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DEVELOPMENT OF FILTER-BASED SURFACE ENHANCED RAMAN SPECTROSCOPIC ASSAYS FOR RAPID DETECTION OF CHEMICAL AND BIOLOGICAL CONTAMINANTS IN WATER

A Thesis Presented

By

SIYUE GAO

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

MASTER OF SCIENCE

September 2016

Department of Food Science

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DEVELOPMENT OF FILTER-BASED SURFACE ENHANCED RAMAN SPECTROSCOPIC ASSAYS FOR RAPID DETECTION OF CHEMICAL AND BIOLOGICAL CONTAMINANTS IN WATER

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SIYUE GAO

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I am very grateful to have accomplished all these works during the two year of my master's study to put together in this thesis. I would like to thank my advisor Dr. Lili He for her patience and guidance throughout the entire process of the project. I could not have made this much improvement during the past two years without her help. I would also like to thank Dr. Park and Dr. McClements for being my committee member and put through time for my proposal and thesis. At last, I would also like to thank all of my lab mates for their kindness and unconditional help. Their passion for research has always been a great motivation for me. I am very grateful for all people that have kindly gave me help during the two years.

ABSTRACT

DEVELOPMENT OF FILTER-BASED SURFACE ENHANCED RAMAN SPECTROSCOPIC ASSAYS FOR RAPID DETECTION OF CHEMICAL AND BIOLOGICAL CONTAMINANTS IN WATER

SEPTEMBER 2016

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Directed by: Professor Lili He

Surface enhanced Raman spectroscopy (SERS) has been widely applied for rapid and sensitive detection of various chemical and biological targets. Here, we incorporated a syringe filter system into the SERS method to detect pesticides, protein toxins and bacteria in water. For the detection of chemical and protein targets, silver nanoparticles (Ag NPs) were aggregated by sodium chloride (NaCl) to form nanoclusters that could be trapped in the pores of the filter membrane to from the SERS-active membrane. Then a coating of capture (e.g. aptamer) was integrated on the nanoparticle substrate if needed. Then samples were filtered through the membrane. After capturing the target, the membrane was taken out and air dried before measuring by a Raman instrument. The developed filter SERS method was able to detect fungicide ferbam as low as 2.5 ppb level and had a good quantitative capability, which could also be carried out on site using a portable Raman instrument. The aptamer integrated filter SERS was able to detect ricin b chain in water at 100 ppb level. The filter membrane was then applied to detect bacteria E.Coli with the

integration of 4-mpba as a capture and indicator. With SERS mapping, we can detect E.Coli down to 10^1 CFU/ml and the viability of bacteria on the membrane could be confirmed by incubating the membrane on TS agar down to 10^2 CFU/ml. This study shows the filter based SERS methods improve the detection capability in water samples, with a great versatility for various types of assays.

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LIST OF ABBREVIATIONS

Ag NPs- Silver nanoparticles
SERS- Surface-enhanced Raman spectroscopy

CHAPTER 1

INTRODUCTION

1.1 Surface enhanced Raman scattering (SERS)

1.1.1 Theory

Surface enhanced Raman Scattering (SERS) was first observed in 1974, by Fleischmann et al, where the Raman spectrum of molecule pyridine adsorbed from aqueous solution onto a silver electrode roughened by means of successive oxidation reduction cycles displayed an intensity that was 10^5 - 10^6 times as strong as the intensity of pure liquid pyridine[1].

Two enhancement mechanisms are generally accepted nowadays: electromagnetic mechanism and chemical effect. The electromagnetic mechanism propose that an electromagnetic resonance, so called surface plasmon exists surrounding the metal surface when an incident light interact with the target on the surface[2], resulting in enhanced scattering due to the excitation of localized surface plasmons (LSP)[3]. The first serious estimates of enhanced fields near metal nanoparticles were done in the late 1970s and early 1980s by Gersten and Nitzan, Kerker and others, respectively[4]. The charge transfer mechanism (also called chemical effect) is induced by the direct contact between metal surface and adsorbate, forming new chemical bonds and accompanied by a metal–molecule or molecule–metal charge transfer[5-6]. The Chemical effect is believed to contribute to a minor part of the enhancement. Charge transfer model has been reported of evidence by a lot of researchers, one of them was a charge transfer band observed by Yamada et al within the pyridine-Ag system in 1987[7].

1.1.2 SERS Substrates

One of the essential properties of a good SERS substrate is nano-scale roughness,

normally on a noble metal surface. By far, SERS substrates have exclusively been associated with three metals, silver (by far the most widely used), gold and copper[3]. A wide variety of nano materials have been utilized for SERS, including silver dendrites, metal island films [8-9], colloids[10-11], and recently reported nanostructured substrate[12-13]. The two approaches to make nanosubstrates are the top-down and bottom-up techniques, which represents patterning large scales dimensions to nanoscales or arrange atoms and molecules in nanostructure, respectively[14-17]. There are two common ways to use these nanosubstrates to prepare a SERS sample: solution-based and substrate-based methods[18]. The solution-based method uses nanoparticle (NP) colloids to mix with samples. Then the NP-analyte complex is collected using centrifugation, and deposited onto a solid support for Raman measurement after drying. The substrate-based method is usually applied by depositing several microliters of liquid sample onto the pre-fabricated solid substrate[19]. However, neither of these two methods is effective and applicable for a large amount of sample volume.

Silver nanoparticles (Ag NPs) are by far the most widely used SERS substrates due to its high enhancement ability[3]. Up to now, various synthetic methods of Ag NPs have been developed[20-23]. Ag NPs can be used alone as effective SERS substrates, or combined with other materials and structures to enhance its sensitivity and/or functionality[24-28].

1.1.3 Advantages and disadvantages of SERS

Surface enhanced Raman spectroscopy (SERS) is a combination of Raman spectroscopy and nanotechnology. Traditional Raman spectroscopy is capable of providing unique spectral information at molecular level rapidly. Nanotechnology improves the sensitivity of the traditional Raman spectroscopy and extends the application to detection areas. By using a

portable Raman spectrometer instead of the traditional bench-top instrument, on site detection is feasible, further extending its application outside of laboratory analysis.

Despite all the advantages that make SERS technique very promising, some drawbacks remain unsolved that prevents further applications of SERS. One of the drawbacks is the structure of the nanosubstrate, associated with the roughened metal surfaces or nanoparticles arrays. While high enhancement efficiency is achieved, the distribution of the hot spots is random and structural reproducibility is hardly achieved[29]. As one of the most commonly used and flexible SERS substrate, colloidal silver particles display several problems including easy oxidation and sulfuration of silver nanoparticles[30] Producing a reliable, inexpensive substrates that can generate uniform sensitive signal remain a problem to be addressed. Another drawback is the reproducibility of the spectra from the target molecule since being statistically reliable is critical to any analytical technique[31]. Since SERS is sensitive to a large variety of target molecules, specificity problem become serious when applying in a complex matrices. Therefore, extraction or separation of target molecules from matrices is needed before SERS measurement. Direct modification of substrate with capturers is another solution, such as introducing antibody or aptamer into SERS substrate. The relatively high cost of the instrument and commercial substrates is another issue that restricts its applications.

1.1.4 Applications

Based on the unique characteristic of SERS and its ability to be integrated with other techniques, SERS has been employed into the analysis of various chemical and biological targets in many different disciplinary, such as pesticides, protein toxins and allergens, food additives, bacteria and virus.

1.1.4.1 Small chemical contaminants

Chemical residues in food have exposed great threat to human health. The applications of SERS on small chemical contaminants have mainly focused on pesticides, antibiotics, illegal drugs and food additives[18, 32]. SERS has also been used as an effective tool for sensitive and quantitative analysis of melamine in food since the infamous intentional adulteration of melamine in foods such as infant formula[33-36].

1.1.4.2 Protein contaminants

Another category of chemical hazards is foreign allergenic or toxic proteins, due to cross-contact or intentional adulteration. SERS has been coupled with biosensors to detect protein targets. Those techniques include IMS-SERS (immunomagnetic separation)[19], antibody-based SERS[37] and aptamer-based SERS[38].

1.1.4.3 Pathogens

SERS offers an alternative for the sensitive detection and identification bacteria cells because of its capability to provide highly enhanced Raman spectra at single cell level[39]. So far, various methods have been developed to detect pathogens, including gram-positive bacteria Bacillus cereus and gram-negative bacteria Escherichia coli and Salmonella typhimurium, some even showed potential for discrimination among strains[40]. Over the years, great effort has been made to fabricate novel nanostructure for SERS biosensing to differentiate between types of pathogens and virus[31, 41].

1.2 Filtration system

A filtration system is designed for the separation of solids from fluids (liquids or gases), which contains a medium through which only the fluid can pass. The fluid that passes through

is called the filtrate. A filtration system normally consists of a sieve, or a filter paper, and a container to hold the filtrate. In order to achieve a fluid flow, a pressure different must exist between the feed side and the filtrate side. This is achieved either by applying pressure on the feed side or by applying vacuum on the filtrate side. Filtration has been employed in various areas, not only in industry, but also in scientific research. For analytical researchers, filtration systems have mainly been used for purifications of sample or as a carrier for analyte.

1.3 Filter based SERS

1.3.1 Substrates

The most significant feature of filter based SERS is to fabricate SERS substrate on a filter paper or membrane. Nanoparticles can be integrated onto the filter paper/membrane either by silver mirror reaction[42], by filtration of silver/gold colloid[43] or simply by depositing nanoparticle colloid[44]. The filter paper/membrane is flexible and easy to be integrated with samples.

1.3.2 Advantages and disadvantages of filter based SERS

One of the limitations associated with traditional SERS methods is the limited sample volume that could be applied with a SERS substrate, which could be overcame by the integration of a filter system into SERS methods [42-48]. The filter based SERS assays have shown great enhancement in sensitivity and feasibility for monitoring the chemical contaminants in large water samples because analyte can be concentrated onto the substrate. Another advantage of using the filter-based system over the solution-based method is the ease of operation and fieldable measurement, as no centrifugation is needed to collect the NP-analyte complex. The substrates fabricated on filter paper/membrane are also relatively

uniform and has the potential to be utilized for quantitative analysis. However, this filter based technique has some limitations when used alone since it cannot be utilized in complex liquid sample. Pretreatment is needed to remove interfering components before passing the membrane.

1.3.3 Applications

Filter based SERS has been utilized for highly sensitive detection of various targets, including small chemical toxins, such as pesticides[49], large target including vitamins[50] and biological targets including pathogens[47].

1.4 Goal and objectives

The goal of this study is to develop a method to fabricate highly sensitive SERS substrates by integrating a filter syringe system that can be applied to the detection of chemical toxins and biological contaminants. According to The EPA administers the Safe Drinking Water Act of 1974, contaminants associated with drinking water are certain inorganic and organic chemicals, turbidity, certain types of radioactivity, and microorganisms.

Herein, there are four objectives in study listed as follows:

Objective 1: Optimization of the parameters in performing filter SERS.

Objective 2: Use filter SERS to detect chemical toxins in water.

Objective 3: Use filter SERS to detect protein toxins in water.

Objective 4 (future study): Use filter SERS to detect pathogens in water.

CHAPTER 2

DEVELOPMENT AND APPLICATION OF FILTER BASED SERS FOR RAPID AND SENSITIVE DETECTION OF CHEMICAL TOXINS IN WATER

2.1 Introduction

2.1.1 Chemical toxins

Chemical toxins in food and environment are mainly from anthropogenic contaminants and have long been a great concern with regard to food and environmental safety. Most of them are organic chemicals and the best-known examples are the halogenated aromatic compounds, dioxins, and organochlorine pesticides such as dichlorodiphenyltrichloroethane (DDT)[51]. Those toxins are exposed to food and environment from several sources including industrial compound or byproducts, pesticides, food additives, veterinary drug residues, etc[52-53] US federal regulator such as Food and Drug Administration (FDA) and US Environmental Protection Agency (EPA) has enforced several regulations on food components to ensure to human health and environmental safety. The Miller Pesticide Amendment that was added to FD&C in 1954 has specified the tolerate level of pesticide residues on fresh produce; the Food Additives Amendment enacted in 1958 was designed to ensure the safety of all food additives added to food products. Other two acts relevant to the protection of food from pesticide residues are the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA), and the Food Quality Protection Act of 1996[54].

Ferbam is a dithiocarbamate fungicide that has been widely used against a variety of plant pathogenic fungi[55]. Since a serious ecological accident due to fungicide pollution in 1991, the safety use of fungicide has raised increasing public awareness. EPA issued an advisory on the use of fungicides in 45-crop applications[56].

2.1.2 Traditional ways of detection and their limitations

Gas and liquid chromatography methods, and their coupling to mass spectrometry, currently are standard methods suggested by the United States Environmental Protection Agency (EPA) for the detection of chemical toxins in drinking water[57]. While being sufficiently sensitive and accurate, most of the methods currently being used are labor intensive, expensive, and/or time consuming because of the complicated sample preparation or analyzing time. Another issue is the lack of portability since currently techniques exist rely on laboratory-based procedures.

2.1.3 Objective of this study

The objective of this study is to develop a rapid, sensitive technique in detecting fungicide ferbam in water samples using SERS by integrating a filter syringe system. In order to develop the filter based SERS, ferbam is used to optimize all the parameters that affect the performance of this method, including nanoparticle size and salt concentration, sample volume and times of reusing the filtrate. Then the sensitivity and quantitative analysis were evaluated. Later on, this developed method is applied to test ferbam in three water samples, including double distilled water, tap water, and pond water (collected from the university pond) to test its performance in applications to real world samples. The integration of this filter-based method with portable Raman was performed on site.

2.2 materials and methods

2.2.1 Sample preparation

Citrate-coated Ag nanoparticles with sizes of 40 nm, 60 nm, 80 nm with a concentration of 20 mg/L were purchased from Nanopartz. Ferbam (Chem Service) is dissolved in 50%

acetonitrile to make a 100 ppm stock solution, and diluted with 50% acetonitriles for series of concentrations. 20 μl of 1 ppm ferbam solution was spiked into three kinds of water samples, which are tap water, pond water and double distilled water, making the final concentration 10 ppb. EMD Millipore Durapore PVDF Membrane Filters and polycarbonate Filter Holders from Cole-Parmer was used in this experiment.

2.2.2 Fabrication of SERS active membrane and detection of liquid sample

The first step was to aggregate Ag NPs by NaCl solutions to produce nano clusters that could be trapped in the pores of the filter membrane and which forms a layer of SERS active nanoparticles. To perform the filter SERS assay, 1 ml of commercial Ag nanoparticles was mixed with the same volume of sodium chloride and incubates on a nutator for 10 minutes. A syringe was connected to the filter holder, with a filter membrane inside. After loading the nanoclusters into the syringe and passing through the membrane, the liquid went through the membrane but not the nanoclusters, forming a SERS-active membrane. The filtered liquid was reused once to ensure all aggregated NPs are utilized. When testing the analyte, a volume of ferbam sample was filtered through the Ag NPs coated membrane. The analyte bound and concentrated on the Ag NPs coated membrane. The membrane was then detached from the filter holder, air dried and measured by a Raman instrument.

2.2.2.1. Optimization of several parameters in filter SERS

Three different sized Ag nanoparticle colloid (purchased from Nanopartz, 20 ppm) with size of 40 nm, 60 nm, 80 nm were aggregated by sodium chloride solution with four different concentrations. 10 mM, 25 mM, 50 mM and 75 mM salt concentration were used for each nanoparticle size.

For each combination of NPs size and salt concentration, 2ml of 1 ppm ferbam solution was used and 2 separate trials were performed with 8 randomly chosen spots within each membrane. The combination with best signal intensity and consistency was chosen as the optimized size and salt concentration.

The influence of sample volume and times of time of filtration were also evaluated. 60nm Ag NPs and 50mM NaCl was used to conduct the experiment. 100 ppb ferbam with the sample volume of 2 ml and 5 ml were tested, with 1, 2, 3 and 4 times of filtration, respectively.

2.2.2.2. Characterization using SEM

SEM images of membranes coated with 60 nm Ag NPs aggregated by 3 different salt concentrations (10 mM, 50 mM, 75mM) were taken to investigate the effect of salt on the aggregation of NPs. The prepared Ag-coated membranes were coated with Au to eliminated surface charge and observed using SEM (FEI Magellan 400 XHR-SEM), with a current of 13 pA and voltage of 5 kV. Pictures were taken with 5 μm scale and 1 μm scales, respectively.

2.2.2.3. Study of limit of quantification and limit of detection

Series concentrations of ferbam solution were tested with one trial performed for each concentration and 8 spots chosen within each trial. 2 ml sample was used and the sample was filtered for 2 times. A standard curve was plotted using the means of the highest peak intensity with regard to each ferbam concentration and the standard deviation was used as error bars. Limit of detection is determined by calculating the concentration in the regression curve that equals to the mean of the control plus 3 times of its standard deviation[58-59].

2.2.2.4. Detection of environmental water samples

20μl of 1 ppm ferbam solutions were spiked into three different water samples, including tap water, DD water, and pond water, making 2 ml of ferbam sample with the final concentration of 10 ppb. Each ferbam sample was reused 3 times upon detection to achieve maximum binding. Two separate trials were performed for each sample with 8 spots chosen within each trial.

2.2.3 Instrumentation

Raman scanning was performed using a DXR Raman microscope (Thermo Fisher Scientific, Madison, WI, U.S.A). A 780 nm Laser with a laser power of 5 mW and sample exposure time of 1 s was used to scan the samples under a 10x objective and a 50 μm slit. A TruScan Raman Handheld Spectrometer (Thermo Fisher Scientific, location) with a 785 nm laser and laser power of 300 mW or lower was used to test samples on site as an alternative to the bench top instrument. The raw data was import to Omnic software and TQ analyst for further analyze.

2.3 Results and discussion

2 ml of 1 ppm ferbam was used to test the influence of NaCl concentration with regard to each sized nanoparticle. 10 mM, 25 mM, 50 mM and 75 mM salt concentration were used because concentration below 10mM is not able to aggregate nanoparticles enough to be trapped in the pores of the membrane and whereas a concentration too high will result in dark Ag solid that precipitated and lost its colloid property. Both spectral result and the most significant peak intensities plotted as column graph are shown. For the 40 nm Ag, 75 mM NaCl achieved the strongest signal, whereas for 60 nm Ag, 50 mM NaCl has shown the best

result, and 75 mM NaCl is the most suitable salt concentration for 80 nm Ag. Generally speaking, higher salt concentration is able to aggregate Ag NPs to larger clusters, which contain more hotspot and are more easily deposited onto the membrane. With sufficiently more Ag and hotspots in the substrate, stronger signals are achieved. This is most obvious with the 40 nm Ag as smaller sized colloid has higher molar concentration with the same weight concentration, thus require more ions for aggregation.

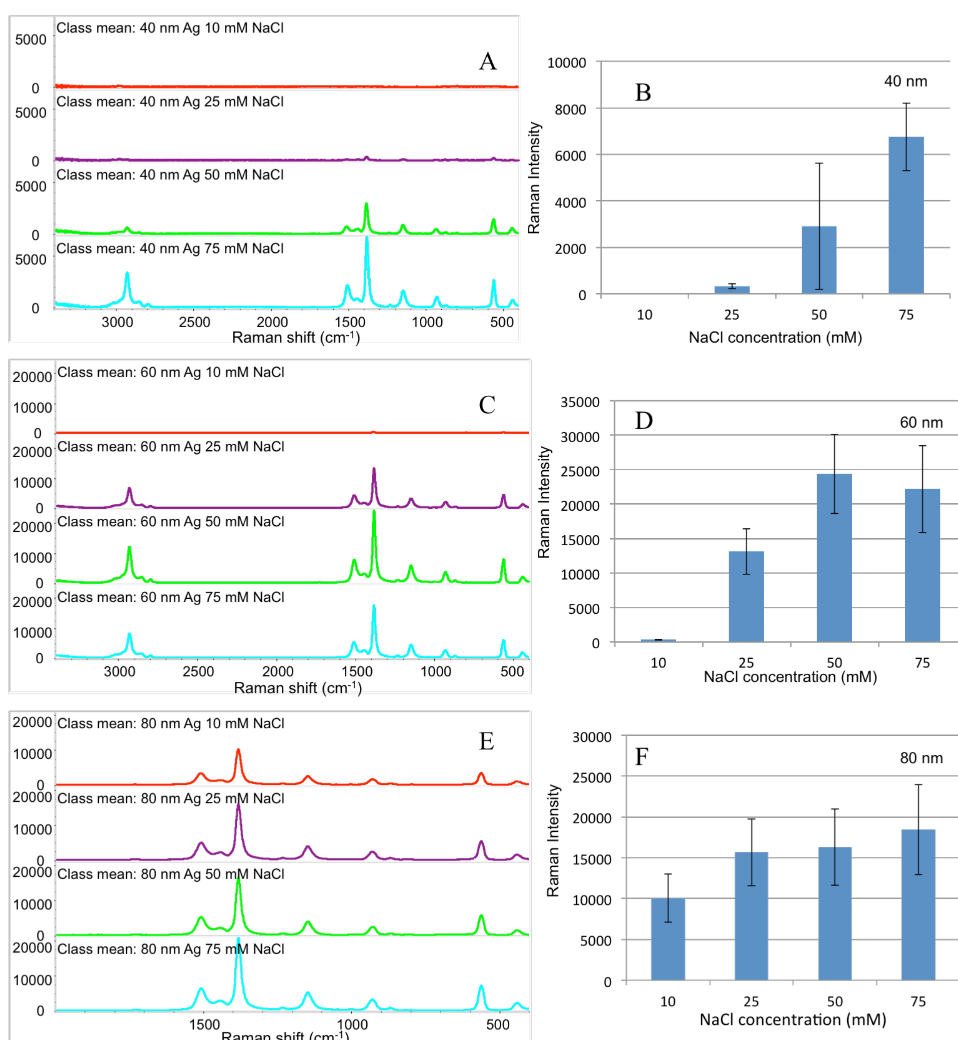

Figure 1. SERS spectra of 2 ml 1 ppm ferbam using 40, 60 and 80 nm Ag NPs with NaCl concentration of 10mM, 25mM, 50mM, 75 mM, (A, C, E) respectively. Peak intensities at 1380 cm-1 using the three sizes of Ag NPs at different salt concentrations with standard deviation displayed as error bars (B, D, F).

In order to compare among three sizes of nanoparticles, the optimal salt concentrations with the strongest peaks with regard to each particle size were put together in a column graph. It's easily recognized that the 60nm nanoparticle with 50 mM NaCl concentration displays the strongest peak intensity and tolerable standard deviation.

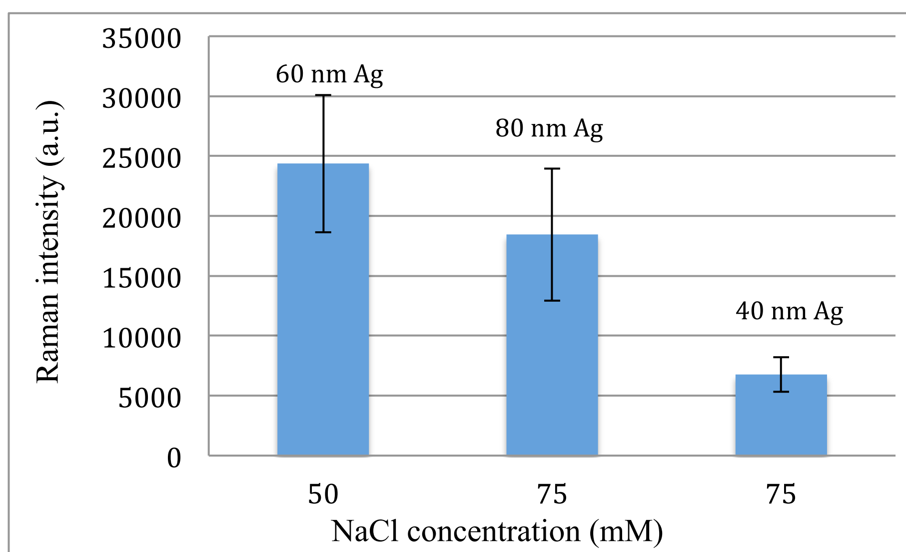

Figure 2. Maximum peak intensities with regard to each sized nanoparticles. Standard deviation is displayed as error bars.

The prepared membranes were further evaluated by SEM on the coating thickness and uniformity of the membranes with different salt concentrations. From the SEM image (figure 3, B-F) of membranes coated with 60 nm Ag NPs aggregated by 10, 50 and 75 mM NaCl, we are able to observe the drastic changes due to different salt concentrations. With increasing NaCl concentration, Ag NPs were aggregated to larger clusters and more effectively covers the surface, the Ag NPs are also distributed more evenly on the membrane and generates stronger signal. However, with the use of 75 mM NaCl, Ag NPs are almost fully aggregated and result in heterogeneous distribution on the membrane. As shown in figure 3 E, there are barely any NPs clusters, whereas in figure 3 F, large Ag clusters forms a thick layer of silver

instead of a structured monolayer, which result in decreased SERS signal. We can conclude from the optical images taken from the Raman instrument under the 10× objective that although NPs distributions are uneven in different areas, there are no significant differences among the whole membrane in the optical images (figure 3 A).

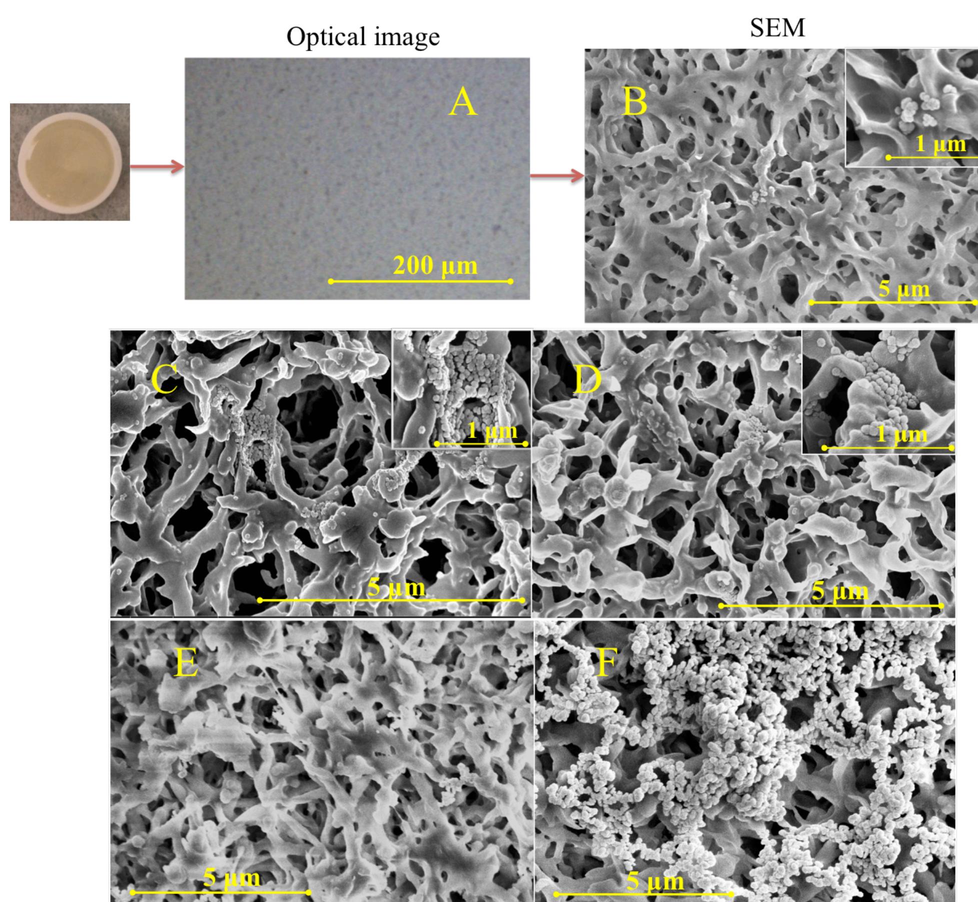

Figure 3. Optical and SEM (A) of a PVDF membrane coated with 60 nm Ag aggregated by 10 nm NaCl. SEM image of PVDF membrane coated with 60 nm Ag NPs aggregated by 50 mM NaCl (B and C), and 75 mM NaCl (D and E), respectively.

Number of times that sample is filtered through the membrane also affects the intensity of SERS signal. The SERS signals were detected when 2 ml or 5 ml 100 ppb ferbam sample was filtered through the membrane for 1, 2, 3 and 4 times, respectively (figure 4). When sample volume was 2 ml, signal intensity increases as the times filtration increased at the beginning, which means the binding of target molecules on the Ag NPs surface being more

efficient. However, when filtered for the fourth time, the signal intensity dropped as too much times of filtration may interrupt the surface distribution of Ag NPs and damage the membrane. In the case of 5 ml of ferbam, this phenomenon was more obvious as 2 times of filtration being sufficient to reach its maximum effect. For future experiments, Ag NPs was filtered through for 3 times to make sure all NPs were coated onto the membrane although the color of the filtered liquid remained visually unchanged after the first time.

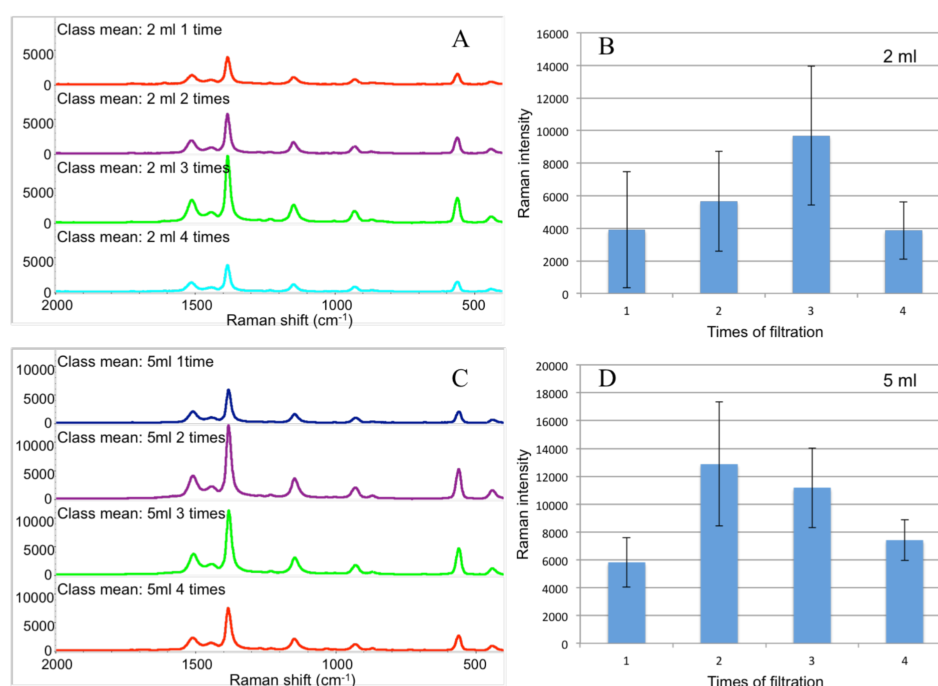

Figure 4. SERS spectra of 2 ml and 5 ml 100 ppb ferbam with 1, 2, 3, 4 times of filtration (A, C), and the peak intensities at 1380 cm-1 with standard deviation displayed as error bars (B, D).

60 nm Ag and 75 mM NaCl was used for the quantitative study, in which 2 ml sample was filtered through the membrane for 3 times at each concentration. The means of the highest peak intensity of 8 spots within each concentration were calculated and plotted, with standard deviation displayed as error bars. A linear regression exists between the concentration of analyte (from 2.5 ppb to 75 ppb) and the SERS intensity of the 1380 cm-1

peak (figure 5). The limit of detection was calculated to be 1.16 ppb.

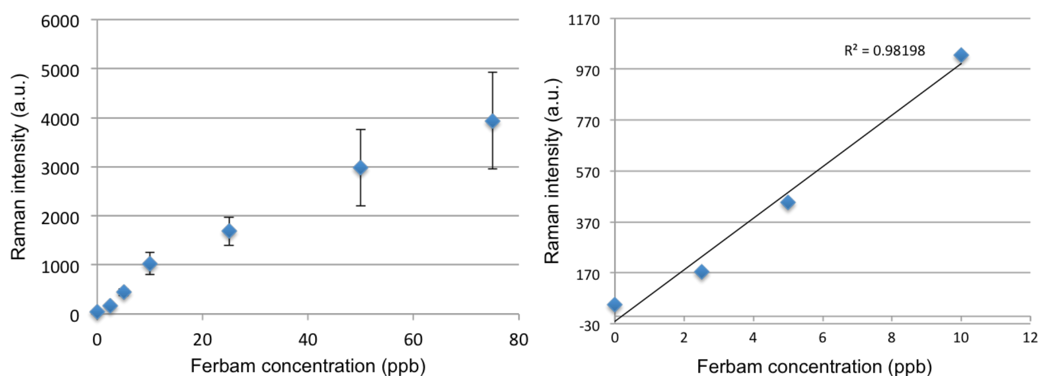

Figure 5. Plots of SERS peak intensities at 1380 cm^{-1} with regard to different ferbam concentrations with standard deviation displayed as error bars (left), and linear regression plotted near lowest detection concentrations to show the calculation of LOD (right).

When spiked into three environmental samples, SERS signal of ferbam decreased to different levels. With the final concentration of ferbam being 100 ppb, the SERS intensity in DD water and tap water experienced approximately 60% decrease compared to the control in 50% acetonitrile, whereas SERS intensity in pond water only decreased approximately 40% (figure 6). The decrease of water samples compared to the control in 50% acetonitrile may be because of the decreased solubility of ferbam in water compared to organic solvent. The higher intensity displayed in pond water compared to other water samples is possibly due to the organic compounds that exist in the pond water, including salts and minerals that enhance the signal in a unknown way.

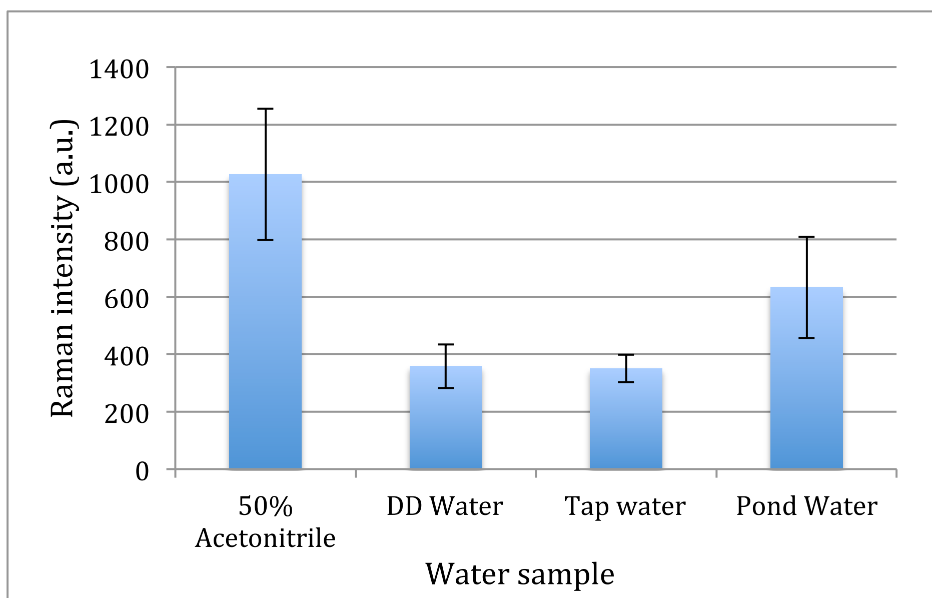

Figure 6. The maximum peak intensities of ferbam in 50% acetonitrile (control), DD water, tap water and pond water, respectively. Standard deviation is displayed as error bars.

The spectrum of 100 ppb level ferbam in pond water using a handheld Raman spectrometer shows almost identical signature peaks as by the bench top instrument (figure 8). Though with lower signal intensity, the portable Raman instrument is able to generate a strong signal within only a few minutes and most importantly, it is most suitable for on-site detection because of its portability.

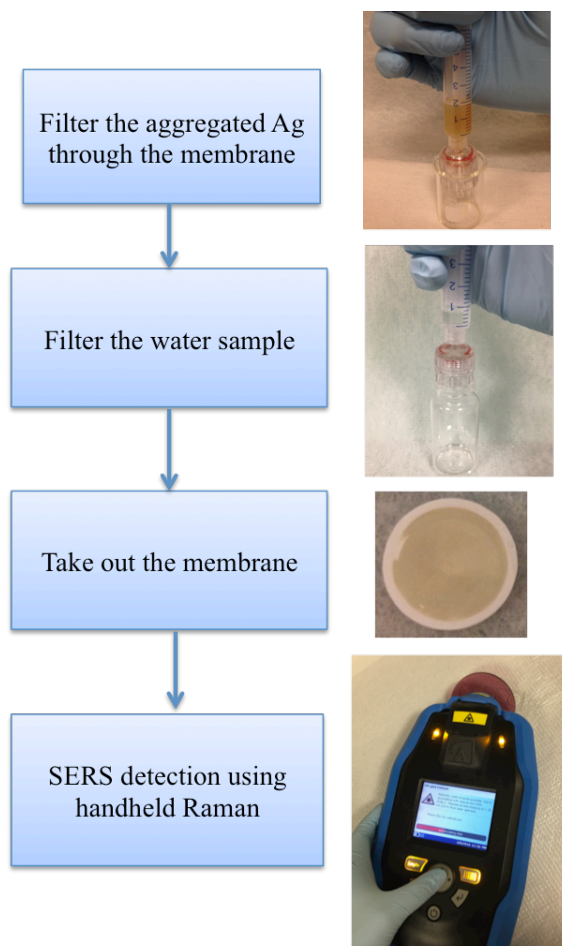

Figure 7. Schematic diagram of performing filter SERS with handheld Raman spectrometer.

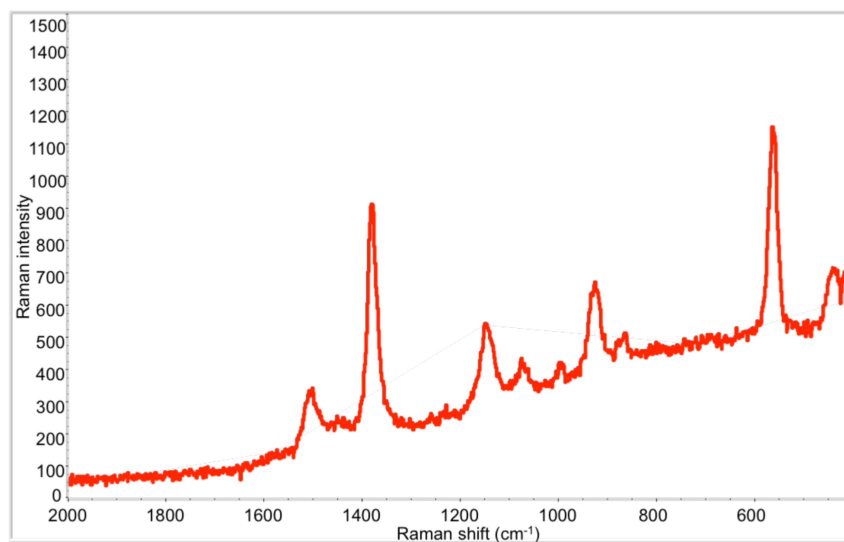

Figure 8. SERS spectrum of 100 ppb ferbam generated by the handheld Raman spectrometer using the developed filter SERS method.

2.4 Conclusion

The optimization of Ag NPs size, salt concentration, sample volume and pass times is important to achieve a sensitive and reliable result. The optimized filter based SERS method is able to detect 2.5 ppb ferbam in 50% acetonitrile and increasing sample volume could raise the sensitivity of this method, it also shows good potential for quantitative analysis. Based on its capability to detect pesticide ferbam in environmental water samples with a handheld Raman spectrometer, the developed method could be applied to detect various chemical contaminants in real water samples on site. One limitation involved with this method is its restriction on liquid samples that do not incorporate matrix interference. Pretreatment of samples are required for the applications in complex matrixes such as food and modifications of the substrate need to be developed for selective capture and detection of large molecules such as proteins.

CHAPTER 3

DEVELOPMENT AND APPLICATION OF FILTER BASED SERS FOR RAPID AND SENSITIVE DETECTION OF PROTEIN TOXIN IN WATER

3.1 Introduction

3.1.1 Ricin

Ricin is a heterogeneous proteinaceous toxin that naturally present in the castor bean (Ricinus communis). The ricin molecule is composed of two chains, A and B, connected by a disulfide bond. Both chains are needed for vivo toxicity[60]. The estimated LD50 for ricin in humans is approximately 5-10 µg/kg through inhalation and 1-20 mg /kg or 8 castor beans through ingestion[61-62]. The Centers for Disease Control and Prevention (CDC) categorizes ricin as a Category B agent (second-highest priority), which requires specific enhancement of the CDC's diagnostic and disease surveillance capacity[63]. It has been considered one of the most toxic compound known, and has been a popular subject in both medical and basic research since its discovery in the 1880s. While ricin shows potential applications in cancer therapy, it can be exposed to human by aerosol release into the environment or adulteration of food and beverages[64]. Due to its easy accessibility and severe toxicity, there is an urgent need for rapid and sensitive detections and quantitation method on the trace amount of ricin.

3.1.2 Traditional ways of detection and their limitations

Current methods for Ricin detection are mainly immuno-based method, including enzyme-linked immunosorbent assay (ELISA) and immunoassay using radioactive labeling[65-69], and toxicity based methods[70-71], which mainly utilize antibody for the capture of ricin molecule. Although offering high sensitivity, these methods could be labor-intensive which often involves complex assay procedures, plus false positive and negative results are

often encountered.

The aptamer-based label-free SERS method used in this study exhibited many advantages over the other methods. The aptamers are single-stranded DNA or RNA that can bind to a specific target molecule, which was proven to be more user-friendly, economic, sensitive, and more suitable for rapid detection[72].

3.1.3 Objective of this study

The objective is to develop a rapid, sensitive and flexible method for detection of non-toxic ricin B chain in water samples by first fabricating a sensitive SERS substrate using Ag nanoparticles by a filter-syringe system and then integrating aptamer specifically designed to capture ricin B chain on the substrate for detection. The result of a good capture by the ricin b chain aptamer will be a potentially good biosensor for capturing Ricin whole molecule.

3.2 materials and methods

3.2.1 Sample preparation

Citrate-coated Ag nanoparticles was with size of 40 nm, 60 nm, 80 nm and 20 mg/L were purchased from Nanopartz. Ferbam (Chem Service) is dissolved in 50% acetonitrile. The aptamer that was used to capture ricin is a thiol-modified SSRA-1 ricin aptamer (sequence thiol-5' ACACCCACCGCAGGCAGACGCAACGCCTCGGAGACTAGCC 3'). Ricin B chain was purchased from Vector Labs and was diluted with double distilled water or 1xPBS solution. EMD Millipore Durapore PVDF Membrane Filters and polycarbonate Filter Holders from Cole-Parmer was used in this experiment.

3.2.2 Ricin detection using aptamer-conjugated SERS substrate

2mL 20 ppm Ag colloid with 80nm particle size was aggregated by 2ml of 50 mM NaCl, the mixture was put on a nutating mixer at 20 rpm for 10 minutes. Then the aggregated Ag colloid was filtered through a filter membrane to fabricate the SERS substrate. After rinsing the membrane with 2 ml double distilled water, the Ag NPs-coated membrane was immersed in 1000 μl of 100-μM aptamer and put on a nutating mixer at 20 rpm for 5 hours. The membrane coated with Ag NPs and aptamer could be directly detected with Raman for the aptamer background. For ricin detection, 5 mL ricin b chain solution was filtered through the Ag-Ap coated membrane for 2 times and air dried before SERS detection.

SERS spectrum of Ricin b chain in water was collected using solution-based method, which was done by mixing 0.5mL 20ppm 80 nm Ag nanoparticles with 100ul 10 ppm ricin b chain aqueous solution for 2 hours, then centrifuging the mixture at 10 g for 5 minutes and collecting 5 μL concentrated solution on a gold slide. The sample is then air dried before it's ready for SERS detection.

3.3 Results and discussion

The results for the detection of 100 ppb ricin b chain in aqueous solution are shown in figure 14-16. From the spectra in figure 14 and 15, we are able to spot some changes in the spectra of the aptamer before and after capturing ricin b chain molecule, the changes are more obvious in the second derivative spectra. Unique peaks in figure 10 at 925, 1127, and 1269 cm^{-1} are only visible after ricin capture. Combining the spectrum of Ricin control on Ag (figure 10), we can confirm that those changes come from specific binding of ricin molecule. The changes in spectra after ricin b chain binding could be further verified by principle

component analysis (PCA) of the second derivative spectra, which clearly distinguish three groups of data, namely aptamer on Ag, aptamer on Ag after specific binding of ricin b and ricin on Ag.

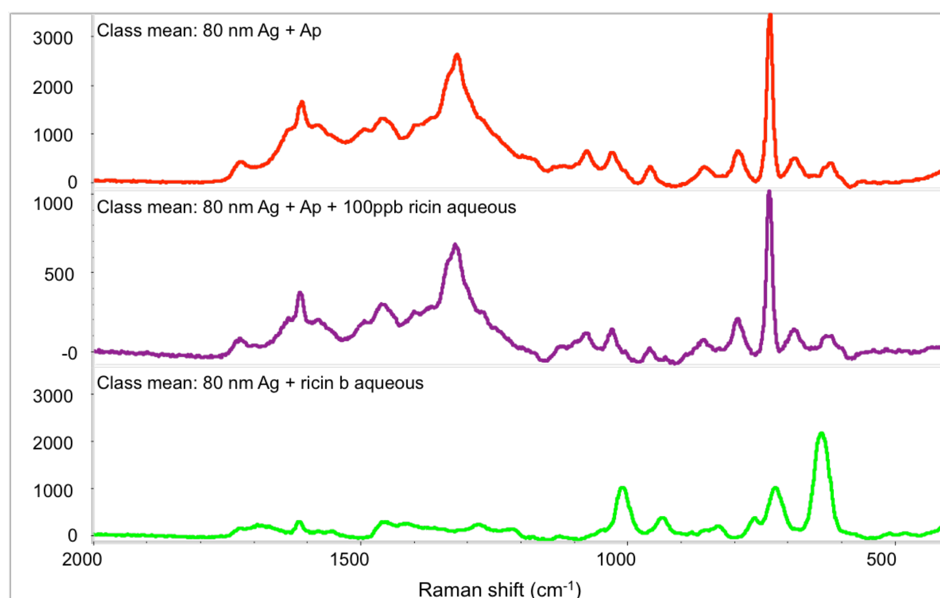

Figure 9. Aptamer on Ag, 100 ppb ricin B chain in aqueous solution captured by aptamer on Ag substrate, spectra of 10 ppm Ricin b chain on Ag.

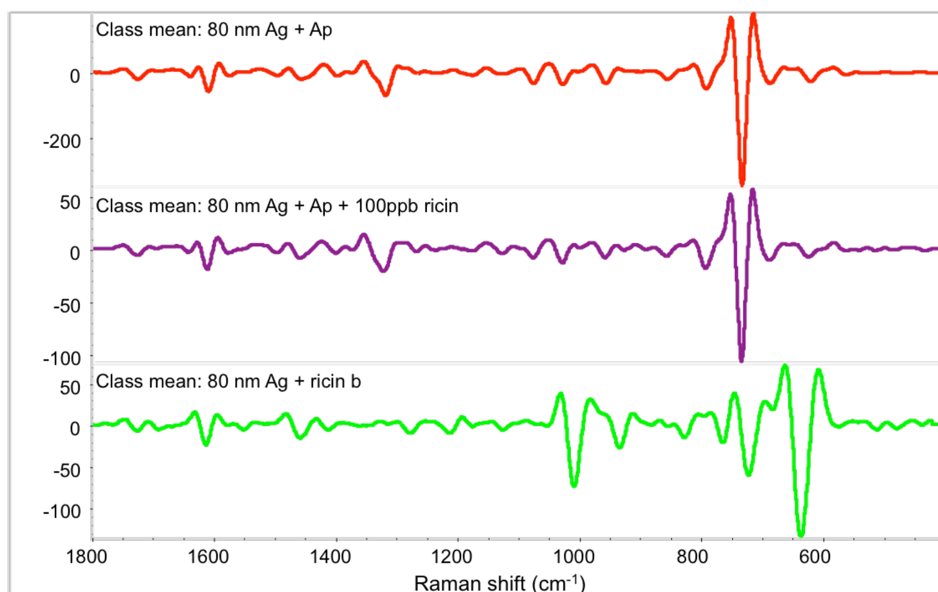

Figure 10. Second derivative spectra of aptamer on Ag, 100 ppb ricin B chain in aqueous solution captured by aptamer on Ag substrate, spectra of 10 ppm Ricin b chain on Ag.

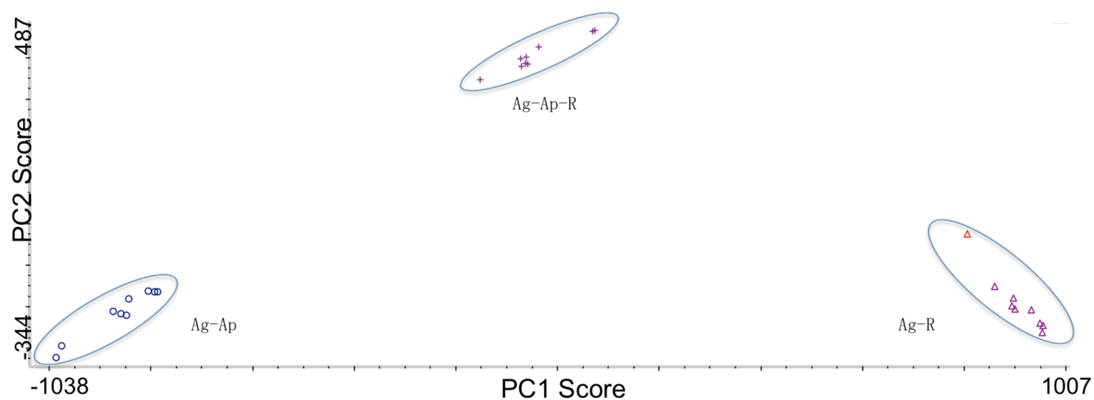

Figure 11. PCA plot from the second derivative spectra of Ag-Ap (circle), Ag-Ap-R (cross) and Ag-R (triangle). Ag: silver, Ap: aptamer, R: ricin B chain.

3.4 Conclusion

This aptamer-based filter SERS method has successfully detected ricin b chain in water down to 100 ppb level. It has shown potential application to detect various protein toxins in water samples. However, there are some barriers existing within this filter SERS method for detecting proteins in complex food matrix sample purification and the interference of other biological molecules.

CHAPTER 4

DEVELOPMENT AND APPLICATION OF FILTER BASED SERS FOR RAPID AND SENSITIVE DETECTION OF BACTERIA CELLS

4.1 Introduction

4.1.1 Bacteria in food

Bacteria are prokaryotic microorganisms that widely exist soil, water, acidic hot springs, radioactive waste, and the deep portions of Earth's crust[73]. Bacteria are involved in human life through various pathways and one important pathway is through food. They could be microbial cultures deliberately added during food production[74] or pathogenic microorganisms that are caused by contamination or poor hygiene. Microbiological hazards have long been a serious issue concerning public health and safety. There are approximately 65 million cases of food borne disease in the US annually. According to centers for disease control and prevention (CDC), Escherichia coli, Listeria monocytogenes, Campylobacter jejuni, and Salmonella have been identified as the major food borne pathogens[75].

4.1.2 Bacteria in water

The safety of drinking water is essential to human well-being. However, in some developed countries, people do not have access to clean, sanitized drinking water, in which pathogenic bacteria can survive and can cause bacterial transmitted diseases. According to the WHO, the mortality of water-associated diseases exceeds 5 million people per year with more than 50% categorized as microbial intestinal infections. The sources of microbial contamination in drinking water is mainly associated with wastewater discharges in freshwater and coastal seawater, which is the major source of fecal microorganisms, including pathogens. Some of the pathogens responsible for the main bacterial infections in water are Salmonella, Vibrio, E.

Coli. EPA has set the standards to monitor fecal indicator bacteria such as E.Coli to indicator the presence of pathogenic microorganisms and the maximum contaminant level goals are zero in most cases. However, there are no standard techniques available to detect particularly low levels of indicator bacteria[76].

4.1.3 Traditional ways of detection and their limitations

Culture method followed by standard plate count has been the most developed and widely used method for pathogen detection, however it usually take days to produce result, and not able to isolate viable but nonculturable organisms[77]. New and advanced technologies have been developed for the detection of foodborne pathogens aimed at overcoming disadvantages associated with traditional microbiological detection techniques, including immunomagnetic separation (IMS)[78-79], polymerase chain reaction (PCR)[80-81] and a variety of biosensor based methods[82]. Unfortunately, these methods can be labor intensive or normally involve complicated procedures and time consuming, false-positive results could be obtained.

4.1.4 Objective of this study

Surface enhanced Raman spectroscopy is a powerful tool to detect and study biological systems on the molecular level because of its ability to provide a unique vibrational signature of the biological species[39]. There have been many attempts to utilize SERS for the detection and identification of bacterial pathogens[83-85]. The objective of this study is to detect some of the most common food pathogens (e.g. *Salmonella* and *E. Coli*) in liquid samples using the developed filter SERS method using 4-mercaptophenylboronic acid (4-mpba) as probe and indicator for bacteria cells. The reason for using an indicator molecule is because that the SERS spectra of bacteria are very weak and inconsistent. 4-MPBA has several unique

properties for detecting bacteria cells: The boronic acid structure consists of a diol group that selectively binds to the peptidoglycan in the bacteria membrane; 4MPBA is capable of producing strong and characteristic SERS signal; it can react with both gram negative and positive bacteria. The reason for using a filter membrane is to isolate and concentrate bacteria cells directly from water sample because of the unique dimension of the membrane pores and its inertness to most chemicals, including 4-mpba. The developed technique will be a rapid and sensitive method to screen bacteria cells at low levels, and have a potential to quantify the bacterial levels in water.

4.2 Materials and methods

4.2.1 Materials

Citrate coated Ag nanoparticles with size of 50 nm and 20 mg/L concentration were purchased form Nanopartz. 4-mercaptophenylboronic acid was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA) and ammonium bicarbonate was from Fisher Scientific (Fair Lawn, NJ, U.S.A). Durapore PVDF filter membranes with 0.1 μm pore size were from EMD Millipore Inc. (Billerica, MA, USA). Nitrocellulose membrane filters with 0.2 μm pore size are purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA) and polycarbonate Filter Holders from Cole-Parmer Inc. (Vernon Hills, IL, USA) was used in this experiment. *Escherichia Coli* 43888 was used in this assay.

4.2.2 Sample preparation

E. Coli is inoculated in TSB media for 16 hours to obtain an approximately 10^9 CFU/ml concentration. 1 ml bacteria culture is taken from the culture and washed 3 times with 0.9% NaCl at 6.5 rpm for 3 minutes. The washed bacteria were diluted to make serial dilutions with

0.9% NaCl (10^7 CFU/ml to 10^2 CFU/ml). The concentration of the bacteria is determined by enumeration in triplicate on tryptic soy agar plate at 37 °C for 24h. 50 mM ammonium bicarbonate solution was made by dissolving 0.1976 g ammonium bicarbonate in 50 mL water. 4-mpba is dissolved in ethanol to make 50mM stock solution and diluted to 10mM with ammonium bicarbonate, and could be diluted with ammonium bicarbonate to 100 μM upon detection. 0.5 ml Au nanoparticles is diluted with 0.5 ml water to make 10 mg/L solution.

4.2.3 SERS measurement of bacteria using 4-MPBA as probe and indicator

4.2.3.1 Immersion method

1 ml of bacteria solution was filtrated onto a membrane. The membrane was first filtrated with 1 ml water to eliminate any small molecules and then detached and put onto a dry tissue to absorb excess liquid. Then the membrane was immersed in 1 ml of 50 mM and 100 mM 4mpba solution for 30 minutes to allow binding between bacteria and 4mpba molecule. After 30 minutes, transfer the membrane to a clean, dry filter holder and wash the membrane with 2.5 ml ammonium bicarbonate solution by filtration. This step is to wash away any excess 4mpba molecules that is not binding to the bacteria surface. Separate the membrane and rinse with 1 ml ammonium bicarbonate solution before filtrate 1 ml of Au nanoparticles onto the membrane. To perform the control, filtrate 1 ml 0.9% NaCl solution instead of bacteria to the membrane followed by the same procedure in the bacteria sample preparation.

4.2.3.2 Filtration method

After integrating bacteria sample onto the membrane (same as 4.2.3.1), the membrane was transferred to a clean, dry filter holder. Start to filtrate 1 ml of 100 μM 4-MPBA solution

through the membrane and stop filtration when there is approximately 0.5 ml solution left in the syringe and let the rest of the 4-MPBA solution incubating with bacteria cells for 30 minutes. The rest of the procedures are the same as the immersion method.

To perform the control, filtrate 1 ml 0.9% NaCl solution instead of bacteria to the membrane followed by the same procedure in the bacteria sample preparation.

4.2.3.3 SERS measurement

For SERS detection, a 780 nm laser with 3 mW laser power and 1s exposure time was used to scan the sample under 20 × objective using a 50 nm slit. For each bacteria sample, three 10 × 10 and one 20 × 20 mapping was scanned with 10 μm step size and all spots are randomly selected. For the control with 0.9% NaCl, three 10 × 10 mappings were scanned from three individual samples to study the variation of background signal. SERS mapping images were integrated based on the characteristic peak of 4-mpba at 1072 cm^{-1} using the OMNIC software (Fisher Scientific). SERS spectra is obtained by randomly selecting 10 points from a sample and data is analyzed with OMNIC software (Fisher Scientific).

4.2.4 Culture of the membrane containing bacteria in agar plate

Prepare a membrane sample containing bacteria followed by the same procedure in 4.2.3.2. Put the membrane on a tryptic soy agar plate with the side containing bacteria facing up. Press the membrane against the agar to make the membrane fully moisturized. The agar plate was incubated at 37°C for18h.

4.3 Results and discussion

4.3.1 Membrane type and effect of washing

Two types of membrane (PVDF and nitrocellulose) were compared for the performance

of the assay. Controls with water were performed in which 100 mM 4-mpba solution were filtrated onto each type of membrane and washed with ammonium bicarbonate. SERS spectra indicated that some of the 4-mpba molecules were retained on PVDF membrane even after the washing step which displayed the characteristic peak at 1072 cm^{-1} . This is possibly due to the hydrophilic coating on the PVDF material that interacts with 4mpba molecules. In the case nitrocellulose membrane, washing with ammonium bicarbonate greatly decreases the amount of 4-mpba on the membrane, and no SERS signals of 4mpba are visible, thus suitable for this assay.

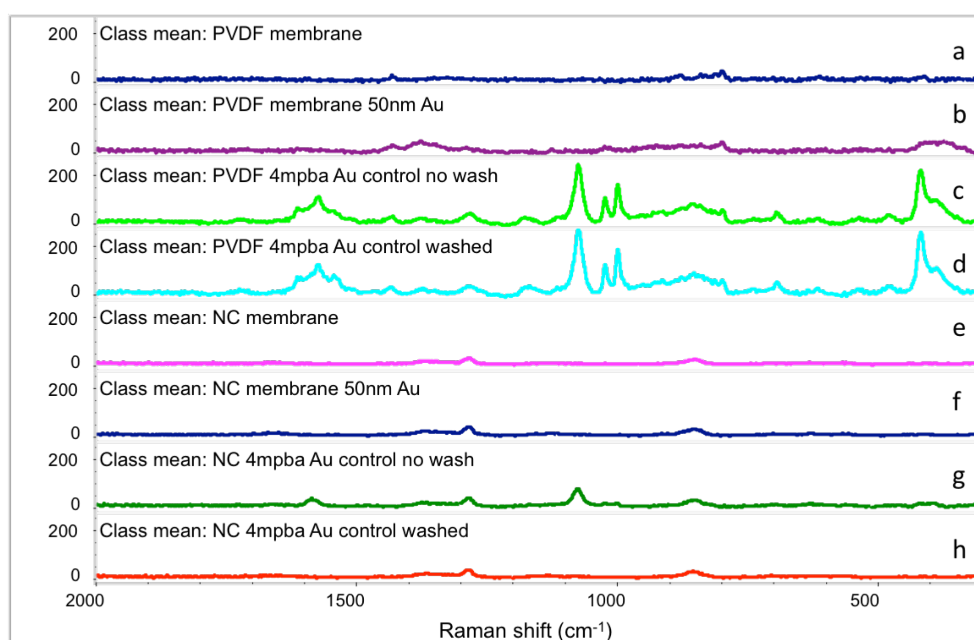

Figure 12. From top to bottom: SERS spectra of membrane, membrane with Au NPs, control with 4MPBA without washing, and control with 4MPBA with washing on PVDF (a-d) and nitrocellulose (e-h) membranes, respectively.

4.3.2 Methods of integrating 4-mpba

4.3.2.1 immersion method

Nitrocellulose membrane was used in this method and control was first measured to see if all 4-mpba molecules could be eliminated. After that, 1 ml of 10^7 CFU/ml E.Coli was tested.

With 50 mM 4-mpba, there are no visible positive points in the control, which indicated no 4-mpba after washing (figure 13 A). However, mapping of the bacteria sample shows only 20% positive points, which is not convincible for such a high bacterial concentration (figure 13 B). When increasing 4-mpba concentrations to 100 mM, positive spots are visible in the control, with only 8% positive points in the bacteria sample (figure 13 C and D). Most possible explanation for those results is that most of the bacteria cells were detached from the membrane surface during the immersion process and the interaction between bacteria and 4-mpba remain unknown.

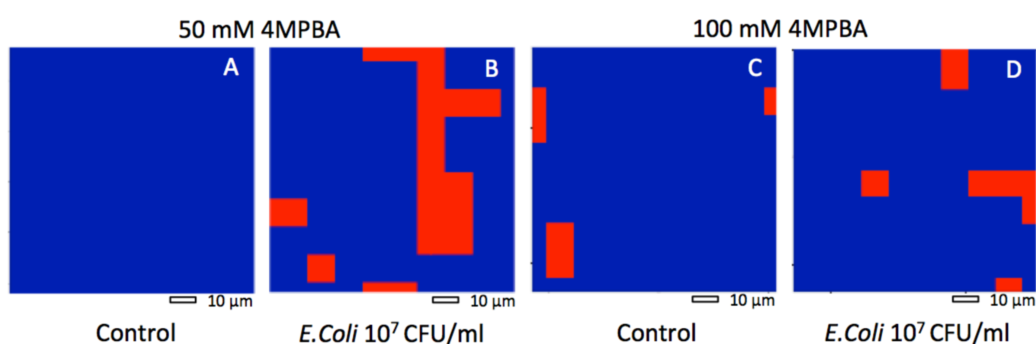

Figure 13. SERS mapping (10 × 10) of control and 10^7 E.Coli obtained from the immersion method using 50 mM 4-mpba (A and B) and 100 mM 4-mpba (C and D), respectively.

4.3.2.2 Filtration method

In the second approach, in order to retain the bacteria cells on the membrane, we tried filtration of the 4-mpba instead of immersion. From the SERS spectra obtained from this assay (figure 14), no 4-mpba peaks are shown in the controls whereas in the bacteria sample, the characteristic peak was visible.

Mapping results from three individual control samples was shown in figure 15, the highest value of each control were used to calculate the mean and standard deviation. The

threshold value for the background is set to be the mean plus three times the standard deviation, which was 17.1 in this assay.

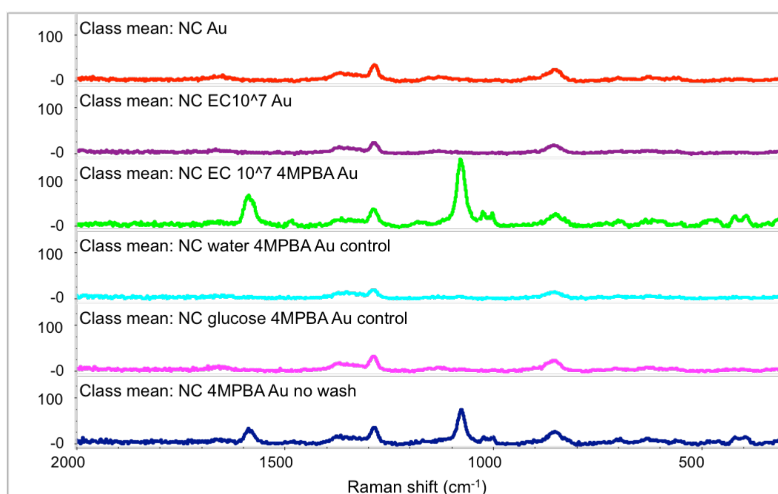

Figure 14. From top to bottom: SERS spectra of nitrocellulose membrane with Au NPs, membrane with E.Coli and Au, membrane with E.Coli integrated with 4-mpba and Au, 0.9% NaCl control with 4MPBA, 1 mM glucose with 4MPBA, membrane with 4-mpba and Au without washing.

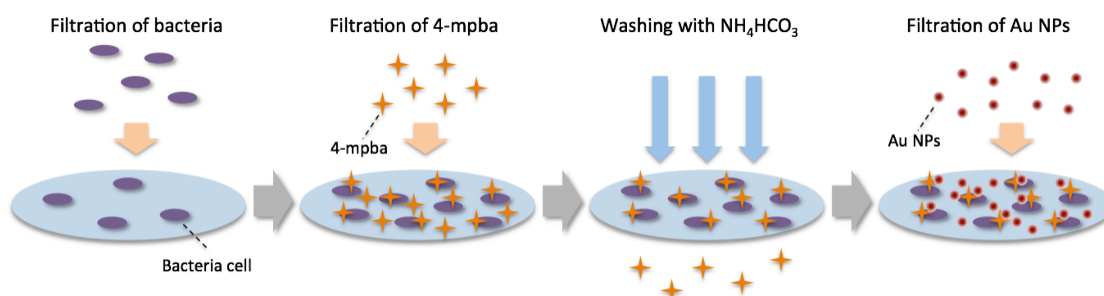

Figure 15. Schematic illustration of the filtration method to detect bacteria using 4-mpba as capture and indicator.

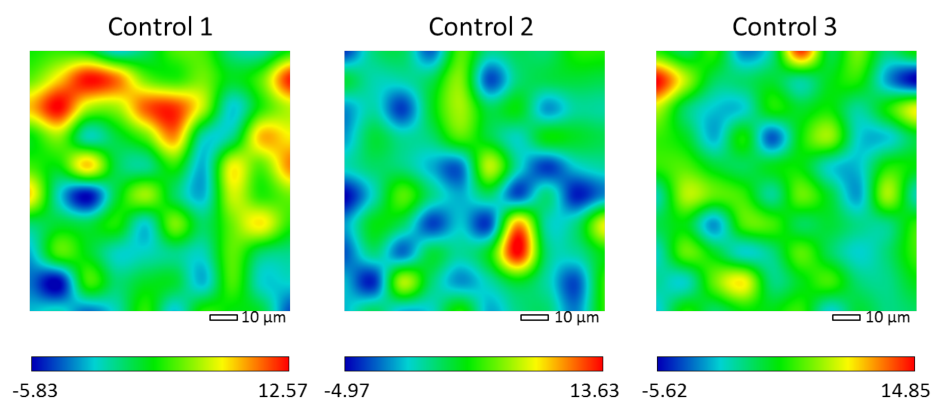

Figure 16. Mapping of three controls using 0.9% NaCl with intensity range displayed at the bottom of each map.

E.Coli of series concentrations are measured by SERS, with 20 × 20 mapping results shown in figure 17. Three 10 × 10 mappings were scanned for each concentration (percent positive points plotted in figure 18). The percent positive points of 20 × 20 mappings (figure 17) correlate well with the results from three 10 × 10 mappings (figure 18). The mappings shows the capability of this method to detect E.Coli down to 0.67×10^1 CFU/ml. The plot in figure 18 shows small variation in different spots on the membrane and the trend of increasing bacteria concentration.

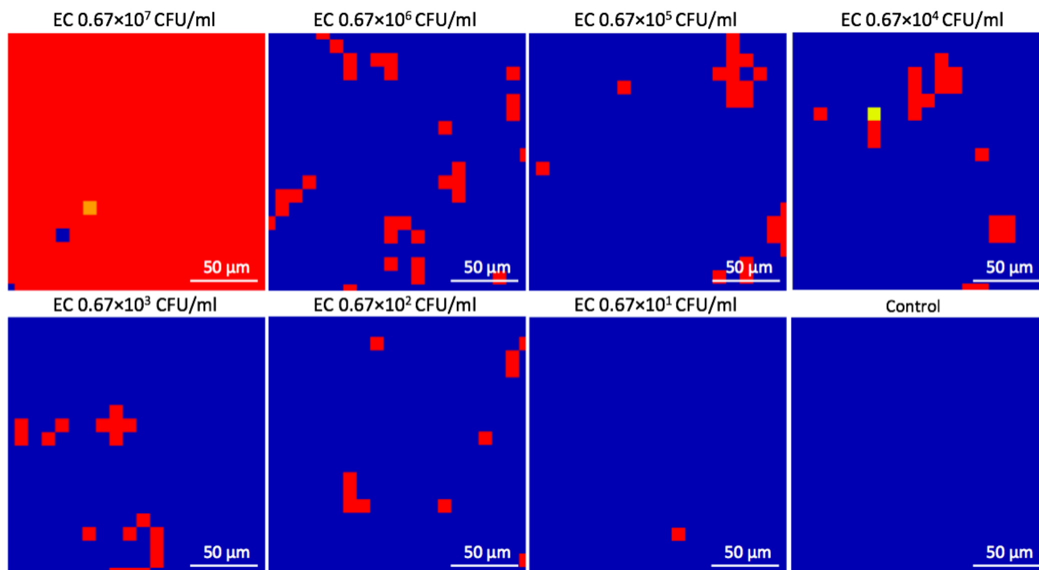

Figure 17. SERS mapping of *Escherichia. Coli* suspended in 0.9% NaCl (concentration from 0.67×10^7 to 0.67×10^1) and control with 0.9% NaCl solution.

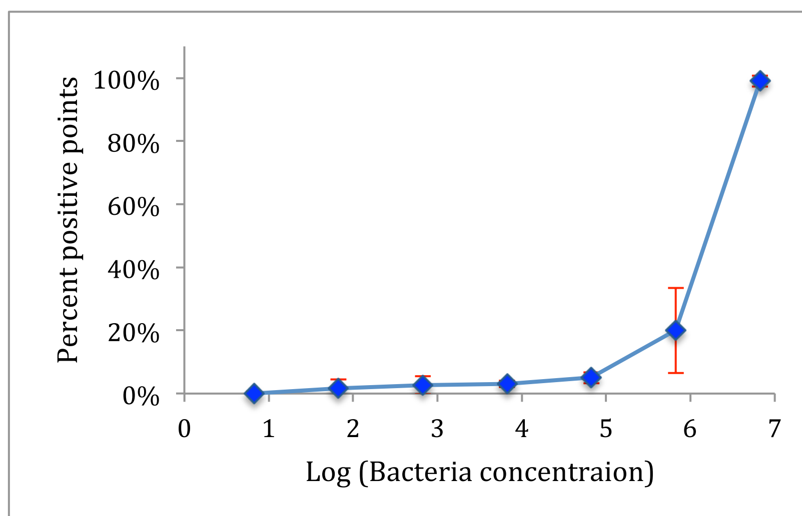

Figure 18. Means of percent positive points calculated from 3 mappings corresponding to different bacteria concentrations (displayed in log form), with standard deviation plotted as error bars.

4.3.3 Culture of bacteria containing membrane

Figure 18 shows the results of cultured membrane sample containing E.Coli of 10^3 CFU/ml and 10^2 CFU/ml. This confirms the viability of the bacteria cells after the interaction with 4-mpba and Au NPs although the number of cells significantly decreased compared to the spread plate results of the bacteria solution. This is partially due to the basic PH (around 9.2) of the 4mpba solution, also because the membranes were dried in between each step. The culture of membrane sample could serve as a validation for the positive signals from SERS.

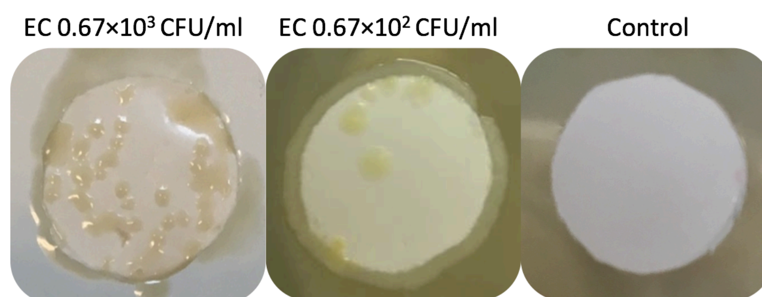

Figure 19. Membrane sample containing E.Coli of 10^3 CFU/ml and 10^2 CFU/ml and control incubated in TSA plate for 24h.

4.4 Conclusion

By integration 4mpba molecules, we are able to get mapping results directly from the filter membrane with consistent and sensitive signals. The percent of positive points in the mappings could be utilized to estimate the number of bacteria cells present in the sample. The membrane containing bacteria could be incubated in TSA plates for validation of the presence of bacteria. The developed method could detect E.Coli down to 10^1 CFU/ml, and SERS results could be validated with membrane culture down to 10^2 CFU/ml.

CHAPTER 5

CONCLUSION AND FUTURE WORK

Filter based SERS techniques offers flexibility and great sensitivity to detect varies targets in water including pesticide, protein toxin and bacteria. The filter membrane could be directly used to fabricate SERS substrate with metal nanoparticles, or be further modified with aptamers to detect large biomolecules like proteins. It could also serve as a substrate to concentrate target (e.g. bacterial cells) and utilized for detection after integrating capture and nanoparticles.

The filter-based method has shown great prospect to detect other pesticides and toxins with of without utilizing captures. The developed assay for bacteria detection could be further optimized to detect Gram positive bacteria and other species of Gram negative bacteria, even bacteria mixture to estimate the total bacterial level in water samples. By integrating captures specifically designed for a type of microorganism (e.g. bacteria, virus), the filter-based method could detect and distinguish different types of microorganism. With proper sample preparation, the developed filter based assays could be applied in simple liquid food matrixes such as fruit juices and skim milk. The membrane substrates could be analyzed not only with Raman spectroscopy, but also has the potential to be coupled with FT-IR to realize surface enhanced IR which may offer features that are not possessed by SERS.

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