recently, a number of outbreaks of Salmonellosis occurred associated with low moisture foods (CDC, 2007; Koch et al., 2005; Smith et al., 2004), and the safety issue of low moisture foods started to become a major concern. For example, Spices, such as chili powder and pepper powder, are usually used as an additive to other foods after cooking/processing. They generally have a high probability of being eaten as raw. Contaminated spices could hence lead to foodborne disease outbreaks [17]. Although the Salmonella content left in spices is usually low (4 to 45 cells per 100g), these levels can
still cause illness [17]. LMFs like milk powder and egg powder are even more susceptible to *Salmonella* contamination, as pathogen cells tend to have higher survival chances in these foods as they may provide sufficient nutrients and protection to the cells. Examples of *Salmonella* survival in LMFs are listed in Table 1.1. Generally speaking, *Salmonella* contamination in LMFs cannot be neglected. It poses serious threat to public health if not handled properly. Epidemiological and environmental investigations of these outbreaks have suggested that cross-contamination plays a major role. Bacterial contamination may come from soil, insects, bird or rodent droppings, or the water used in processing. Cross-contamination may also occur during the packaging of dried food materials.

Table 1.1: Examples of *Salmonella* survival in foods with low water activity

<table>
<thead>
<tr>
<th>Food</th>
<th>Salmonella serotype(s)</th>
<th>Inoculum (log cfu/g)</th>
<th>Water activity</th>
<th>Length of Survival</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried milk products</td>
<td>Naturally contaminated with 3 serotypes</td>
<td>Up to 10 months</td>
<td>Bell and Kyriakides, 2002</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pasta</td>
<td>Infantis, Typhimurium</td>
<td>0.12%</td>
<td>Up to 12 months</td>
<td>Bell and Kyriakides, 2002</td>
<td></td>
</tr>
<tr>
<td>Milk chocolate</td>
<td>Eastbourne</td>
<td>8.0</td>
<td>0.41</td>
<td>&gt; 9 months at 20°C</td>
<td>Tamminga et al., 1976</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>0.38</td>
<td>9 months at 20°C</td>
<td></td>
</tr>
<tr>
<td>Bitter chocolate</td>
<td>Eastbourne</td>
<td>7.0</td>
<td>0.51</td>
<td>9 months at 20°C</td>
<td>Tamminga et al., 1976</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5.0</td>
<td>0.44</td>
<td>76 days at 20°C</td>
<td></td>
</tr>
</tbody>
</table>
Table 1.1 Continued

<table>
<thead>
<tr>
<th>Food</th>
<th>Salmonella serotype(s)</th>
<th>Inoculum (log cfu/g)</th>
<th>Water activity</th>
<th>Length of Survival</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peanut butter</td>
<td>A composite of Agona Enteritidis</td>
<td>5.7</td>
<td>0.20-0.33</td>
<td>Up to 24 weeks</td>
<td>Burnett et al., 2000</td>
</tr>
<tr>
<td></td>
<td>Michigan Montevideo Typhimurium</td>
<td>1.5</td>
<td>0.20-0.33</td>
<td>Up to 6 weeks at 21°C</td>
<td></td>
</tr>
<tr>
<td>Paprika powder</td>
<td>Multiple serotypes</td>
<td>&gt; 8 months</td>
<td></td>
<td></td>
<td>Lehmacher et al., 1995</td>
</tr>
</tbody>
</table>

Since low moisture food tends to have a longer shelf life, industries usually produce a large amount of them, so early detection of pathogens in low moisture food is essential for the food industry. Moreover, ideally the detection method would be able to detect the pathogens within short amount of time so that the production line can stop once the pathogens are detected. There are some methods already in use in the industry right now like ELISA, PCR which give high accuracy and reliability. More methods are still under development but show great potential in reducing detection time and sample preparation time.

1.3 Detection Methods for foodborne pathogens

1.3.1 Culture-Based Method

The oldest traditional method for pathogen detection is the culture-based method. This method is used to detect a particular bacterium and has a really high success rate. Usually, this method allows bacteria to grow in an agar plate and confirm the target
pathogens by the characteristics of the colony. The disadvantage of this method is it is time-consuming. The expected time for a culture-based method is around 18-24 hours, and this duration will cause a large amount of loss of the contaminated product being produced. [2]

1.3.2 Enzyme Linked Immunosorbent Assay (ELISA)

Enzyme linked immunosorbent assay is a very popular molecular assay that is used to detect and quantify target proteins, antibodies and hormones. ELISA was first described by Engvall and Perlmann (1972) and has been used widely in the food and health industry since then. HIV and hepatitis are a couple of examples of ELISA tests that have been developed and widely used. ELISA typically is performed in 96-well polystyrene plates which passively bind proteins and antibodies [13]. Since the ELISA involve the binding and immobilization of reagent, it is easy to design and operate. A tag can be linked directly to the primary antibody or introduced secondary antibody which can recognize the primary antibody. The enzyme can also be linked to a protein like streptavidin if the primary antibody is biotin labeled. There are several formats for ELISA as illustrated in

Figure 1.1: Illustration of ELISA (https://biomedicool.tumblr.com/post/186800673962/elisa)
figure 1.1, and each format has advantages and disadvantages. Two general formats are direct and indirect detection of ELISA strategies. The advantages direct ELISA equips are quick response time due to one antibody used and cross-reactivity of secondary antibody is eliminated. The disadvantages are 1: labeling primary antibodies for each ELISA system become time-consuming and expensive, 2: minimizing the amplification of the signal and 3: there is no flexibility in the choice of primary antibody label from one experiment to another. Those disadvantages lead to less application using the direct ELISA method. The advantages for indirect ELISA method are more: 1: increasing the sensitivity because each primary antibody contains several epitopes that can be bound by the labeled secondary antibody which simplify the signal; 2: retain the maximum immunoreactivity of the primary antibody because it is not labeled; 3: wide range of selection of secondary antibody commercially; 4: different markers can be used on the same primary antibody. Two disadvantages of indirect ELISA are cross-stage contamination, which will lead to false positive results, and indirect ELISA requires extra washing steps, which can be time-consuming. Sandwich ELISA is another format that is modified from the indirect method. The sandwich method will use two antibodies against one antigen directly which increases the sensitivity and specificity [13]. Competitive ELISA is a method used when the target antigen is small and only has one antibody binding site. Unlabeled antigen from samples and labeled antigen will compete for binding to the capture antibody. A lot of ELISA kits sold in the market and indirect ELISA are the
most common ones that people use to detect pathogens now. For powder and viscous food, if using ELISA, multiple washing steps are required so duration using ELISA is also long.

Apparently, ELISA only works for aqueous samples. It cannot be directly applied to LMFs. LMFs would need to be washed to produce wash water which can then be analyzed with ELISA. In this process, only a certain percentage (30%-70%) of the microbes in the LMFs will be transferred into the wash water for analysis. More importantly, LMFs cannot be interrogated/tested in a non-invasive way which would rule out ELISA as a method for in-line application.

1.3.3 Polymerase Chain Reaction (PCR)

Kary Mulls first discovered PCR in 1983 (Mulls, 1983) which has been considered as one of the milestone discoveries in DNA technology. The principle of PCR is to single out a specific gene sequence of the target pathogens to be amplified for further study. In PCR, a specific DNA segment in the target pathogen will be copied and exponentially amplified to generate thousands to millions of more copies. PCR is now commonly used in the medical and clinical laboratory and there are many commercial testing kits on the market. PCR is extremely concentration sensitive, meaning even if there is only one bacterium in food, PCR is able to detect it when enough copies of the target sequence are generated. PCR offers advantages of specificity, sensitivity, accuracy for pathogen detection in food [13]. The target DNA segment can be amplified one million-fold within one hour, so it is relatively fast to see the result for PCR. However, the sample preparation
for PCR is much longer than the testing stage. Before amplifying the target DNA segment, we first need to find the target DNA. For different foodborne disease pathogens, the DNA segment is unique. Moreover, a different subgroup of bacteria will also have different DNA segments so to detect a wide range of pathogens, a large database for a unique DNA segment is required. In addition, before DNA folding begins, DNA segment extraction also requires a long time to do. These limitations mean that PCR is still a lab-based method and cannot be readily used for in-line or on-site deployment to be operated by non-skilled workers.

1.4 Biosensors

Biosensors are analytical devices that can convert a biological response into an electronic signal; it is a modern method to detect target microorganisms, viruses and enzymes, etc. Biosensors usually consist of two main parts which are the bioreceptors site and biotransducer. The bioreceptors are designed to interact with a specific analyte of interest to create an effective measurement by the biotransducer. There are several types of bioreceptor for specific use: antibody, enzyme, cell, DNA, etc. Biotransducer determines the classification of the biosensor; based on the type of the biotransducer; biosensors can be classified into electrochemical, optical, electronic, piezoelectric, gravimetric, and pyroelectric.
Biosensors can be used in food processing, fermentation process and medical fields. The reason biosensor is good for food monitoring is it could provide real-time, sensitive detection and it is inexpensive. Optical biosensors are the most widely used biosensors in foodborne pathogen detection. In this section, biosensor technologies that could work better in powder and viscous pathogen detection will be introduced and proposed.

1.4.1 Surface Plasmon Resonance (SPR)

Surface plasmon resonance (SPR) is the resonant oscillation of conduction electrons at the interface between negative and positive permittivity material stimulated by incident light [12]. SPR spectroscopy has been developed to measure material adsorption onto planar metal (gold or silver) surfaces or onto the surface of metal nanoparticles. In SPR biosensors, upon absorption of pathogen onto a SPR-active surface, the electromagnetic light wave of the biosensor can be coupled with surface plasmon wave and change the angle shift of the reflected light, which in turn yield a signal correlated to the binding
between surface-immobilized antibodies and the pathogen targets [11]. With this feature, SPR provides single-step, rapid and real-time target pathogen detection.

Figure 1.3: Surface Plasmon Resonance Implementation [12], reproduced with permission.

The localized surface plasmon resonance (LSPR) is another applicable approach for label-free and real-time pathogen detection. LSPR is a phenomenon based on the interaction between a specific light wavelength and plasmonic nanoparticles [12]. However, both SPR and LSPR could have low analytical sensitivity which may result from a small refractive index, slow diffusion-driven mass transfer or the insufficient depth of the influenced layer [12].

Another method is called long-range SPR, which was developed in combination with magnetic nanoparticles to detect pathogens. For E. coli detection in liquid food, long-range SPR can achieve 30 min detection time with an average 74% recognition percentage and 2–4.8 log CFU/mL detection range [12]. Magnetic nanoparticles have been utilized to separate and concentrate target analytes from aqueous solution. Even though magnetic
nanoparticles work better in aqueous solutions, it is recognized as a simple and powerful technique to detect target pathogen [12]. Magnetic nanoparticles can provide advantages such as large surface areas that directly influence the microbial adherence efficacy of particles and surface/volume ratio. For low moisture food (peanut butter, spice, etc.), dilution is required for this method. At the liquid phase, magnetic nanoparticles show outstanding performance for pathogen detection.

1.4.2 Surface-Enhanced Raman Spectroscopy

Raman spectroscopy has emerged as a powerful whole-organism fingerprinting tool for rapidly identifying different chemical and biological analytes in recent years (Kudelski. 2008) [28]. Raman spectroscopy enables the label-free, non-destructive and dynamic chemical analysis of living cells based on the inelastic scattering of photons reflecting specific molecular vibrational and rotational modes of the compositional molecules. However, Raman scattering is a rare event that results in low signal intensity. Typical Raman cross-sections are between $10^{-30}$-$10^{-25}$ cm$^2$ per molecule with the larger values occurring only under favorable resonance-Raman conditions when the excitation light matches the related electronic transition energy in the molecules (Soga. 2000; Kneipp et al. 2008) [29][30]. The rare Raman scattering events, especially when using visible light excitation, usually translate into weak signal intensities as only a low number of scattered photons are available for detection. One method to amplify the weak Raman signals is to employ surface-enhanced Raman scattering (SERS). Surface-enhanced Raman spectroscopy is a technique that enhances Raman scattering by molecules adsorbed on
rough metal surfaces or by nanostructures such as metal nanospheres, nanorods, nanocubes and nanocages. SERS amplifies the Raman signal of molecules nearby plasmonic nanoparticles, yielding a significant 6- to 112-fold log increase as shown in fig.1.4, which means this technique provides high sensitivity at low concentration of target analyte (Kneipp et al., 2008). Usually, the advantages of SERS can be explored on any Raman system and the actual measurement is made in the standard way. Typically, it is necessary to use a laser wavelength that is compatible with the chosen SERS metal, but further more, there are no major difficulties. SERS spectra are sometimes different than “normal” Raman spectrum of the same material. So the interpretation of data must be considered. The basis of SERS is using metal nanoparticles which increase the chances of signal enhancement and create a detection probe with molecular recognition agents (MRAs) such as antibodies conjugated to the probe for pathogen detection. If a pathogen is present, the enhanced signal shows the bonding event between probe and pathogen. Silver and gold nanoparticles are widely used for probe fabrication. When probes with different MRAs are used, the detection of multiple targets via SERS fingerprint differences is possible.
Further refined SERS technique with filter membrane (0.45 μm pore size) can trap nanoparticle probe-labeled pathogens [6]. This method enabled the multiplex, efficient and sensitive detection of bacteria in PBS buffer with a series of simple steps. According to Wang et al. (2016), a range of 2-3 log CFU/ml level of detection (LOD) was reported [31]. SERS technique can be used in powder and viscous food as long as the bonding event occurs, and the results are highly sensitive with short detection time. Raman signals can be chemometrically and statistically analyzed for microbial identification, and automatic Raman spectroscopy-based biosensors could be developed which would be field-deployable for on-site pathogen detection.

1.4.3 Electrochemical Detection Methods

Electrochemical transducers have also been used for identifying and quantifying foodborne pathogens. Electrochemical biosensors can be classified into four categories

![SERS Example](image-url)
depending on the observed parameters such as current, potential, impedance and conductance: amperometric, potentiometric, impedimetric and conductometric. Metallic nanoparticles with intrinsic electronic properties have been used to enhance electrochemical signals. Impedance biosensors were used by Varshney et al. (2008) to detect bacteria in phosphate buffered saline and supernatants of centrifuged ground beef homogenates. Using the principle of impedance, they detected 4.9 log CFU/mL and 5.9 log CFU/mL of E. coli O157:H7 from PBS and the supernatant within 35 min from the beginning of sampling to the end of measurement [8]. That being said, an electrochemical biosensor can also be used for pathogen detection in powder and viscous food; however, washing would be needed as the wash water of the original food may be the only way for such detection to be carried out. Wash water may not contain all the bacteria that adhered to food so low concentration detection of pathogens with electrochemical biosensor may not be feasible.

1.4.4 Mechanical Biosensor

Another biosensor that is also very popular is the mechanical biosensor. Mechanical biosensors are usually very mass sensitive and also can provide a short detection time. For most of the mechanical biosensors, the change of height of the biosensor is converted into electronic signals for further analysis. The main element in many mechanical biosensors is a small cantilever that is sensitive to the attachment of biomolecules/biotargets of interest, as shown in fig.1.5. There are two types of mechanical biosensors: surface-stress mechanical biosensors and dynamic-mode mechanical biosensors. The sensors measure
the quasistatic deflection of a miniature mechanical device for surface-stress mechanical biosensors, typically a cantilever. As the target pathogen binds to the sensor, the surface stress is developed and can induce the deflection of mechanical elements [14]. Dynamic-mode mechanical biosensors are not quasistatic. They oscillate with a resonance frequency and this frequency changes when target pathogens land on the cantilever.

Both types of mechanical biosensors provide high sensitivity and accuracy no matter the concentration of the target bacteria. A cantilever sensor would be another method to detect the pathogens in powder with higher accuracy and lower LOD (limit of detection); however, since the cantilever sensor is super sensitive, there are chances the results are false positives.

1.5 Conclusion

To detect foodborne pathogens usually requires high specificity (only detect the pathogen that we are interested in), high sensitivity (can detect as low as a single bacterial cell), short detection time (for food, the detection time is critical considering public health
and industrial waste) and cost effectiveness. Conventional methods are more completed at this time, providing more accurate and stable results but taking a longer time to get the results. In the past two decades, developed methods show great potential in a short time, high accuracy detection. Even though most biosensors require more expensive reagent and sophisticated equipment, the biosensor is an excellent tool to detect the target of interest in the long run. Most of the techniques are developed for liquid-based samples. It would be more difficult for powder and viscous food to operate a pathogen detection directly with the sample unless a washing step was added to prepare the sample. Every method has both advantages and disadvantages, depending on the specific situation, choosing the proper method to best fit the purpose and expectation of the task at hand needs to be conducted with skill. As a whole, as of today, there is no method that can provide a direct and rapid test on LMFs with reliability to screen for pathogens.
1.6 Summary of the thesis

This thesis consists of four chapters; the first chapter provides background information and literature review on the safety concerns in low moisture food and how foodborne pathogens can be detected with technologies that are currently available, with an emphasis on biosensors. Chapter 2 discussed the results of a feasibility study on the application of the DIRECT method with antibody conjugated GNRs (Gold Nanorods) as the molecular probes on the detection of non-pathogenic E. coli in model LMFs (black pepper powder and egg powder). Chapter 3 discussed a more advanced DIRECT approach in which probes were functionalized with target recognition peptides specifically selected for Salmonella; the peptide-based probes were more stable and more sensitive than the antibody-based systems, which offered better performance in the detection of Salmonella in model LMFs. Finally, Chapter 4 offered some discussion on future perspectives, and further improvement I envisioned can be added to the current approach to address the critical needs of pathogen detection in LMFs.
1.7 References


27. Kudelski A 2008 Analytical application of Raman spectroscopy *Talanta* 76 1-8


CHAPTER 2: A DUAL IMMUNOLOGICAL RAMAN-ENABLED CROSSCHECKING TEST (DIRECT) FOR DETECTION OF BACTERIA IN LOW MOISTURE FOOD *

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Abstract

Among the physical, chemical and biological hazards that could arise with respect to food safety, bacterial contamination has been one of the main concerns in recent years. Bacterial contamination in low moisture food (LMF) was an emerging threat that was used to draw less attention as LMFs were considered at low risk of such hazards. Bacteria can survive in a low moisture environment and cause foodborne diseases once they enter the digestive system. Common detection methods such as ELISA and PCR are not well suited to low moisture food, as most of them operate under an aqueous environment. In this study, a Dual Immunological Raman-Enabled Crosschecking Test (DIRECT) was developed for LMFs using a nano-scaled Surface-enhanced Raman scattering (SERS) biosensor platform with a portable Raman spectrometer. It could provide a limit of detection (LOD) of $10^2$ CFU/g of bacteria in model LMFs, with a detection time of 30-45 minutes. It has the potential to become a quick screening method for on-site bacteria detection for LMFs to identify food safety risks in real-time.

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2.1 Introduction

For many years, low moisture food (LMF) is considered safe from microbial contamination because the low water content is supposed to prevent microbes' growth. Low moisture food (LMF) is defined as food with less than 0.85 water activity, and most bacteria such as *Salmonella* and *E.coli* need water activity of at least 0.91 to grow [1]. However, bacteria can still survive in an environment with a low water activity (<0.85).

Many cases of foodborne disease outbreaks have been linked to the contamination of LMFs by pathogenic bacteria, such as *Salmonella* spp. in peanut butter, spice, milk powder [2]; Shiga toxin-producing *E. coli* (STEC) strains in nuts, flour, rice [2], etc. These bacteria can retain their viability for a long time in a low moisture environment. Given the opportunity and suitable condition, they can start to grow inside the human body and cause different levels of illnesses. Studies have shown that the long term survival of pathogenic bacteria like *Salmonella* spp. can be up to days to years under a low moisture environment, which may even increase their resistance to heat treatment [1]. Moreover, low moisture environment may improve these pathogenic bacteria's tolerance to other stresses such as low pH, UV radiation, and disinfectants [2]. The infectious dose for the pathogens in low moisture food can be very low (10 to 100 CFU) [2], so even only a small number of viable bacterial cells existed in the LMF, they can still cause illness when consumed.

Prevention of biological contamination in food is often regulated by the guidance provided by FDA which usually includes hazard analyses for preventive control in human food. When manufacturers are processing any raw materials like meat, vegetables, grains,
they should follow the Good Manufacturing Practices (GMP) to prevent any physical, chemical or biological contamination. For low moisture food, most of the pathogens can be killed during heating and dehydration processes. However, chances still exist that some pathogens with a high tolerance can survive after those treatments and pose as a food safety threat. Reported foodborne disease outbreaks are on the rise for the past decade; the main pathogen culprits are *Salmonella spp.*, *Bacillus cereus*, *Cronobacter sakazakii* (formerly *Enterobacter sakazakii*), *Clostridium spp.*, *E. coli O157:H7*, and *Staphylococcus aureus*. Among them, *Salmonella spp.* are the most important in LMFs [3].

Numerous methods have been investigated to detect pathogens in foods; including culture-based detection [4, 5], Enzyme Linked Immunosorbent Assay (ELIZA) [5], Polymerase Chain Reaction (PCR) [5, 6] and biosensor methods such as Surface Plasmon Resonance (SPR) [4-7], Nanoparticle enabled bacterial capturing and detection [8-10], Surface-Enhanced Raman Spectroscopy (SERS) [11], mechanical biosensors [12], and electrochemical sensors [13]. Most of the existing methods based on molecular recognition or genetic sequencing operate in an aqueous environment. To use them for LMFs, food samples are collected and typically subjected to multiple washing steps, and the wash water is then subject to testing. In general, these methods are not suitable for detecting pathogens directly in LMFs.

Among the variety of detection methods developed, Surface-enhanced Raman spectro-sensing (SERS) offers an appealing potential for LMF applications as it could
provide a means to detect molecular recognition events in a nonaqueous environment [14-16]. In SERS-based detection, a molecular recognition agent (MRA) such as an antibody can be conjugated onto a nano-scale metallic structure (e.g., Au nanorods and nanocubes) to make a nanoprobe, and the binding of this nanoprobe to its molecular targets (e.g., epitopes on the bacterial cell surface) can be detected and characterized via the Dual Immunological Raman-Enabled Crosschecking Test (DIRECT) developed in our lab [17, 18] in which no washing/separation steps between target bound and unbound probes are needed. As illustrated in fig.1. In the DIRECT scheme, gold nanoparticles are functionalized with Raman tags (e.g., 4-aminothiophenol, or 4-ATP) and MRAs to make Raman molecular probes (RMPs). In the DIRECT scheme, these would transduce probe signal. When they get in touch with target bacterial cells, they bind to cell surface epitopes via specific MRA-target interactions. These binding events bring enough RMPs onto the cell surface, and Raman spectroscopic signals of the cell wall/extracellular matrix hence

![Figure 2.1: Scheme of the DIRECT Assay](image-url)
get enhanced as well due to their close vicinity to the nanoparticles (<10 nm), which are multiple magnitude more intense than unenhanced signals (SERS enhancement is typically $10^5$-$10^6$ for bacterial cells). The enhanced cell signal becomes detectable (unenhanced cell signal is too weak to be detectable), and this is the target signal in the dual signaling scheme of DIRECT. As illustrated in fig.1, the only scenario under which the dual signals (probe+target) are detected is when RMPs bind to their targets via specific MRA-target interaction. Without enough of the RMPs bound, the signal from cells won’t be enhanced enough to be detectable. Nonspecific binding of RMPs onto nontargets (other cells in the sample) won’t create enough binding events needed for SERS enhancement, hence it won’t create false positives. This DIRECT scheme can work for conditions with low water activity; hence it is suitable for direct interrogation of LMF samples.

In this study, the DIRECT scheme was applied for the direct interrogation of LMF samples to achieve rapid detection of bacteria in the LMFs at $10^{2-3}$ CFU/g level of contamination within 30-45 minutes. Two LMFs were selected as model food systems (black pepper powder and egg powder), as they represent main food safety concerns at the consumer end (black pepper powder, in restaurants/dinners) and production end (egg powder, as a protein additive in various processing lines). A non-pathogenic *E. coli* strain was chosen as a model bacterium to evaluate the limit of detection (LOD) of the method as it was easy to culture and manipulate to prepare spiked LMFs with different contamination levels. As the detection mechanism was based on molecular recognition of
antibody-antigen interaction, it is straightforward to modify the platform to detect other pathogenic bacterial species.

2.2 Experiment Section

2.2.1 Reagents and Antibodies

Hexadecyltrimethylammonium Bromide (CTAB, 99%), benzyl dimethyl ammonium chloride hydrate (BDAC, 99%), sodium borohydride (99%), L-ascorbic acid, gold(III) chloride hydrate (>99%), silver nitrate (>99%), 4-aminothiophenol (4-ATP, >99%), sodium nitrite (>99%) and E. coli serotype polyclonal antibody were all purchased from Sigma-Aldrich. Black pepper powder was bought from Walmart. SERS substrates slides were bought from Ocean Insight. Deionized water (18 MΩ) was used in all the experiments.

Polyclonal anti-E. coli antibodies (NB200-579) were purchased from Novus Biological (Centennial, CO, USA) for E. coli molecular detection. E. coli cultures (ATCC#25922) were provided by a colleague (Dr. Brehm-Stecher).

2.2.2 Fabrication and Functionalization of Gold Nanorods to make SERS Nanoprobes

Gold nanorods with an aspect ratio of 2 were synthesized via the seed-mediated growth method [19]. Details of the procedure were reported elsewhere [17]. The GNRs were further functionalized by 4-aminothiophenol (4-ATP) [20]. Briefly, 4 mL of 6 nM gold nanorods were reacted with 1 mL of 10 mM 4-ATP dissolved in acidic water (pH =
2) and the mixture was stirred vigorously at 60 °C for 3 hours. Centrifuge and wash the solution twice with 3 mM CTAB acidic aqueous solution to eliminate the unbound 4-ATP. Finally, 4-ATP functionalized GNRs were resuspended in 2.5 mL of acidic water (pH = 4).

E. coli antibody has an initial concentration of 4.5 mg/mL. 0.5 mL E. coli antibody was divided into 10 aliquots (each was diluted with PBS buffer to 1 mL). For each conjugation run, 0.5 mL 4-ATP functionalized GNRs were mixed with 0.5 mL 1 mM NaNO₂. Then, the mixture was incubated at 4 °C for 30 min; 200 μL from one antibody aliquot (45 μg antibodies) were added into the solution and incubated at 4 °C overnight. The mixture was then centrifuged (6000g, 10 min, 4 °C) and washed twice to remove the unbound antibodies, then resuspended the pellet 0.5 mL 1x PBS buffer. The final concentration of the nanoprobe was ~5 nM. They remain stable for up to 1 month.

2.2.3 Bacterial Cell Culture and sample preparation

E. coli was incubated in LB medium broth at 37 °C for 18 h. The bacterial cells were then centrifuged and washed with PBS buffer twice and finally redispersed in PBS buffer. The final bacterial cell concentration was determined by optical density (OD) measurement at 600 nm (the concentration of the bacterial cells was ~10⁹ CFU/mL at OD = 1.0). The cell suspension was then diluted to 10³ CFU/mL. 1 mL and 0.1 mL of the cell suspension were then mixed with 1 g of black pepper/egg powder samples to create bacterial spiked samples at 10³ CFU/g and 10² CFU/g levels for the test with the DIRECT
assay alongside with the un-spiked samples. Each sample was mixed with 0.5 mL SERS nanopores and incubated at 4 °C for 30 minutes in an Eppendorf tube to allow probe-target binding.

2.2.4 One-Step Raman Spectroscopic Measurement

As shown in fig.2, the sample in the tube was then subjected to Raman measurement using a portable Raman spectrometer (i-Raman Plus, B&W Tek, Inc., Newark, DE, USA). The tube was put against the head of the fiber optical probe of the spectrometer to allow the laser beam (15 mW, 785 nm NIR laser, 3 cm⁻¹ spectral resolution, 400-2,000 cm⁻¹ range) to penetrate the plastic tube wall. Raman spectra of the sample were obtained directly in one single step. 60 s integration time was used for spectral acquisition. 15 spectra were collected from each sample to calculate an average spectrum, which was used for further analysis. All experiments were conducted in triplicates.

2.2.5 Spectral data processing

Using near-infrared excitation (785 nm) effectively reduced the background sample auto-fluorescence. To further reduce the remaining fluorescence, a polynomial background subtraction method was implemented as described before [17, 18]. The 10-point moving average method was used in this study to smooth the spectra to remove noise.
and other fine-scale variations. All spectra were then area-normalized for intensity consistency in the region between 400 cm\(^{-1}\) to 2000 cm\(^{-1}\). All data processing was conducted using R, a widely used language and software tool for statistical computing and graphics. Peak intensity was calculated based on the integration of the peak area.

### 2.3 Results and Discussion

**The DIRECT assay for LMFs contaminated with model bacteria**

The mechanism of the self-referencing DIRECT assay was illustrated in fig.1. Only specific binding of nanoprobes to targets (i.e., bacterial cells in LMF samples) would generate detectable dual SERS signals for a definitive positive readout for the bacteria. The DIRECT assay has been utilized to provide a 1-10 CFU/mL LOD for waterborne pathogens [18]. Similar to the earlier report, anisotropic gold nanorods were used in this study as well to generate specific SERS nanoprobes with conjugated E. coli antibodies via diazo chemistry through 4-Aminothiophenol (4-ATP) anchors. As shown in fig. S1a, after the conjugation, the nanoprobes showed stronger 4-ATP peaks as their fingerprints. The nanoprobes' color also changed after each step of the conjugation chemistry, indicating the changes occurring on the nanoparticle surfacing were changing the optical properties of the nanoprobes.

It should be noted that when the DIRECT assay was applied to LMFs, a quite unique challenge emerged comparing to aqueous samples. In aqueous samples, the background was not a main interfering concern; the aqueous environment itself did not generate strong Raman signals to be superimposed on top of the signals generated by the target bacteria,
regardless of whether they were bound to nanoprobes. For LMFs, the situation was completely different.

The spectra taken from the LMF samples were always dominated by the peaks associated with the LMFs and the plastic Eppendorf tube themselves. As shown in fig.3, average spectra from egg_probe (un-spiked egg powder with probes added), egg_ecoli (spiked egg powder with no probe added) and egg_probe_ecoli (spiked egg powder with probe added) showed great similarity due to the common egg powder/tube contribution. Nonetheless, a close investigation of the spectra still suggested that in the egg_probe_Ecoli spectra, peaks could still be identified that originated from the presence of nanoprobes binding to E. coli targets. The strong peaks at 1094 cm\(^{-1}\) and 1562 cm\(^{-1}\) undoubtedly were from the nanoprobes (4-ATP, as shown in fig.2a), which were weak on the spectra of egg_Ecoli when the probes were not present. Peaks at ~735 cm\(^{-1}\) and ~1330 cm\(^{-1}\) are typical of

Figure 2.3: Spectral indicators showing presence of E. coli in spiked egg powder at 10^3 CFU/mL

Figure 2.5: PC loadings showing peaks related to the I.D. of presence of E. coli in spiked black pepper powder at 10^3 CFU/mL
adenine [17, 22]. They could come from the adenine part of the fully reduced FAD [23] or other adenine-bearing molecules (NAD, ATP, DNA, etc.). They were attributed to cell origin in our earlier report [17] which could serve as the indication of the probe-cell binding in the DIRECT assay. However, the 735 cm\(^{-1}\) peak was quite weak, and the 1330 cm\(^{-1}\) peak could be associated with probe/egg powder as it was also seen in the spectra of egg_probe samples with a little wavelength shift. It should be noted that some of the strong E. coli peaks (1260 cm\(^{-1}\) and 1578 cm\(^{-1}\)) were not seen in the egg_Ecoli and egg-_probe_ecoli samples. When nanoprobes bound to the E. coli cells in the spiked samples, only molecules on or right beneath the cell walls were sitting within the effective surface enhancement range, and peaks associated with other cell components would not be enhanced; hence became “invisible”. These observations with only a couple of E. coli peaks seen in the DIRECT assay were consistent with our earlier work with waterborne pathogens as the enhancement to the Raman signal decayed rapidly as the distance between the nanoprobes and the cellular components became larger than 10 nm. The presence of the dominant LMF background obviously complicated the identification of DIRECT signals by simple visual inspection of the spectral signals.

Two different types of LMFs were tested; one was egg powder, a major protein and lipid rich ingredient used in the food processing industry; and the other was black pepper powder, a common LMF of specific food safety concerns in the restaurant industry. The test with the black pepper powder turned out to be even more challenging. The black
pepper powder, as it was black, absorbed the laser energy much more effectively comparing to the egg powder, and the energy absorption led to strong localized heating

![Spectral indicators showing presence of E. coli in spiked black pepper powder at 10^3 CFU/mL](image)

Figure 2.4: Spectral indicators showing presence of E. coli in spiked black pepper powder at 10^3 CFU/mL to occur, which, combined with the reduced scattered light, weakened the SERS signal. Nonetheless, as shown in fig.4, the DIRECT assay could still provide spectral indicators for the detection of the presence of E. coli in the spiked samples. Similar to the results for egg powder, peaks at 1094 cm^{-1} and 1562 cm^{-1} could be attributed to the nanoprobe, and peaks at 1260 cm^{-1} and 1440 cm^{-1} appeared to be associated with the presence of E. coli in the spiked samples.
Since the complication brought by the LMF backgrounds made simple visual inspection of the spectra less reliable, to better differentiate the signals from the DIRECT assay between contaminated (spiked) samples vs. control (unspiked) samples, a support vector machine (SVM) based discriminant analysis was conducted to differentiate between the two groups of samples. The SVM model was constructed with principal components calculated from the spectral set of 40 randomly selected spectra from each group (e.g., spiked black pepper powder vs. un-spiked black pepper powder), another set of 12 spectra from each group (also randomly selected) were used for testing the SVM model. Principal component analysis is routinely used to reduce the complexity of spectral data to facilitate discriminant model construction. This study showed that the first 5 PCs accounted for over 90% of the total variance among the data sets for both egg powder and black pepper powder assays. As shown in fig.5, the first 3 PCs’ loadings for the black

Figure 2.5: PC loadings showing peaks related to the I.D. of presence of E. coli in spiked black pepper powder at $10^3$ CFU/mL.
pepper powder assays showed that these three PCs (1260 cm$^{-1}$ peak in PC1 and PC2, 1440 cm$^{-1}$ peak in PC3) were associated with the DIRECT identification of E. coli in the samples.

Based on the PC loadings study, a discriminant model could be constructed using the first 5 PCs. The discriminant model provided a 100% differentiation accuracy between spiked and un-spiked black pepper powder for the testing set (12 spectra in the set). The black pepper powder was among the worst system for Raman spectroscopic analysis due to its strong light absorption; the high differentiation accuracy certainly demonstrated the potential of the DIRECT assay armed with PCA-SVM modeling.

The PCA-SVM model for the egg powder samples yielded the same high differentiation results (also at 100%). Clearly, at $10^3$ CFU/g level, the contaminated black pepper powder samples and egg powder samples could be clearly differentiated from un-spiked ones with the DIRECT assay with the help of PCA-SVM discriminant analysis. The PCA-SVM in this study was conducted with a limited dataset (40 spectra in the model set, 12 in the testing set); we deliberately chose this small dataset to see if the assay needs to be developed on the flight with limited data collection time, how well it would work. It is fully understood that analysis with a limited dataset tends to be susceptible to overfitting; hence the 100% differentiation accuracy obtained could be biased. Further validation with a greater dataset is certainly needed. As reported earlier [18], multiplex epitope recognition should improve the accuracy of the DIRECT assay, which is to be explored in future work.
Results obtained for the egg powder samples were better than that for the black pepper powder samples since egg powder did not absorb the excitation laser energy as much as the black pepper powder samples. At $10^3$ CFU/g level of contamination, as shown in fig. 3, the DIRECT assay revealed specific Raman peaks that were associated with E. coli+nanoprobe binding events which provided a quick, single-step detection of bacterial contamination in the egg powder samples.

To further test the limit of detection (LOD) of the DIRECT assay on LMFs, both LMF samples were also spiked with E. coli at $10^2$ CFU/g level. For waterborne samples, it has been shown that the DIRECT assay can reach a LOD of 10 CFU/mL; but for the LMFs, that level of LOD would not be realistic. For $10^2$ CFU/g, however, the DIRECT assay appeared to work well, with only a slight decline in the differentiation accuracy.

![Figure 2.6: Classification of spiked vs. un-spiked black pepper powder using PCA-SVM discriminant modeling.](image-url)
became 94.4% (comparing to 97.2% at $10^3$ CFU/g); and for egg powder, it became 83.3% (comparing to 87.5 at $10^3$ CFU/g). As shown in fig.6, PCA-SVM discriminant analysis revealed a clear separation of un-spiked vs. spiked black pepper powder samples at $10^2$ CFU/g contamination and $10^3$ CFU/g contamination. The SVM separation suggested potentially a semi-quantitative assay could be developed that would allow a quick estimate of the contamination level.

Ideally, the DIRECT assay result (i.e., the dual bands associated with both bacteria and nanoprobes) shall be obvious with a simple visual inspection of the spectra, as has been shown for waterborne samples [17]. However, the presence of LMFs ruled out such possibility as the original Raman signatures of the LMFs would always be much more visible than that of the bacteria and the probes, so the enhanced dual signals of the bacteria+probes could be hidden within the Raman spectrometer readings. Another reason the enhancement was not strong is that the binding between the bacteria and the probes could be restricted due to the low flowability of the material. Using a multiplex epitope recognition scheme [18] could further improve the detection accuracy of target bacteria. Nonetheless, the results still suggested that the DIRECT assay can be used to detect pathogens in LMFs qualitatively with a very quick turnaround time to determine whether or not contamination has occurred, with a LOD of $10^2$ CFU/g, which is a big step forward for LMFs. However, further improvements are certainly needed to enhance the outcomes and to evaluate how well the scheme would work when multiple bacterial species were in presence simultaneously.
2.4 Conclusion

Using a DIRECT assay to detect bacteria in low moisture foods is feasible according to the experiment results of this work. For spices like black pepper, detection could be hard due to the high absorbance of light in the material which leads to unstable readings. Other powder foods like egg powder could be more readily analyzed. Nonetheless, good detection results could be obtained via statistical (i.e., SVM) discriminant analysis. Further improvements could be made on the current experiment design to increase sensitivity and stability. Overall, The SERS-based DIRECT technique can deliver a rapid detection of bacteria in low moisture food without damaging the format of the food comparing to other conventional methods now available in the market, which in the future may offer an effective alternative for a quick screen of LMFs for food safety concerns in the industry.

2.5 Acknowledgments

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2.6 Conflict of Interest

The authors declare no conflict of interest.
2.7 Supplement materials

Fig. S1. Making of DIRECT molecular nanoprobes via functionalization of gold nanorods

(a)

![Graph showing Raman spectra of GNRS-4ATP and GNRS-4ATP-E.coli Antibody, with peaks at 1090, 1394, and 1435.](image)

(b) (c) (d)

Figure S1: Comparison of GNRS-4ATP and GNRS-4ATP-E.coli Antibody
(a). Raman spectra; (b). Gold Nanorods (aspect ratio≈2); (c). GNRS-4ATP; (d). GNRS Final Probes
Fig. S1 shows the Raman spectra of 4-ATP-coated GNR and anti-E. coli antibody-conjugated GNR. A layer of 4-ATP molecules was anchored on the surface of the GNR due to Au-S bonding. As shown in fig S1a, band at 1090 cm\(^{-1}\) is the stretching vibration of C-S bond and band at 1590 cm\(^{-1}\) is the C-C stretching vibration of the benzene ring in 4-ATP [1-5]. The appearance of these bands indicated the successful replacement of CTAB with 4-ATP on the GNR surface. Another notable difference between the spectra of pure 4-ATP and that of 4-ATP labeled Ag-cube is the intensity of 4-ATP characteristic peak at 1590 cm\(^{-1}\). The apparent enhancement of the mode at 1590 cm\(^{-1}\) can be ascribed to a charge transfer between the metal and the 4-ATP molecules [6], further confirms the binding of 4-ATP to the GNR surfaces.

The Ag-4-ATPs were then reacted with nitrite ions in acid condition to form diazonium salt, which subsequently reacted with histidine residues of the antibodies. The strengthening of the 1394 cm\(^{-1}\) and 1435 cm\(^{-1}\) diazonium peaks (N=N stretching) proved the conjugation of the antibodies.

Panels b-d showed the change of color of the GNRs after each step of the surface chemical modification.
2.8 References


CHAPTER 3: RAPID DETECTION OF SALMONELLA IN LOW MOISTURE FOODS USING SURFACE ENHANCED RAMAN SPECTROSCOPIC MAPPING WITH MOLECULAR PROBES FUNCTIONALIZED WITH TARGET-BINDING PEPTIDES

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Abstract

Salmonella contamination poses a serious food safety concern for low moisture foods (LMFs). Direct detection of Salmonella in LMFs is difficult with conventional molecular methods as the lack of an aqueous environment creates a major constraint. In this paper, a rapid Salmonella detection method based on surface-enhanced Raman spectroscopic (SERS) screening and mapping was developed, in which nanomolecular probes functionalized with Salmonella-recognizing peptides were applied to yield SERS signals upon binding to Salmonella targets in the LMFs. It was demonstrated that the peptide probes were superior to antibody-based probes as the small size of the peptides significantly increased the surface-enhancement effects to improve the reliability that increase the reproducibility for the experiment and sensitivity which reach the limit of detection as low as \(10^3\) CFU/mL of the detection method. With SERS mapping, visualization of detection of Salmonella was demonstrated for a model LMF of black pepper powder. The method developed in this study could potentially be used for the rapid screening of LMFs directly for Salmonella detection.
3.1 Introduction

Low moisture foods (LMFs) are usually defined as foods with water activity lower than 0.85 ($a_w < 0.85$), such as chocolate, peanut butter, spices, dried vegetables, and meat, etc. [1]. Water activity is partial vapor pressure in a substance divided by the standard partial vapor pressure of water. It is often used in food safety cases to regulate the critical control point and demonstrate the ability of bacteria growth. In common sense, people tend to think LMFs are safer than other types of food because, with low moisture content and water activity, it would be more difficult for microbial pathogens to survive in LMFs. Unlike high moisture content food such as meat and vegetables, LMFs usually do not require cooking (i.e., heat treatment) before consumption, which may result in higher risks to foodborne diseases if the foods are pathogen-contaminated.

*Salmonella* strains are the most important pathogens that are of concern for LMFs. Reported outbreaks of Salmonellosis have occurred related to LMFs [2-4] in recent years, raising the awareness of the food safety concerns associated with LMFs. It is known that *Salmonella* cannot always grow in LMFs; however, it can survive for a long period once the food is contaminated. Depending on the type of LMFs and temperature, the survival time may vary. Although the optimal temperature for *Salmonella* is around 35-37 °C, it can grow at a temperature as low as 2°C and as high as 54 °C [5]. *Salmonella* can survive in chocolate for up to 15 months at room temperature, up to 29 weeks at 22 °C in honey, up to 24 weeks at 21°C in peanut butter, up to 10 months in spray-dried milk and egg, up to 1 year in spices, pasta and flour [5]. When the water activity decreases, the heat
tolerance of *Salmonella* increases markedly, which indicates that it could be more difficult to eliminate *Salmonella* from LMFs with heating [5,6]. According to CDC (Centers for Disease Control and Prevention) and RASFF (Rapid Alert System for Foods and Feeds), hundreds of cases have been reported associated with *Salmonella* in LMFs since 2000 [23,24].

In recent years, biosensors have been widely explored in pathogen detection. A biosensor usually has two key functional parts: bioreceptors and biotransducers. Bioreceptors are usually built on molecular recognition agents (MRAs) such as antibodies, enzymes, aptamers, etc. Biotransducers are signal-transducing units that translate the MRA-target binding events into signals that can be analyzed for detection, which can be classified into electrochemical, optical, mechanical, etc. [8]. Biosensors can provide real-time, highly sensitive, and inexpensive detection of various biological targets. For foodborne pathogens, optical biosensors are the most popular [7].

Surface-enhanced Raman scattering (SERS) offers a good biotransducing method that has been explored effectively for foodborne and waterborne pathogen detection in real-time with high sensitivity and specificity [15]. In the SERS approach, metal nanoparticles are functionalized with MRAs such as antibodies to make molecular probes, and the binding of bacterial targets to the probes are then analyzed for target detection [17, 18]. Silver and gold nanoparticles are widely used for probe fabrication. When probes with different MRAs are used, the detection of multiple targets via SERS probes has also been demonstrated [21]. As shown in our previous work (chapter 2), with a Dual
Immunological of Raman-Enabled Crosschecking Test (DIRECT), the SERS technique can be directly applied to screen LMFs such as black pepper powder and egg powder to detect bacterial contamination at $10^2$ CFU/g level.

However, the DIRECT approach was not without its problems and limitations. One key feature in SERS is that the enhancement effects of the nanoprobes decay rapidly as the distance between the probe surface and the analytes (i.e., targets) increases. With antibodies as the MRAs, the size of the antibody molecules (MW~150 kDa) would limit the enhancement to the Raman signals from the probe-bound targets and hence would reduce the accuracy of the DIRECT assay. Another concern when using antibodies as the target recognition handle is that antibodies are usually susceptible to environmental factors such as temperature and pH, which could reduce or even neutralize the antibodies' binding capacity. In LMFs, the pH and moisture content might not be suitable for antibody to maintain high binding affinity towards the targets, which may lead to inaccurate detection results. Some recent studies have suggested that certain short peptides originated from bacterial phages could be utilized as MRAs to detect Salmonella [11-14] because peptides are short-chain protein with a much smaller size (MW=1-2 kDa, <1% of antibody), nanoprobes functionalized with them would generate stronger enhancement for the targets. They are also more stable than antibodies. Therefore, in this paper, two specific peptides were selected from the literature that was shown to be Salmonella-specific MRAs [13, 14]. They were then used to make molecular nanoprobes and used in DIRECT detection of Salmonella in black pepper powder at $10^3$ CFU/g. To overcome the
difficulties arisen from sampling the LMFs, a Raman color-mapping approach was
developed in conjunction with the DIRECT sensing scheme to detect Salmonella in black
pepper powder samples effectively. The method potentially can become a powerful tool
to help to address the critical need for pathogen detection in LMFs.

3.2 Experiment Section

3.2.1 Reagents and Peptides

Hexadecyltrimethylammonium Bromide (CTAB, 99%), benzyl dimethyl ammonium
chloride hydrate (BDAC, 99%), sodium borohydride (99%), L-ascorbic acid, gold(III)
chloride hydrate (>99%), silver nitrate (>99%), 4-aminophenol (4-ATP, >99%),
sodium nitrite (>99%) and E. coli serotype polyclonal antibody were all purchased from
Sigma-Aldrich. Black pepper powder was bought from Walmart. SERS substrates slides
were bought from Ocean Optics Inc. (Orlando, FL, USA). Deionized water (18 MΩ) was
used in all the experiments.

Peptides specifically made to target Salmonella with a sequence of
NFMESLPRLGMH (Peptide 1) and NRPDSAQFWLHH (Peptide 2) were purchased
from ChinaPeptides (Shanghai, China) with a molecular weight of 1432 g/mol and 1507
g/mol. Salmonella cultures (ATCC 49214) were provided by a colleague (Dr. Carmen L
Gomes).
3.2.2 Fabrication and Functionalization of Gold Nanorods to Make SERS Nanoprobes

Gold nanorods with an aspect ratio of 2 were synthesized via seed-mediated growth method [20]. Details of the procedure were reported elsewhere [21]. The GNRs were further functionalized by 4-aminothiophenol (4-ATP) [22]. Briefly, 4 mL of 6 nM gold nanorods were reacted with 1 mL of 10 mM 4-ATP dissolved in acidic water (pH = 2) and the mixture was stirred vigorously at 60 °C for 3 hours. Centrifuge and wash the solution twice with 3 mM CTAB acidic aqueous solution to get rid of the unbound 4-ATP. Finally, 4-ATP functionalized GNRs were resuspended in 2.5 mL of acidic water (pH = 4).

Conjugation of peptides and antibodies to the 4-ATP anchored GNRs was conducted with the same procedure of diazonium-linkage chemistry. In a typical operation, 4.5 mg peptide is dissolved in 10 mL DI water to make peptide stock solution. For each experiment, dilute the peptide stock solution with a ratio of 1:100 to get the concentration needed for the conjugation. For each conjugation run, 0.5 mL 4-ATP functionalized GNRs were mixed with 0.5 mL 1 mM NaNO$_2$. Then, the mixture was incubated at 4 °C for 30 min; 100 µL of diluted peptide solution was added into the solution and incubated at 4 °C overnight. The mixture was then centrifuged (6,000 g, 10 min, 4 °C) and washed twice to remove the unbound peptides, then resuspended the pellet 0.5 mL 1x PBS buffer. The final concentration of the nanoprobe was ~5 nM. They remain stable for up to 1 month.
3.2.3 Bacterial Cell Culture and Sample Preparation

*Salmonella* was incubated in LB medium broth at 37 °C for 18 h. The bacterial cells were then centrifuged and washed with PBS buffer twice and finally redispersed in PBS buffer. The final bacterial cell concentration was determined by optical density (OD) measurement at 600 nm (the concentration of the bacterial cells was \( \sim 10^9 \) CFU/mL at OD = 1.0). The cell suspension was then diluted to \( 10^3 \) CFU/mL. 1 mL of the cell suspension was then mixed with 1 g of black pepper sample to create bacterial spiked samples at \( 10^3 \) CFU/mL for the test with the probe assay alongside the un-spiked samples. Each sample was mixed with 0.5 mL SERS nanoprobes and incubated at 4 °C for 30 min in an Eppendorf tube to allow probe-target binding.

3.2.4 One-Step Raman Spectroscopic Measurement

As it is shown in Fig 3.1, the sample on the gold slide was then subjected to Raman measurement using a dispersive Raman spectrum microscope (DXR Raman Microscope, Thermo Scientific, Waltham, MA, USA). Raman spectra of the sample were obtained directly in one single step. 90 s integration time was used for spectral acquisition. Fifteen spectra were collected from each sample to calculate an average spectrum, which was used as the spectral representation for the sample. All experiments were conducted in triplicates which include fifteen spectra for each replicate.
3.2.5 Raman color-mapping for Salmonella detection

The LMFs are not easy to sample for bacterial contamination. Unlike liquid samples, the bacterial cells cannot move around freely, and their distribution in the LMFs usually is not uniform. At the relatively low contamination level of $10^2$-$10^3$ CFU/mL, difficulty in sampling could translate into inaccuracy in the detection results. To overcome the sampling difficulty, an easy-to-use tape-based Raman color-mapping approach was developed.
The Raman color mapping approach is illustrated in fig.3.2. A special made tape was peeled off from the tape roll and used in a “stick and peel” step to collect a thin layer of LMFs on its surface. Then, the tape-surface was subjected to Raman mapping. The pixel size of the mapping scan was chosen to be 20 μm × 20 μm, which offered a good enough spatial resolution to capture the presence of probe-bound *Salmonella* cells, yet large enough for a quick scan to be conducted. Raman spectra were acquired from each pixel. Then, pseudo-color heatmaps of the tape surface were constructed based on selected Raman peaks that were identified via statistical analysis to represent the probe-*Salmonella* binding events. As shown in fig.3.2, three heatmaps for three peaks were to be constructed. For the spots where *Salmonella*-probe binding occurred, overlaps among the three heatmaps were to be expected. Hence, combing the heatmaps, we would be able to spot on the tape where the presence of Salmonella cells could be identified with high fidelity. In turn, an accurate detection result could be obtained for the LMFs in question.
3.3 Results and Discussion

3.3.1 SERS Characterization of Molecular Probes with Gold Nanorods Conjugated with Peptides

Figure 3.3: SERS spectra indicating surface conjugation of peptides to GNRs

After linking the 4-ATP to GNRs, the Raman spectra of the probes showed a strong peak around 1578 cm\(^{-1}\), which indicated the presence of 4-ATP. Further strengthening of the diazonium peaks (N=N stretching) at 1394 cm\(^{-1}\) and 1435 cm\(^{-1}\) confirmed the conjugation of target-binding peptides to the GNRs via 4-ATP linkage. It should be noted that color change could be observed after each step of the conjugation chemistry, which could also be used as a visual indicator for evaluating the progress of the surface conjugation of the MRAs (e.g., antibodies and peptides) to the GNRs.
Salmonella cell suspension (10⁹ CFU/mL, in PBS buffer) was drop-coated onto the SERS active-spot of a commercial SERS substrate slide (ARM-SERS-AG, Ocean Insight, Orlando, FL) as shown in Fig. 3.1. SERS spectra of the cells were then acquired. It should be noted that SERS spectra of cells usually differ from that of bulk cells due to the enhancement to Raman signals originated from certain surface elements of the cells, which were brought into close vicinity of the SERS substrate. To identify DIRECT peaks of the cell origin, SERS spectra of the cells were needed, as shown in Fig. 3.4.

3.3.2 Comparison of DIRECT performance with peptides and antibodies as MRAs

Student’s t-test was conducted to identify statistically significant differences among all the spectra measured from samples with/without Salmonella and nanoprobes at the confidence level at 99%. The LMFs studied was black pepper powder. In general, nanoprobes with peptide 2 (NP2, seq: NRPDSAQFWLHH) tended to yield more reproducible and clear results comparing to nanoprobes with peptide 1 (NP1, seq:
NFMESLPRLGMH), and both NP1 and NP2 were better than nanoprobe antibodies (NPA). Results with NP1 showed too many peaks with significant differences (Fig. 3.5(A)) between LMF (black pepper powder) with/without spiked Salmonella at $10^3$ CFU/mL, many of which could not be easily attributed to known peaks of Salmonella/probe origin, hence it was not straightforward to associate these peaks to probe/Salmonella binding events. By eyeballing the differences, it was clear that the peaks of the probes (4-ATP peaks at ~1578 cm$^{-1}$ and 1035 cm$^{-1}$) changed quite significantly, which could be caused by the uneven distribution of the probes in the probe/sample mix. Nonetheless, Fig 3.5C indicated that SERS signatures of Salmonella cells at around 1000-1100 cm$^{-1}$ and 1200-1400 cm$^{-1}$ were also correlated to significant differences between LMF with/without Salmonella, indicating that some of the differences observed were to be caused by probe/target binding which could be utilized for Salmonella detection.
Figure 3.5: Results with NP1 (Peptide 1 Conjugated Probe).

Antibody conjugated probes were also tested to compare the results to the peptide group. As it is shown in Fig 3.4, it appeared that the differences between two groups: probe-pepper-Salmonella at $10^3$ CFU/mL (i.e., positive) and probe-pepper (i.e., negative) were minimal, and most of the differences seemed to arise from the probes, not Salmonella cells. As mentioned earlier, the size of the antibodies determined that enhancement to the cells signal would be limited, which could be the main reason for the cell signatures not to show. The NPAs seemed not to be reliable enough to provide reproducible results.
Among the three nanoprobes studied, NP2 provided the most accurate results and reproducible results from multiple experiments, as shown in Fig 3.7. Specific peaks related to Salmonella cells were significantly different between the positive (+ Salmonella) and negative (- Salmonella) black pepper powder samples, including peaks at 811 cm$^{-1}$, 1033 cm$^{-1}$, 1266 cm$^{-1}$ and 1432 cm$^{-1}$, as shown in fig.3.4 and 3.7C, in addition to the 1578 cm$^{-1}$ peak which was clearly originated from the probes.
Figure 3.7: Peptide 2 Conjugated Probe Results

A comparison among figs 3.5-3.7 revealed that the peptide-functionalized probes (NP1 and NP2) performed better than the antibody-functionalized probes (NPA). As discussed earlier, it could be attributed to the stronger enhancement of the cell Raman signals due to the much smaller size of the peptides. Between NP1 and NP2, it appeared that the results of NP1 groups were not as consistent and clear-cut as that of the NP2 groups, and the underlining reasons remained to be discovered. Overall, NP2 was the better choice between the two peptide-based probes as reliable and consistent DIRECT signals could be obtained with them for *Salmonella* positive samples.

### 3.3.3 Detection of Salmonella contamination in LMFs with Raman color-mapping

As discussed earlier, one of the main difficulties that we had in pathogen detection in LMFs was the uneven distribution of the pathogens in the LMFs, especially at low
concentration levels. When testing the presence of *Salmonella* in LMF, sampling has to be carefully conducted to avoid false negative results.

With tape-based sampling, we were able to identify the presence of *Salmonella* cells in LMFs. As the LMF particles were uniformly spread out on the tape surface, Raman maps constructed via mapping the tape surface for Raman peaks with a clear correlation to probe/cell-binding events revealed the presence of the target cells on the spot.

![Raman Mapping to reveal Salmonella in LMFs](image)

As shown in fig.3.8, using DXR Raman microscope, we scanned the tape surface of 200×200 μm, with a pixel size of 20×20 μm. With 100 pixels, the total scanning time was ~3-4 hrs. In order to assure that *Salmonella* cells would be present in the viewing area of 200×200 μm, we used a high *Salmonella* level at $10^5$ CFU/g. In Fig 3.8, two unique peaks associated with *Salmonella* alongside with one peak associated with the probe showed
high intensity at the same spot on the map, which could be used as multiplex verification of the presence of *Salmonella* at the marked location on the tape.

With tape-based sampling, Raman mapping was shown to be a very useful tool for the interrogation of LMFs; with the multiplex verification based on the DIRECT approach, the stability and sensitivity of the detection scheme can be further improved with easy readout from the result visualization. Although the initial concentration of *Salmonella* we set in the experiment was relatively high, the approach could be improved by reducing the time for each scanning step so that a larger area could be scanned quickly, translating into a lower contamination level be detected.

### 3.4 Conclusion

Typically, antibodies are the most common MRAs used nowadays in biosensors to detect target pathogens in food. This work demonstrated that for the SERS-based DIRECT approach, short peptides could be used instead for *Salmonella* which would provide better and more reproducible detection performance. Peptides have much shorter structures which would render the SERS molecular probes more effective in teams of generating enhanced signals from the bound target cells. As a feasibility study, we showed that with the peptide probes, *Salmonella* contamination in LMFs such as black pepper powders could be detected at $10^3$ CFU/g level. The most effective peptide probes were made with NRPDSAQFWLHH, which provided reproductive results over multiple trials and can be further tested on different strains of *Salmonella* to check the consistency. Combined with
tape-based sampling and Raman mapping, a biosensor platform can be developed for accurate and reliable detection of *Salmonella* in LMFs.
3.5 References


CHAPTER 4: FUTURE IMPROVEMENT AND PERSPECTIVE UPON THE DIRECT METHOD

4.1 Multiplex Recognition

Through Chapter 2 and Chapter 3, only one MRA was used in each experiment and when the food composition is complicated, it is not easy to recognize the presence of the target pathogen. One alternative to try in future research can be using multiple detection probes to target the pathogens to increase the sensitivity and specificity. With multiple biological markers, it is also possible to detect a lower concentration of pathogens in low moisture food. Multiplex recognition also decreases the chances of false positive or false negative by adding more verification agents in the detection system. Theoretically, if one of the Raman molecular probes (RMPs) has the detection accuracy of 80% and if three probes are used simultaneously, the accuracy can be improved by 19.2% which achieved a 99.2% final accuracy rate. With such high detection accuracy, the limit of detection can also be improved to $10^{1-2}$ CFU/g or even cell detection in low moisture food which tends to have minimal flowability.

4.2 TERS and DIRECT Mapping Tool for Faster Salmonella Detection

Current techniques were mainly used on power food which is capable of adding small amount of water to create paste-like format. When the low moisture food is solid and larger in size such as dried vegetable, meat jerky, DIRECT may not be applied to those foods like the method we used we tried in Chapter 2 and 3. With the Tape-based Salmonella Enrichment and Repair (TERS) method, target pathogen can be collected from
the surface of the low moisture food and transferred to a single tape which is a simple and
easy stick-and-peel process. Then the tape would be transferred to a selective agar to
create a chamber for the bacteria to grow so that the initial cells contained in the low
moisture food can be repaired and enriched over the growth which increases the detection
rate using the DIRECT method. DIRECT Mapping method was briefly mentioned in
Chapter 3 which allows people to see the location of the source bacteria visually. Once
the tape is ready with repaired and enriched pathogens, we can directly take measurement
from the tape which reduces a lot of time for sample preparation. Another advantage of
using the mapping tool is that the contaminated level can be clearly seen and the location
of the pathogen can be precisely selected from the original source of food. There are more
potential further improvements that can be tested on this project and so far, we have
sufficient data and knowledge to establish the feasibility of this technique.