

LABEL-FREE MICRO-RNA DETECTION USING SURFACE-ENHANCED RAMAN
SCATTERING AND DEVELOPMENT OF A LAB-ON-A-CHIP SERS DEVICE

by

JUSTIN LOUIS ABELL

(Under the Direction of Yiping Zhao and Ralph Tripp)

ABSTRACT

Label-free surface-enhanced Raman scattering (SERS) analysis of nucleic acids has been previously impeded by issues relating to the reproducibility of SERS measurements and SERS substrates, interpretation and quantification of the SERS signal of DNA, and development of suitable SERS-based devices. This dissertation will therefore focus on addressing these issues for microRNA (miRNA) biomarker detection.

Improved quantitative SERS analysis using silver nanorod (AgNR) substrates fabricated by oblique angle deposition (OAD) is demonstrated using a dynamic measuring technique. To process and analyze multiple DNA and RNA sequences in parallel, a multi-well array SERS chip is fabricated onto a large-area AgNR substrate using a molding method.

To detect miRNA with SERS, DNA is immobilized onto the AgNR substrate to act as a probe to specifically capture complementary miRNA. A linear least squares (LS) regression analysis is developed to decompose the resulting SERS spectrum of DNA to the spectra of the pure nucleotides, and determine the relative contribution of each type of nucleotide within the measured DNA signal. This can be used to distinguish if hybridization/capture has occurred or not. Despite the high degree of spectral similarity observed for the immobilized DNA probe before and after capture of the miRNA, the LS

method is capable of quantitatively determining if, and how much, hybridization has occurred.

In addition, the effect of solvents on the AgNR SERS response is evaluated using thiol test analytes. The AgNRs are discovered to bundle together upon drying of the solvent, which induces the formation of SERS hot spots. With proper surface functionalization of the AgNRs, the nanorod bundling/de-bundling is shown to be a reversible process that depends on the solvent polarity.

The feasibility of utilizing the AgNRs as electrodes is also investigated. Applying a negative voltage to the AgNRs yields improved SERS signal of a test analyte, yet does not induce electrochemical degradation of the AgNRs. The OAD fabrication technique is also integrated with traditional lift-off processes to form AgNR microelectrodes, and a novel lab-on-a-chip (LOC) SERS device is constructed.

INDEX WORDS: Surface-enhanced Raman spectroscopy, silver nanorods, nucleotide, label-free, DNA functionalization, microRNA detection, linear least squares regression analysis, microelectrode, lift-off, lab-on-a-chip

LABEL-FREE MICRO-RNA DETECTION USING SURFACE-ENHANCED RAMAN
SCATTERING AND DEVELOPMENT OF A LAB-ON-A-CHIP SERS DEVICE

by

JUSTIN LOUIS ABELL

B.S., University of Georgia, 2004

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2012

© 2012

Justin Louis Abell

All Rights Reserved

LABEL-FREE MICRO-RNA DETECTION USING SURFACE-ENHANCED RAMAN
SCATTERING AND DEVELOPMENT OF A LAB-ON-A-CHIP SERS DEVICE

by

JUSTIN LOUIS ABELL

Major Professors:	Yiping Zhao Ralph Tripp
Committee:	Leidong Mao Jason Locklin Yao-Wen Huang

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December, 2012

ACKNOWLEDGEMENTS

I would like to thank my advisor Dr. Yiping Zhao for years of scholarly guidance, scientific insight, and financial support. He has proven to be an exceptional mentor and a great deal of my success as a student would not be possible without him. I am also grateful to my co-advisor, Dr. Ralph Tripp, who has offered scientific and financial assistance. In addition, he was a major influence on my ultimate decision to pursue the graduate school career that I am now completing. I would like to thank my advisory committee members Dr. Leidong Mao, Dr. Jason Locklin, and Dr. Yao-Wen Huang for guidance and helpful advice over the years.

I also owe special thanks to Dr. Jeremy D. Driskell, who has offered a significant amount of invaluable technical insight during my years as graduate student. I am also grateful to Dr. Richard Dluhy for helpful scientific discussions, in addition to his generosity to allow me to use instruments in his lab. Mr. Pierre Negri and Mr. Gareth Sheppard are also acknowledged for their helpful technical assistance.

The Instrument Shop at the University of Georgia has also played an integral role in my research, much of which would not have been possible without them. Mr. Matthew Ritz is also acknowledged for lending his programming skills, which has directly made my research much more productive. I thank Jeonifer Garren not only as an academic peer for invaluable insight about statistics and research, but also thank her personally for years of moral and emotional support.

Finally, I would like to acknowledge many of my fellow lab members, both past and present, for much help over the years: Dr. John Gibbs, Dr. Wilson Smith, Dr. Yongjun Liu, Dr. Junxue Fu, Dr. Vivien Chu, Dr. Yu Zhu, Dr. Yuping He, Dr. Jianguo Fan, Dr. Slade Jokela, Mr. George Larson, Mr. Xiaomeng Wu, Ms. Jing Chen, and Dr. Kun Yao.

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	iv
LIST OF TABLES	vii
LIST OF FIGURES	viii
CHAPTER	
1 INTRODUCTION	1
1.1 The Current State of Nucleic Acid Detection	1
1.2 Label-Free miRNA Detection	7
1.3 Surface-Enhanced Raman Scattering (SERS)	9
1.4 Silver Nanorod Array SERS Substrates Fabricated by Oblique Angle Deposition.....	22
1.5 Organization of this Dissertation.....	26
2 TOWARDS QUANTITATIVE SERS MICROARRAYS.....	28
2.1 Dynamic Rastering Measurements for Improved Detection Reproducibility	28
2.2 Design, Fabrication, and Evaluation of AgNR Array Chip Device for High Throughput SERS Screening	34
2.3 Conclusions	44
3 LABEL-FREE MICRO-RNA HYBRIDIZATION AND DETECTION USING A SILVER NANOROD SERS CHIP.....	45
3.1 SERS Analysis of miRNA Building Blocks: A, C, G, and U	47
3.2 Functionalizing AgNRs with DNA Probes and Spacer Molecules	50

3.3	Hybridization and SERS Detection of miRNA on AgNRs	61
3.4	Quantitative SERS Detection of miRNA Using Least Squares Regression Analysis	71
3.5	Conclusions	84
4	<i>IN SITU</i> SILVER NANOROD SERS MEASUREMENTS IN SOLVENT.....	85
4.1	Effects of Water on the SERS Spectrum of Thiol-Based Analytes	86
4.2	The Affect of Solvent Polarity on the SERS Intensity	92
4.3	Solvent-Induced Bundling of AgNRs	95
4.4	Reversible AgNR Nano-Bundling: The Effect of Solvent Polarity	105
4.5	Conclusions	109
5	SILVER NANOROD LAB-ON-A-CHIP ELECTROCELL FOR ELECTRIC FIELD-ASSISTED SERS ANALYSIS.....	110
5.1	E-field Assisted Detection for Label-Based Nucleic Acid Analysis	111
5.2	Integration of AgNR Substrates into a Electrocell Device	114
5.3	E-field and Electrical Potential Effects on SERS	128
5.4	AgNR Lab-on-a-Chip Microfluidic Electrocell Device	144
5.5	Summary and Conclusions	152
6	Conclusions and Future Work.....	154
	REFERENCES	158
	APPENDICES	177
	Appendix A.....	177
	Appendix B.....	180

LIST OF TABLES

	Page
Table 1.1. Comparison of experiments and results of selected publications of label-free SERS DNA analysis.....	21
Table 3.1. Ribonucleic acid nucleotide structures of A, G, C, and U with the nucleobases highlighted in red. The deoxyribonucleic acid of thymidine (T) is also shown.....	48
Table 3.2. Sequences, absolute nucleotide ratios, and relative nucleotide ratios of DNA probes and miRNA targets.....	53

LIST OF FIGURES

	Page
Figure 1.1. Illustration of traditional DNA detection technologies. A) Quantitative real-time PCR: 1) A double stranded sequence of DNA is separated into two single stranded sequences with thermal treatment. 2) A probe sequence (with fluorescent signal quenched) hybridizes with complementary portions of the ssDNA. Primer sequences are also added (not shown) that hybridize with one end of the ssDNA allowing polymerase to transcribe complementary sequences (light blue). 3) During transcription, the probe sequence is degraded, releasing the fluorescent marker from the quenching agent (black circle); the transcription process has doubled the amount of DNA. B) Microarray: specific DNA probe sequences are immobilized onto a substrate at known locations. Then fluorescently labeled miRNA targets are added and allowed to hybridize to complementary DNA probes. After washing away excess miRNA targets, a fluorescent signal can be detected where miRNA targets have hybridized with the complementary probes.....	6
Figure 1.2. Illustration of infrared, Rayleigh, and Raman scattering.....	8
Figure 1.3. Calculated E-field enhancement of spherical shell dimer under optical excitation. Analytes experiencing a large field enhancement (white star) and lower field enhancement (green star) are presented for illustrative purposes. <i>(Adapted with permission from Ref [18]. Copyright (2010) American Chemical Society.)</i>	11

Figure 1.4: Electron micrographs of common SERS substrates: A) ORC electrode, B) colloids, and C) AgFON. (*A adapted with permission from Ref [21]. Copyright (2010) American Chemical Society. B adapted with permission from Ref [26]. Copyright (2008) John Wiley and Sons. C adapted with permission from Ref [28]. Copyright (2006) American Chemical Society.*).....14

Figure 1.5. A) Extrinsic detection of DNA hybridization using an immobilized probe and a SERRS-active Raman label covalently bound to the target DNA. Excitation with an appropriate wavelength (red) generates a strong SERRS signal (green). B) Intrinsic detection of DNA hybridization. Excitation produces a SERS signal of all the DNA components, e.g. nucleotides.....15

Figure 1.6. Selected SERS spectra of DNA from literature: A) 70-nt thiolated DNA thermally pretreated and adsorbed onto Au-nanoshells, B) 30-nt unmodified ssDNA on Ag colloids synthesized with hydroxylamine and aggregated with MgSO_4 , and C) 12-nt unmodified dsDNA on Ag colloids aggregated with spermidine. The x-axis scale is the same for A, B, and C, but the spectra are not scaled equally on the y-axis. (*A adapted with permission from Ref [46]. Copyright (2008) American Chemical Society. B adapted with permission from Ref [51]. Copyright (2012) John Wiley and Sons. C adapted with permission from Ref [45]. Copyright (2006) American Chemical Society.*).....20

Figure 1.7. Diagram of OAD deposition configuration showing the Ag vapor originating from Ag source material and a glass substrate rotated 86° relative to the incident vapor flux; 1) before rotating, a Ti adhesion layer and then Ag film is first deposited onto the glass substrate with the substrate surface directly facing the incident vapor; 2) the substrate is rotated 86° relative to the source vapor and nucleation points begin to grow due to the shadowing effect; 3) Ag deposition is continued, allowing the nucleations to grow into a tilted nanorod array.....	23
Figure 1.8. Top and side-view scanning electron micrographs of OAD-generated AgNR film..	24
Figure 2.1. Picture of the stepper motor translation stage: 1) translating metal stage, 2) stationary Raman probe, 3) AgNR SERS substrate, and 4) stepper motor.....	30
Figure 2.2. Representative SERS spectra of EZ-link Biotin HPDP. The peak near 1052 cm^{-1} is used for quantitative analysis. The structure of the molecule is also presented. (From Ref [64]).....	31
Figure 2.3. The RSD calculated from 15 substrates, each measured at multiple locations ($n = 5$ or 9) using standard static measurements or rotating rastering measurements. The rastering measurements demonstrate a strong tendency to produce a lower RSD for the substrates. The dark and light dashed lines show the average RSD for the static and rastering measurements, respectively. (From Ref [64].).....	32
Figure 2.4. Effect of rotation A) radius r and B) frequency f on the average RSD of $n = 6$ AgNR substrates using a chopper motor to rotate the substrates. (From Ref [64].).....	33

Figure 2.5. Normalized I_{SERS} versus cumulative exposure time over a period of ~15-30 minutes for A) standard static measurements and B) dynamic rastering/translating measurements. Each plot represents a different location on the substrate or a different substrate. All plots are normalized to the first data point of each data set and the plots are vertically offset for clarity. (From Ref [64].)	34
Figure 2.6. A- Components for of the well patterning mold assembly: a) 1 x 3 in ² AgNR substrate, b) well patterning plate, c) well molding posts, d) top plate, and e) base plate. B - Fabrication process of PDMS wells: i) AgNRs are first uniformly deposited onto a glass microscope slide, ii) attach the aluminum mold so that molding posts are in direct contact with the AgNRs, iii) injecting PDMS, and iv) after curing the mold is removed. C - Picture of a completed PMDS-patterned 4 x 10 well AgNR SERS chip.	37
Figure 2.7. Mapping of average I_{1200} acquired from all 40 wells for each treatment A) untreated, B) MeOH only, C) 1×10^{-8} M, D) 1×10^{-7} M, E) 1×10^{-6} M, and F) 1×10^{-5} M BPE. The average I_{1200} is represented by the logarithmic gray scale with units of counts (shown at the right). Each rectangular panel represents a single well. The well in the lower left corner can be seen to be unresponsive and is excluded from the analysis. (From Ref [75].)	39
Figure 2.8. A) Bar graph showing the mean I_{1200} of 39 wells for each BPE concentration C treatment from Figure 2.7. The error bars represent the standard deviation, and the RSD is provided for each treatment. B) A high resolution spatial I_{1200} mapping after addition of drying of BPE sample in the well. The mapping is composed of ~5000 points measured at 50 μ m steps; the I_{1200} is expressed by the gray scale shown to the right. (From Ref [75].)	40

Figure 2.9. A) The I_{1200} vs C_{BPE} for the 39 active wells each fit with a linear regression (thin lines). The average linear regression is plotted as the bold line. B) The average apparent sensitivity (from part A; with respect to N_{BPE}) compared to the apparent sensitivity calculated from BPE data from Driskell et al. and Chu et al. (From Ref [75].).....	42
Figure 2.10. TOP: The I_{1200} of each of the 40 BPE-treated wells from a cylindrical and conical well chip. The solid horizontal lines represent the average I_{1200} acquired from the whole chip, and the dotted lines represent one standard deviation above and below the average. (From Ref [80].) BOTTOM: Cross sectional diagram showing the profile of the cylindrical and conical wells (not drawn to scale).....	43
Figure 2.11. The 730 cm^{-1} adenine peak intensity (I_{730}) plotted as a function of thiolated let-7f DNA probe concentration for both the cylindrical and conical wells. (Data courtesy of Jeremy Driskell.).....	44
Figure 3.1. Average SERS spectra acquired for each MNT from Ar^+ plasma-cleaned AgNR substrates. The spectra are all scaled equivalently and vertically offset for clarity. (From Ref [82].).....	49
Figure 3.2. SERS spectra of thiolated let-7f DNA probe immobilized onto AgNRs using water and 300 mM Na_2SO_4 pH~7. Five measurements from the same well are shown for each sample.....	54
Figure 3.3. A) Raw SERS spectra of four different DNA probes. The dotted lines highlight some of the primary spectral features that indicate the presence or absence of specific nucleotides. Spectra are vertically offset for clarity. B) Spectra of miR-224 probe (black, which contains all four nucleotides) overlaid with individual nucleotide (colored, scaled for easier comparison).....	56

Figure 3.4. Comparison of spectra of the let-7f probe treated with MH in A) water, and B) 30 mM Na ₂ SO ₄ . The structure of MH is also included. The let-7f probe spectra are presented before MH treatment (black), and after MH treatment (gray). The structure of MH is also presented.....	57
Figure 3.5. SERS spectra of let-7f probe as a function of increasing MH-treatment concentration. The bottom spectrum shows the pure MH spectra (no DNA probe); the dotted lines highlight the primary MH spectral features, which can be seen to increase in intensity with the increase in MH concentration. Spectra vertically offset for clarity. (From Ref [82].).....	59
Figure 3.6. Let-7f probe spectra before (black) and after washing (gray).....	63
Figure 3.7. Raw hybridization spectra of let-7f probe.....	64
Figure 3.8. Normalized spectra of probes: A) let-7f, B) Test, C) miR-224, and D) rev let-7f after treatment with targets: let-7f (red), Test (green), rev miR-224 (blue), and SSC buffer (black).....	66
Figure 3.9. SERRS spectra of wells with different probes treated with 1 μ M let-7f-FAM target. The 1180 cm ⁻¹ peak is highlighted.....	69
Figure 3.10. A) The intensity of the signature FAM peak at 1180 cm ⁻¹ (I_{1180}) measured from the spectra in Figure 3.8. B) The measured I_{1180} of let-7f probe treated with increasing concentrations of let-7f-FAM target. Both plots share the same y-axis scale.....	70
Figure 3.11. Direct, unscaled comparison of the raw miR-224 probe spectrum with the artificial mixture spectrum ($S_A+S_C+S_G+S_U$)/4.....	71

Figure 3.12. Estimated LS fitting coefficient ratios for four different DNA probes using the entire spectral region from 350-1800 cm^{-1} . The results of additional experiments are presented for let-7f and Test probe. The pink stars represent the theoretical values based on the number ratio of each nucleotide in the sequence. (From Ref [82].).....	75
Figure 3.13. The residual spectrum R_{DNA} of let-7f probe for three different experiments showing a large residual at 1318 cm^{-1} . The bold trace is the portion of the spectrum used after region X is removed. (From Ref [82].).....	76
Figure 3.14. Estimated LS fitting coefficient ratios for four different DNA probes after using the same spectral data from Figure 3.11, but removing region X from the spectra. The pink stars represent theoretical nucleotide ratios for each probe. (From Ref [82].).....	78
Figure 3.15. Estimated LS fitting coefficient ratios for let-7f or Test probes from Experiment 1 after treatment with complementary target, non-complementary target, or SSC buffer. The pink stars represent theoretical nucleotide ratios for probes after complementary hybridization (A and C) or non-complementary hybridization/negative control treatment (B and D). (From Ref [82].).....	79
Figure 3.16. Estimated LS fitting coefficients for let-7f probe versus increasing concentration of let-7f miRNA target. The solid horizontal lines represent the approximate values to which the LS estimates appear to be converging to, and the dotted lines represent the theoretical values to which the nucleotide ratios should converge. (From ref [82].).....	83

Figure 4.1. I_{wet} and I_{dry} vs immersion time t in MPh solution for concentrations of A) 10 μM and B) 1 mM. Each time point represents one substrate. The red and blue plots represent the I_{400} and I_{1070} (scaled by the left y-axis), respectively, while the green plots represent the I_{400}/I_{1070} ratio (scaled by the right y-axis). The solid symbols represent the I_{dry} , while the hollow symbols represent the measurements acquired when water was applied back onto the AgNR film, I_{wet} . The dashed line is a reference for the $I_{400}/I_{1070} = 1.0$	89
Figure 4.2. $I_{\text{wet}}/I_{\text{dry}}$ ratio (of the 1070 cm^{-1} peak) vs immersion time t in 10 μM and 1 mM MPh solutions.....	90
Figure 4.3. I_{wet} or I_{dry} of AgNR substrates treated with A) MPh and B) MH under different conditions. The x-axis represents the process flow of the processing/measurement conditions; W = water, I = IPA, T = toluene, and H=hexane; the values in between are the I_{dry} measurements. The connected gray squares represent the I_{wet} and I_{dry} (plotted with respect to the log scale of the left y-axis). The black circles represent the $I_{\text{wet}}/I_{\text{dry}}$ ratios for each liquid measurement and its preceding dry measurement (plotted with respect to the right y-axis).....	94
Figure 4.4. Overhead SEM images of AgNR film A) before and B) after treatment with water. Both images have the same magnification. These substrates were from the bottom of the sample holder in the evaporation chamber.....	98
Figure 4.5. Cross sectional SEM image of AgNR film A) before and B) after treatment with water. The thickness T and tilting angle ϕ parameters used for quantitative analysis are also illustrated. The scale bar is 3 μm	98

Figure 4.6. Morphology Statistics of AgNR film thickness T (squares) and tilting angle ϕ (circles) acquired from SEM images for two groups of substrates (bottom and top of sample holder) for as-deposited and water-treated.....	99
Figure 4.7. Conductance measured on AgNR films before (gray) and after (black) treatments with different solvents.....	102
Figure 4.8. SERS spectra of MPh vapour-deposited onto AgNRs before adding water (red), while immersed in water (blue), and after drying (green).....	105
Figure 4.9. I_{wet} and I_{dry} of AgNR substrates treated with A) MPh and B) MH under different conditions. The x-axis represents the process flow of the processing/measurement conditions; W = water, I = IPA, T = toluene, and H=hexane, D = dry. The gray squares represent the I_{wet} or I_{dry} (plotted with respect to the left y-axis). The arrow in (A) indicates the change in SERS intensity expected to result strictly from the dielectric effect. The black circles represent the $I_{\text{wet}}/I_{\text{dry}}$ ratios for each liquid measurement and its preceding dry measurement (plotted with respect to the right y-axis).....	108
Figure 5.1. A) Schematic diagram showing a single cell of the SERS chip electrocell. B) Low and high magnification SEM images of AgNRs after treatment with $V = -2.8$ V in DI water with 10^{-5} M BPE. Both SEM images are from the same cell.....	116
Figure 5.2. I_{SERS} of four different peaks (1010, 1200, 1610, and 1640 cm^{-1}) vs time t and with different applied step voltage V_{step} . The I_{SERS} is scaled by the left y-axis, and the V_{step} (blue) is scaled by the right y-axis. Each panel represents a different cell treated to a different V_{step}	118

Figure 5.3. I_{SERS} of four different peaks (1010, 1200, 1610, and 1640 cm^{-1}) vs time t and with $V_{\text{step}} = -2.0$ V for BPE dissolved A) DI water and B) Na_2HPO_4 . The I_{SERS} is scaled by the left y-axis, and the V_{step} is scaled by right y-axis.....	123
Figure 5.4. I_{SERS} of four different peaks (1010, 1200, 1610, and 1640 cm^{-1}) vs time with incremental increases of V_{step} for BPE dissolved Na_2HPO_4 . The I_{SERS} is scaled by the left y-axis, and the V_{step} is scaled by right y-axis.....	123
Figure 5.5. I_{SERS} as a function of multiple short voltage cycles between -1.8 and -2.2 V for BPE in A) electrolyte and B) DI water.....	125
Figure 5.6. Ratios of I_{1010} , I_{1610} , and I_{1640} relative to I_{1200} for V_{step} equal to A) -0.8 V, B) -2.0 V, and C) -2.8V using the same data from Figure 5.2 C), F), and H), respectively, and D) with electrolyte using the same data from Figure 5.3B. The peak ratio is scaled by the left y-axis, and V_{step} is scaled by right y-axis.....	137
Figure 5.7. Peak position shifts ($\Delta\nu$) of ν_{1010} , ν_{1200} , ν_{1610} , and ν_{1640} for V_{step} equal to A) -1.2 V, B) -2.0 V, and C) -2.8V acquired from the same spectral data from Figure 5.2 C), F), and H), respectively. The change in peak position is scaled by the left y-axis, and the V_{step} is scaled by right y-axis.....	138
Figure 5.8. 1 in x 3 in electrode mask pattern for used for positive-tone photoresist lift-off. The white portions represent the metal pattern deposited onto the glass microscope slide; A) the mask for the working electrodes, counter electrodes wires, and contact pads; B) the mask for selective deposition of AgNRs onto central working electrode. Each mask is 1 $\times$ 3 inches in size.....	144
Figure 5.9. Process flow for OAD AgNR deposition onto metal electrodes. See main text for description.....	146

Figure 5.10. SEM images of one of the AgNR working electrode (WE) and a Pt counter electrode (CE) on the glass substrate. A) low magnification showing both WE and CE electrodes, B) higher magnification showing the different layers (Pt, Ag, and AgNRs) and 3) high resolution image showing intact AgNR film.....	149
Figure 5.11. Mask design used to expose SU-8 for fabrication of the microchannel array mold.....	150
Figure 5.12. Picture of completed 1 × 3 inch AgNR LOC device. A - Au connection wire insides PDMS cell, B - 20 pin header, C - PDMS slab containing the microchannels, and D - PDMS slabs for reinforcement.....	151
Figure 5.13. Higher magnification image of individual cells showing A - inlet reservoir, B - outlet reservoir, C - microchannel in PDMS, D - AgNR SERS sensor/working electrode, E - Au counter electrode.....	152
Figure B.1. Spectra (when AgNRs are dry) and molecular structure of MPh and MH. The marked peaks are those used for quantitative analysis in Chapter 4.....	180

Chapter 1

INTRODUCTION

1.1 The Current State of Nucleic Acid Detection

Nucleic acids, i.e. deoxyribonucleic acid (DNA) and ribonucleic acid (RNA), contain a wealth of highly specific biological information. This information is encoded in sequences of four different nucleotides, referred to as A, C, G, and T (or U, in the case of RNA). These sequences are highly unique and very specific for each type of organism, such as an infectious agent, and the sequences are specific enough to allow discrimination between strains within a single species. In addition to providing specific and detailed information about pathogens, nucleic acids also provide a detailed account of the physiological response of an individual suffering from a disease. Their ubiquitous association with disease therefore implies that nucleic acids can be used as indicators of disease, or as *biomarkers*.

In a relatively recent discovery, short non-coding sequences of RNA, known as microRNA (miRNA) have been shown to be involved in many biological processes such cellular development, differentiation, apoptosis, and metabolism [1, 2]. Concordantly, miRNA is intimately involved with many diseases such as viral infections, cancers, or genetic disorders, and miRNA sequences are also highly conserved between individuals. As of 2009, miRNAs have been reported to be involved in over 90 different human diseases [3], and the number will surely increase as further research is performed. In addition to providing critical information for a wide-range of diseases, miRNA is also accessible, as it is not strictly confined to diseased tissue and can propagate through the circulatory system [4]. Thus, miRNA contains many

characteristics of a valuable biomarker. With these facts in mind, the central importance of miRNA biomarkers for the development of next generation disease-sensing devices and applications becomes evident.

Although miRNA is a good biomarker candidate, a reliable way to detect miRNA must be developed before it can be utilized in a practical manner. Beyond providing the necessary sensitivity and specificity, a miRNA detection platform should allow for high throughput analysis where numerous sequences can be screened for in parallel. Fortunately, because miRNA is simply a short nucleic acid sequence, traditional DNA detection strategies can therefore be adapted [5]. However, the established methods have been in general limited to only a few mainstream techniques, namely northern/southern blotting, polymerase chain reaction (PCR), and DNA microarrays ('DNA chips').

Nucleic acid detection, including the aforementioned techniques, is based upon the principle of *hybridization*. This precept states that the nucleotide A can form a specific set of hydrogen bonds with T (or U, in the case of RNA), thus the nucleotides are complementary to each other; likewise, C is complement to G. Therefore two different yet complementary single-strand sequences can hybridize to form a double-stranded helix structure, which is stabilized along the central axis by a network of hydrogen bonds formed between the complementary nucleotides. Because of this property, an appropriately designed probe sequence can be used to hybridize, or 'capture', a specific known target sequence. Hybridization, therefore, imbues these methods with the necessary sequenced-dependent *specificity*. However, in order to detect hybridization, the aforementioned techniques require the use of a label, such as a fluorescent dye or radioactive isotopes. These reporter molecules can be covalently bonded to either the target or probe sequences. Detection of the fluorescent/radioactive signal therefore indicates

hybridization. Thus, the labeling approach instills nucleic acid detection with the necessary *sensitivity*.

Northern blotting is the most common technique for miRNA detection [5, 6]. This technique entails loading a sample of miRNA into a polymer gel slab, and then applying an external electric field where electrophoretic migration separates the RNA molecules according to their sequence length. The separated sequences manifest as a series of spatially resolved bands within the gel. The RNA sequences within the bands are then blotted onto a membrane to which they are covalently bound. Then radioactively labeled probes, which are complementary to specific target sequences, are incubated with the membrane and allowed to hybridize with the immobilized complementary miRNA. After washing the membrane, only the bands on the membrane that contain radio-labeled probe sequences (hybridized with the immobilized complementary miRNA sequences) will produce a radioactive signal. Despite the wide spread use of northern blotting for miRNA detection, it suffers from poor sensitivity and requires time-consuming and laborious processing. Moreover, only one sequence can be assayed at a time, which further prevents this technique from being utilized as a high-throughput, multi-sequence miRNA screening platform.

PCR may also be used for miRNA detection [5, 7]. In this case, the miRNA is used to produce complementary sequences of DNA (cDNA) using reverse-transcriptase (RT) PCR. Then, other PCR techniques such as quantitative real-time PCR (qPCR) can be employed to quantify the amount of cDNA transcribed from the original miRNA sample. One way to perform qPCR is to use a short probe sequence that is complementary to a portion of the cDNA sequence. This probe has a fluorescent label on one end, and a quencher group on the other end (e.g. TaqMan probe); the short length of the probe keeps the label and quencher in close

proximity, so that the fluorescent signal is quenched. The cDNA is initially double-stranded (ds), but heating the DNA solution causes the dsDNA to separate, or melt, into single stranded (ss) DNA. The probe sequence then binds to complementary regions on the ssDNA. Short nucleotide sequences, i.e. primers, are also added to the sample solution. These primers are complementary to specific sequences at the ends of the ssDNA. After hybridization with complementary portions of the ssDNA, the primers allow polymerase enzymes to transcribe a complementary sequence for the ssDNA. During this transcription process, the hybridized probe sequences are degraded, which releases the fluorescent marker (separating it from the quencher), thereby producing a fluorescent signal. After transcription, there are now two dsDNA copies. The entire process is repeated multiple times, each time doubling the number of dsDNA, thereby amplifying the fluorescent signal in an exponential manner with each cycle. Monitoring the increase in the fluorescent signal with each cycle can be used to quantify the amount of cDNA undergoing hybridization. Figure 1.1A shows the qPCR principle. However, considering the extremely short length of miRNA sequences (only ~22 nucleotides long) the PCR primer sequence is typically comparable in length to the miRNA sequence itself, which mitigates the feasibility of the PCR amplification process [5]. To overcome this issue, special techniques and probe designs must be utilized. Additionally, PCR is traditionally hampered by interference from exogenous DNA, and requires a clean, sterile processing environment.

DNA microarrays are a powerful miRNA sensing platform as they allow for a very large number of miRNA sequences (i.e. entire genomes) to be screened for in parallel [5, 8]. The general microarray detection scheme is as follows: probes with known sequences are first immobilized onto a microscope slide at discrete locations using conventional surface (e.g. silane) chemistry. The microarray is then incubated with a sample containing pre-labeled, yet unknown

target sequences, which will hybridize with complementary probe sequences immobilized at various locations within the array. Then excess target solution, along with weakly bound targets is rinsed off from the array, and the fluorescence signal of each probe spot is measured. Because the sequences and locations of each probe are known, the sequences of the miRNA in the sample can be deduced by recording which probe locations produce a fluorescent signal. Figure 1.1B illustrates the general microarray principle. However, due to the extremely short length of miRNA sequences, bonding of a label on the target can actually impede hybridization, therefore reducing the sensitivity. To overcome this, miRNA microarrays require special post-hybridization labeling procedures [5].

Other label-based miRNA detection strategies have also been introduced recently, namely electrochemical biosensors [5]. The general scheme is the same as that for a microarray, i.e. a surface-immobilized probe hybridizes with a complementary target. However, the probe is immobilized onto an electrically conductive surface (i.e. an electrode). Rather than a fluorescent label, a redox-active enzyme is coupled with the target. As with microarray, post-hybridization labeling procedures may be required to overcome the hybridization interference caused by a labeled target, which will ultimately add more processing steps to the detection assay. When the enzyme label is present, an electrochemical signal can be measured when the enzyme's chemical substrate is added. Although the electrochemical method has demonstrated exceptional sensitivity (i.e. limit of detection $< 10^{-12}$ M), this strategy is rather novel, and all of the advantages and disadvantages have yet to be determined.

From this discussion, we have examined how the extremely short sequence of the miRNA can impede detection, and why the aforementioned techniques require a molecular labeling procedure to achieve the necessary detection sensitivity. The use of a label, however,

means that the miRNA target detection is indirect and can only be inferred from the presence of the label's signal. Yet, DNA probes do not necessarily yield 100% specificity, and may hybridize with miRNA sequences that are not completely complementary, which is referred to as cross-hybridization. That being said, label molecules are insensitive to cross-hybridization, and a 'hybridization signal' will still be detected despite the fact that the wrong target sequence has been captured. This will result in false positives and impede detection specificity. Disregarding the analytical drawbacks, the labeling requirement is still an undesirable aspect of molecular

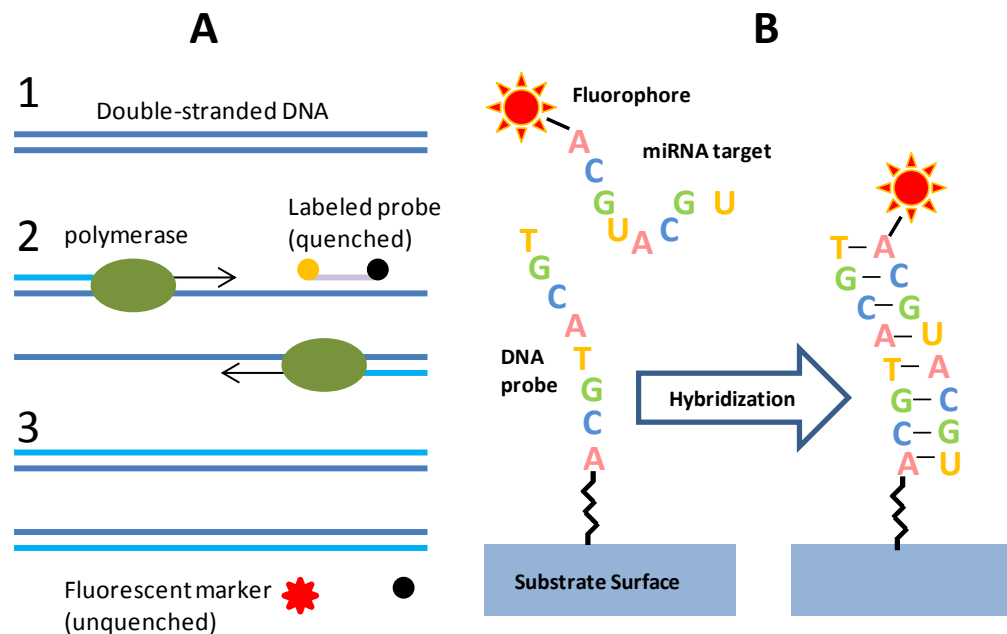


Figure 1.1. Illustration of traditional DNA detection technologies. A) Quantitative real-time PCR: 1) A double stranded sequence of DNA is separated into two single stranded sequences with thermal treatment. 2) A probe sequence (with fluorescent signal quenched) hybridizes with complementary portions of the ssDNA. Primer sequences are also added (not shown) that hybridize with one end of the ssDNA allowing polymerase to transcribe complementary sequences (light blue). 3) During transcription, the probe sequence is degraded, releasing the fluorescent marker from the quenching agent (black circle); the transcription process has doubled the amount of DNA. B) Microarray: specific DNA probe sequences are immobilized onto a substrate at known locations. Then fluorescently labeled miRNA targets are added and allowed to hybridize to complementary DNA probes. After washing away excess miRNA targets, a fluorescent signal can be detected where miRNA targets have hybridized with the complementary probes.

sensing technology. This is because the use of an extrinsic label requires more complexity, extra processing steps, and higher cost, which works against the driving motivation for emerging biosensing technology, where emphasis is placed on simple, rapid, and low cost solutions. In light of this mentality, the need for label-free miRNA sensors is apparent.

1.2 Label-Free miRNA Detection

Direct, label-free detection of miRNA has been demonstrated using methods such as surface plasmon resonance (SPR) [9], which is an optical-based method that measures the changing refractive index at the surface of a thin metal film. Because organic matter such as miRNA has different dielectric properties than that of the surrounding liquid, hybridization of miRNA with an immobilized probe layer causes an angular shift in the optical reflection of the metal-dielectric interface. Therefore the amount of accumulated miRNA at the surface can be measured. In addition, other label-free sensing techniques such as quartz crystal microbalances and micro-cantilevers have been successfully implemented as DNA sensors [10], and could in theory be adapted to miRNA applications. Although label-free, these techniques only measure the accumulation of mass at the sensing surface, and do not elicit the chemical composition of the hybridized probe/target duplex. Even though the labeling procedure has been eliminated, the ability to differentiate between specific and non-specific hybridization still remains elusive.

To address this issue of chemical specificity, chemical composition analysis could be employed. Spectroscopic detection techniques such as mass spectroscopy (MS) is a mainstay technology for this type of analysis, and can be used to ascertain direct chemical information about the composition of a sample, but unfortunately requires large, bulky, delicate, and expensive instrumentation that impedes portable, on-site detection. Fluorescence and absorbance

spectroscopies offer more practical instrumentation, yet the spectral resolution is insufficient to allow for reliable differentiation of similar chemical species, especially within a mixture of nucleic acid sequences. Infrared (IR) spectroscopy benefits from exceptional spectral resolution and chemical specificity, but interference from water makes the integration of IR with general biochemical detection applications a challenging ordeal.

Raman spectroscopy (RS), on the other hand, obviates many of these obstacles. Similar to IR, RS is also a vibrational spectroscopic technique. Whereas IR measures the absorbance of photons by molecular bonds, RS measures the inelastic photon scattering from the bonds [11]. Figure 1.2 is an illustration of Raman scattering compared to Rayleigh scattering and IR absorption. Upon excitation with monochromatic photons, the energy level of a vibrational mode is excited to an intermediate virtual state before relaxing back to a lower energy state. When the vibrational mode relaxes to the same energy state as the initial state, the photon undergoes elastic Rayleigh scattering. If the vibrational mode relaxes to an energy state different than the initial state, the photon's energy shifts and undergoes inelastic Raman scattering; if the relaxed state is of higher energy than the initial state, then the photon scatters with reduced energy and demonstrates a Stokes shift.

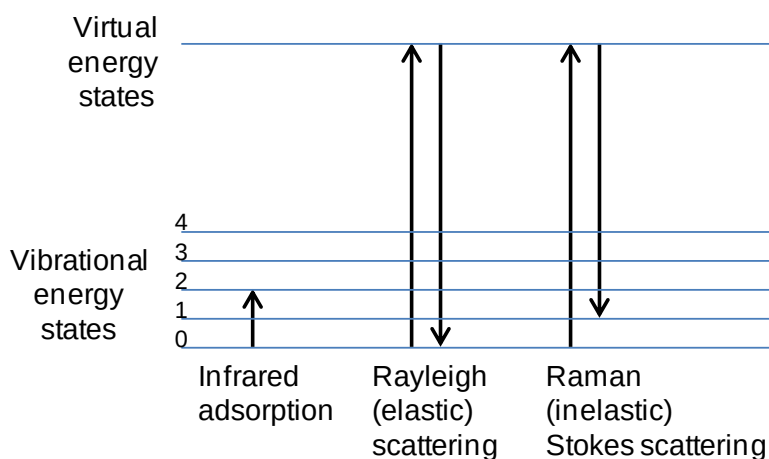


Figure 1.2. Illustration of infrared, Rayleigh, and Raman scattering.

The spectrum of the Raman scattering encodes a wealth of chemical information about the probed molecules, ultimately yielding a unique chemical signature of the sample under analysis. RS also produces superior spectral resolution with very narrow peaks (sometimes only several wavenumbers wide). In addition to providing both a qualitative and quantitative measure of the chemical composition of a sample, information regarding the molecular configuration, orientation, and bond strength can also be ascertained from RS.

The highly specific and information-rich RS signal allows for accurate analysis of complex biological mixtures, such as blood, to be achieved. This exceptional specificity clearly provides distinct advantages over many of the aforementioned analytical techniques. Moreover, RS instrumentation is relatively simple and can be readily miniaturized; in fact, many hand-held Raman systems are already commercially available. Because water has an intrinsically low Raman cross-section, RS is also compatible with biological and *in situ* analysis. RS benefits from non-destructive sample analysis, as well as rapid measurement time, usually on the scale of seconds. Despite this multitude of benefits, however, traditional RS ultimately suffers from very low sensitivity due to an extremely small scattering cross section, thereby disqualifying it from the trace-level analysis needed for miRNA detection. In order to utilize the benefits of Raman spectroscopy for miRNA detection, the sensitivity must be greatly improved.

1.3 Surface-Enhanced Raman Scattering (SERS)

Fortunately, RS sensitivity can be improved by employing a phenomenon known as surface-enhanced Raman scattering (SERS) [11-13]. Under certain conditions, optical excitation of metal nanostructures can induce generation of large, oscillating electromagnetic (EM) fields localized at the metal surface. The incident excitation source used to induce these EM-fields can

simultaneously generate Raman scattering from molecules adsorbed on the metal nanostructures. The intense EM-fields will greatly amplify the Raman scattering process of these molecules, providing enormous enhancement to the resulting Raman signal. The magnitude of the enhancement is referred to as the enhancement factor (EF), which is defined as $EF = \frac{I_{SERS}N_{bulk}}{I_{bulk}N_{SERS}}$, where I_{SERS} and I_{bulk} are the signal intensities generated by an analyte under SERS and bulk Raman excitation, respectively, and N_{SERS} and N_{bulk} are the numbers of analyte molecules being probed with SERS and bulk Raman excitation, respectively. The EF is routinely on the order of 10^5 - 10^6 , and a majority of the EM enhancement is believed to result from points of exceptionally high field enhancement, referred to as ‘hot spots’. The EM enhancement is responsible for a large portion of the observed EF; however, another mechanism known as the chemical (CHEM) enhancement and can further amplify the Raman signal up to another two orders of magnitude [14]. The CHEM effect has been in general more elusive to study and quantify, but is believed to result from the charge transfer between the molecule and the metal surface during excitation. Under special conditions the combined EM and CHEM enhancement is sufficient enough to allow for single molecule detection [15, 16].

By greatly improving the sensitivity, whilst maintaining the aforementioned analytical advantages of traditional RS, SERS is poised as a feasible platform technology for label-free detection of miRNA. However, before SERS can be properly utilized for miRNA detection, the central component to any SERS analysis, i.e. the SERS substrate, must be carefully selected.

1.3.1 Overview of SERS Substrates

Generally speaking there are only two requirements needed to make a useful SERS substrate. The first criteria is that the surface material must have appropriate optical properties in the visible/near-IR range capable of supporting plasmonic excitation [11, 13]. Typically Au and Ag are used as they demonstrate superior optical responses within these wavelengths, that is to say the real and imaginary terms of the dielectric function $\epsilon(\omega)$ have a large negative and a small positive value, respectively, in the wavelength range under consideration. Cu meets this criterion as well, but is prone to chemical corrosion/oxidation, and is therefore less stable. The second requirement is that the surface must have appropriate nanoscale structure, or ‘roughness’, to produce sufficient E-field enhancement during optical excitation [17]. Ideally, a SERS substrate should contain many hot spots capable of large E-field enhancement. Hot spots are believed to be primarily located at points of sharp curvature and within nano-sized gaps. Figure 1.3 shows the calculated E-field enhancement for a spherical shell dimer structure [18] with the star symbols representing adsorbed analytes expected to experience high and low surface enhancement.

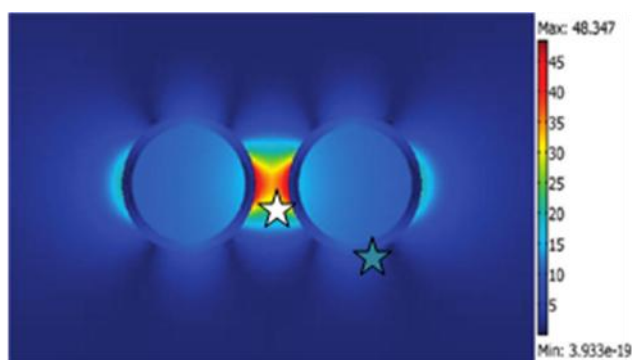


Figure 1.3. Calculated E-field enhancement of spherical shell dimer under optical excitation. Analytes experiencing a large field enhancement (white star) and lower field enhancement (green star) are presented for illustrative purposes. (Adapted with permission from Ref [18]. Copyright (2010) American Chemical Society.)

With these two general criteria, a plethora of SERS substrates have been developed over the years. The SERS characteristics of each type of nano-surface are intimately tied to the nanofabrication technique used [19]. For example, SERS was serendipitously discovered using silver electrodes that had been roughened through oxidation-reduction cycling (ORC) [20, 21]. Although such a method is effective at generating an enhancing surface, it does not allow much rational, directed control over the resulting nano-scale surface morphology. The uniformity of this morphology is also challenging to maintain across a large area of the electrode, or between different electrodes. This technique is therefore currently impractical for routine, high-throughput applications that require precise quantitative measurements.

On the other hand, Ag and Au colloids are attractive due to their cost-effective and straightforward synthesis method. The general synthesis procedure entails mixing a metal precursor, such as HAuCl_4 or AgNO_3 , with a reducing agent such as NaBH_3 and a stabilizing/capping agent such as citrate [22, 23]. The result is a dispersed suspension of metal nanoparticles that demonstrate only minor enhancement. Addition of a salt such as Mg_2SO_4 leads to aggregation of the colloidal particles, which can greatly enhance the SERS signal of analytes caught within the nano-size interstitial space of the aggregate. However, unlike a solid-phase, fixed nanostructure substrate, a suspended colloid may suffer from reduced measurement sensitivity as the colloids are dispersed throughout a three dimensional volume, rather than being fixed within the focal plane of the excitation and collection optics. Moreover, the aggregated colloid is not stable over time, as the colloids eventually precipitate out of solution. This issue may be overcome by casting the nanoparticles onto a solid surface, but control of the resulting surface uniformity over large area is challenging without employing special nanoparticle

immobilization techniques [24]. This lack of control can ultimately lead to poor measurement uniformity/reproducibility, disqualifying these substrates for quantitative studies.

SERS substrates have also been successfully fabricated using electron beam lithography (EBL), which can generate extremely precise and uniform arrays of surface nanostructures [25, 26]. Unfortunately, however, each individual nanostructure on the surface must be fabricated one at a time in a serial manner. EBL is thus restricted from fabrication of large area substrates and is unsuited for large-scale batch manufacturing.

Another popular SERS substrate is fabricated by spin coating a suspension of polymer or silica nanospheres onto a wafer, followed by deposition of a SERS-active metal such as Ag or Au onto the nanospheres [27, 28]. Such a ‘template’ method is referred to as Ag film over nanospheres (AgFON). By subsequently removing the metal-coated nanospheres, the metal deposited in the interstitial spaces between the nanospheres remains on the underlying wafer. This method is called nanosphere lithography and produces a planar array of nanostructures with a triangular-like shape. Although these approaches can yield substrates with good Raman enhancement, controlling the uniformity of the nanosphere dispersion over a large area can be challenging. Additionally, this technique restricts the scope of nanostructure shape and configuration.

Figure 1.4 shows scanning electron microscope (SEM) images of the aforementioned SERS substrates: ORC electrode [21], EBL [26], and AgFON [28]. However, there are still many more different types of SERS substrates, each fabricated using variations of the techniques mentioned above, or using completely different approaches (for representative reviews about SERS substrate fabrication see references [29-32]). Clearly, a broad range of SERS substrate options exist, and each type will have an equally diverse range of benefits and drawbacks.

Meanwhile, the SERS community has not settled on a ‘best’ substrate, nor have any serious attempts been made to establish a standard. In light of this variety and lack of standardization, researchers have employed various types of substrates to evaluate SERS as a label-free DNA/RNA detection platform.

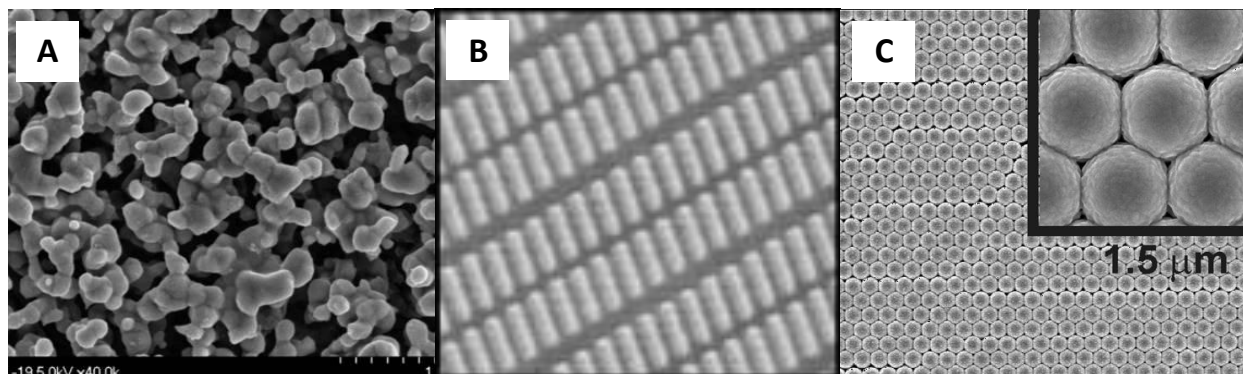


Figure 1.4. Electron micrographs of common SERS substrates: A) ORC electrode, B) colloids, and C) AgFON. (A adapted with permission from Ref [21]. Copyright (2010) American Chemical Society. B adapted with permission from Ref [26]. Copyright (2008) John Wiley and Sons. C adapted with permission from Ref [28]. Copyright (2006) American Chemical Society.)

1.3.2 SERS for Label-Free DNA Detection

Many reports have successfully utilized SERS for DNA detection. However, a vast majority of those reports have employed an extrinsic label to greatly boost the detection sensitivity (see references [33-39] for representative studies). For this strategy, a Raman reporter molecule can be covalently attached to the target sequence, which is then allowed to hybridize with a probe sequence that has been pre-immobilized onto a SERS substrate. The reporter molecule can be excited with a wavelength that induces a very strong surface-enhanced resonant Raman scattering (SERRS) signal. This approach produces exceptionally sensitive analysis, but only the SERRS signal of the Raman reporter is detected. Figure 1.5A presents an illustration of the label-based SERS detection method. Other than providing higher spectral resolution, which

can be very useful for multiplexing, these strategies do not fundamentally differ from the traditional fluorescence label-based detection techniques previously described (e.g. microarrays; see Figure 1.1B). That is to say, a Raman label has simply replaced the fluorescent label, and many of the related disadvantages discussed in Section 1.1 still apply.

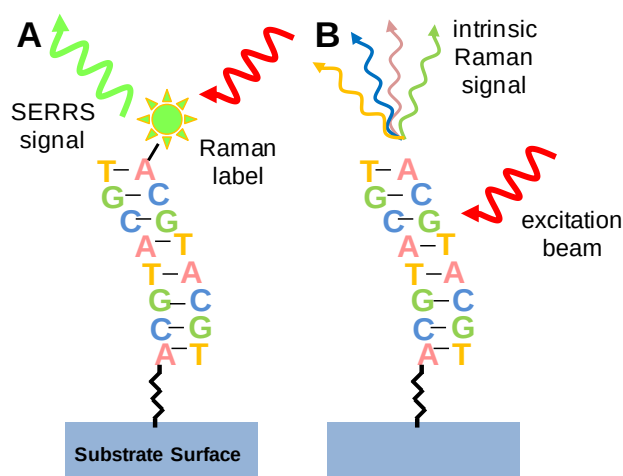


Figure 1.5. A) Extrinsic detection of DNA hybridization using an immobilized probe and a SERRS-active Raman label covalently bound to the target DNA. Excitation with an appropriate wavelength (red) generates a strong SERRS signal (green). B) Intrinsic detection of DNA hybridization. Excitation produces a SERS signal of all the DNA components, e.g. nucleotides.

Only a small handful of reports have broached the prospect of direct, label-free SERS detection of DNA. This strategy relies on the detection of the intrinsic SERS signal of the DNA itself (Figure 1.5B). Brabec and Niki performed one of the earliest SERS DNA studies and compared the DNA spectra of natural calf thymus DNA to that of short synthetic oligonucleotides using an electrochemically roughened electrode [40]. Kneipp et al. also measured the SERS spectra of calf thymus DNA but used Ag colloid [41]. Gearheart et al. compared the of SERS spectra of oligonucleotides single-stranded (ss) DNA to that of double-stranded (ds) DNA using Au colloid as an enhancing medium [42]. Dong et al. studied synthetic thiolated ssDNA oligonucleotides adsorbed onto electrochemically roughened Au electrodes

before and after hybridization with complementary sequences [43]. Green et al. compared the SERS spectra of thiolated DNA oligonucleotides immobilized onto Ag ‘nano-torus’ structures before and after treatment with complementary and non-complementary DNA targets [44]. Wu et al. analyzed the SERS spectra of unthiolated oligonucleotides adsorbed to Ag nanoparticles aggregated with spermine [45]. Naomi Halas’ group has performed several studies on the SERS response of both ss- and ds-oligonucleotides immobilized onto fixed Au-nanoshells via a thiol linker; the effect of a DNA thermal pre-treatment was also investigated [46-48]. Steven Bell’s group has published several reports that discuss the SERS response of both ss- and ds-oligonucleotides on Ag and Au colloids synthesized with various reducing agents [49-51]. Prado et al. measured polyadenosine, polyuridine, polycytosine, and polyguanosine oligonucleotides using Ag colloids aggregated with MgCl_2 [52]. Rusciano et al. investigated the SERS signal of G-quadrexes immobilized to Ag colloidal nanoparticles [53]. Xu et al. used Si nanocones coated with AgNPs to detect thiolated ssDNA [54].

Thus, we can see that most of these studies focused primarily on the detection and analysis of short DNA oligonucleotides, typically < 100 nucleotides in length, for both ss- or dsDNA. Additionally, these studies primarily employed Au or Ag colloidal nanoparticles as Raman-enhancing mediums, while only a few have employed solid-phase fixed support substrates.

Because an extrinsic label is not present in these studies, the detection relies solely on the intrinsic SERS signal originating from the chemical components of the DNA itself, i.e. the nucleotides. However, even qualitative analysis of the intrinsic DNA SERS signal has proven to be a non-trivial matter, as we will see from the following discussion. In general, the literature does not appear to be settled on several fundamental and non-trivial issues such as: 1) relative

Raman cross section of the different nucleotides, 2) vibrational assignment of spectral features, 3) effects of DNA conformation on the resulting DNA SERS signal, and 4) the effects of the substrate nanostructure/composition on issues 1, 2, or 3. Although many of these reports provide very systematic and thorough investigation of DNA SERS analysis, much controversy is still evident.

Substantial disagreement exists in the literature about the relative Raman cross section of the individual nucleotides A, C, G, T and U. Studies by Naomi Halas's group consistently demonstrated that thiolated DNA adsorbed to Au-nanoshell substrates yield a spectrum that is overwhelmingly dominated by the signal of A [46, 47] (see Figure 1.6A). Meanwhile, G produces a detectable signal but is very weak in comparison. C and T are completely undetectable and therefore assumed to have extremely low cross sections. In contrast, Papadopolou and Bell showed that DNA oligomers adsorbed to Ag and Au nanoparticle colloids had the relative cross sections $A \sim C \gg G \sim T$ [49, 50]. Additionally, they also showed that the spectral profile and relative peak intensities varied somewhat between Ag colloid produced with hydroxylamine reduction, and Au colloid produced with citrate reduction. Moreover, spectral changes were strongly dependent on the nucleotide ratios, but only weakly dependent on nucleotide positions within the sequence. The study by Prado et al., using Ag colloids, showed that for sequences containing only one type of nucleotide, A provided the highest cross section, yet the other nucleotides provided comparable SERS cross sections [52]. Brabec et al. demonstrated that the A and U nucleotide components produced similar intensities, yet U was slightly more intense (at an applied potential of -0.6 V vs. Ag/AgCl reference). Yet, under similar conditions, dsDNA only produced strong peaks resulting from A [40].

There has also been some disagreement about the band assignments of the nucleic acid spectra. For example, based on bulk Raman analysis of DNA, Wu et al. asserted that the major band at $\sim 790\text{ cm}^{-1}$ was a result of the symmetric stretching of the deoxyribose-linked phosphodiester bond of the DNA backbone [45]. In addition, they pointed out that the presence of this band is highly dependent on the conformation of the DNA backbone. This is in contrast to the assignment from other reports, which claimed that the band at this location could be assigned to the ring-breathing modes of C, T, and U [44, 51, 55]. Considering that this band is commonly used as the primary diagnostic spectral feature for detection of C, T, and U nucleotides, such a disagreement is by no means trivial. Green et al. claimed that the two bands at 650 and 688 cm^{-1} were both due to ring breathing modes of G [44]. The authors point out that this was the first time to show both of these bands together in the same spectra, which they attributed to different adsorption configurations of the nucleotide to the metal surface.

The interaction and orientation/configuration of ss- and dsDNA with the metal surface of a SERS substrate have not been clearly elucidated. DNA is capable of strongly adsorbing to the surface via the lone-pair nitrogens of the nucleobases [56], leaving the DNA laying flat on the metal surface. For thiol-modified sequences, the DNA is assumed to be capable of obtaining a more vertical orientation allowing for the molecules to adsorb and form a highly packed and structured monolayer. However, the literature has not settled on a clear agreement as to exactly how the DNA-surface interaction affects the SERS signal. For example, Brabec et al. monitored the SERS response of DNA on a roughened Ag electrode. They reported that homogenous poly-A sequences adsorbed much more strongly and yielded a much stronger SERS intensity than that of a homogenous poly-U sequence. Yet, A and U nucleotide monomers produced similar intensity. After hybridization, dsDNA was shown to produce a much weaker signal than ssDNA

[40]. Another study by Dong et al. claimed that the conformation of ds-DNA thiol immobilized DNA changed from primarily A-form in solution to primarily B-form in air. This study also suggested that ssDNA adopted a more flat conformation, but after hybridization, the dsDNA changed to a more vertical or tilted conformation [43]. Gearheart et al. showed that unmodified DNA oligomers adsorbed to AuNPs was only detectable with SERS if the DNA was double-stranded [42]. On the other hand, Papadopolou and Bell demonstrated that unmodified ssDNA sequences were detectable with Au and Ag nanoparticles, and provided a significantly better signal response than their corresponding double-stranded sequences [51]. Prado et al also showed a strong signal of ssDNA [52]. Halas' group demonstrated that both ss- and dsDNA sequences were detectable on Au-nanoshells, but the quality and reproducibility of their spectra were highly dependent on the use a thermal pre-treatment step [46]. In addition, the tilting angle of thiol-immobilized dsDNA was found to be strongly dependent on the packing density of the DNA layer [47]. On the other hand, Bell's group showed that good quality thiolated-dsDNA spectra could be achieved on Au and Ag colloids without thermal pretreatment, yet thermal pretreatment of the thiolated DNA did selectively augment the A signal [51]. Rusciano et al. studied that the conformation and adsorption orientation of DNA oligonucleotides containing sequence regions rich with G [53]. In water, these sequences were proposed to adsorb to the colloid surface via the nucleobases in a 'lying-down' conformation. However, in K^+ solution, these G-rich sequences formed four-stranded structures, i.e. quadruplexes, and were believed to adopted an upright, vertical conformation on the surface of the colloid.

As highlighted from the above discussions, suspensions of Ag and Au colloidal NP's have been the most popular SERS substrate for nucleic acid detection, but other substrates have been used, e.g. Au-nanoshells [46, 47], chemically etched Ag surfaces [57], Au or Ag electrodes

[40, 43], AgNPs fixed to silicon nanocones [54], and Ag nano islands [44]. However, considering the fact that the intrinsic SERS characteristics of a given substrate (e.g. sensitivity and reproducibility) are closely tied to the fabrication technique used to generate the substrate, some discrepancy and disagreement is expected between the aforementioned studies. For example, Xu et al. demonstrated that silicon nanocones coated with AgNPs produced highly

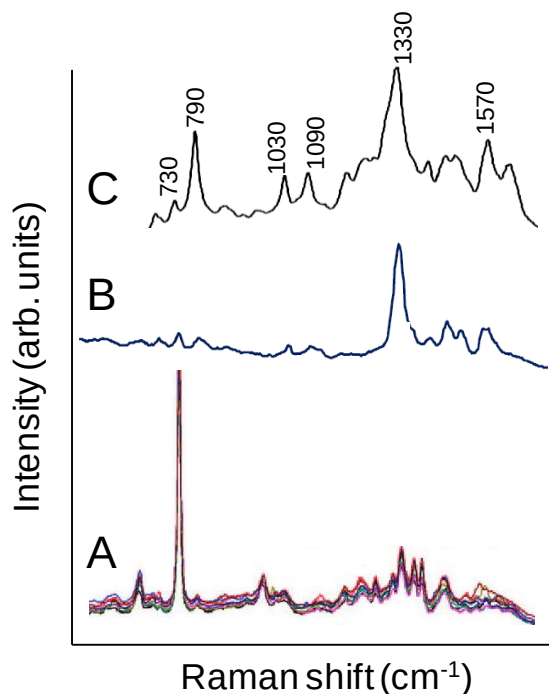


Figure 1.6. Selected SERS spectra of DNA from literature: A) 70-nt thiolated DNA thermally pretreated and adsorbed onto Au-nanoshells, B) 30-nt unmodified ssDNA on Ag colloids synthesized with hydroxylamine and aggregated with MgSO_4 , and C) 12-nt unmodified dsDNA on Ag colloids aggregated with spermidine. The x-axis scale is the same for A, B, and C, but the spectra are not scaled equally on the y-axis. (A adapted with permission from Ref [46]. Copyright (2008) American Chemical Society. B adapted with permission from Ref [51]. Copyright (2012) John Wiley and Sons. C adapted with permission from Ref [45]. Copyright (2006) American Chemical Society.)

reproducible spectra, but the substrates demonstrated only a very weak DNA signal [54]. Dong et al. used electrochemically roughened Au electrodes, but only yielded relatively poor quality spectra [43]. Moreover, DNA oligomers are rather large molecules with a size scale comparable

to the scale of nano-sized features of the substrate's surface. This means that different types of substrates may induce different types of conformational changes when DNA is adsorbed to the surface, which could yield different adsorption behavior thereby affecting the SERS response.

Our discussion thus far has highlighted the fact that much disagreement exists in the literature about the qualitative and quantitative nature of DNA SERS analysis. To summarize, Table 1.1 presents the findings in the literature by listing the substrates, DNA samples, results, and relative nucleotide cross sections of several aforementioned studies. Figure 1.6 shows selected DNA SERS spectra from the literature and illustrates the disparity of DNA SERS measurements achieved by different researchers. Ultimately, before we can address these issues, a highly uniform, reproducible, and sensitive SERS substrate is required.

Type of Substrate	DNA Sample	Results	Notes on Relative Nucleotide Cross Sections and Intensity	Ref
14 nm AuNPs (in solution)	Unmodified 14-mer ssDNA and dsDNA	Only dsDNA could be detected	A > G >> C ~ T	42
ORC Au electrode	Thiolated 20-mer	ssDNA lays more flat on Au surface; becomes more vertical/tilted after hybridizing to dsDNA	T decreases after hybridization	43
80-120 nm AgNPs and Au colloids (in solution)	Unmodified ss- and dsDNA; ≤ 30-mers	ss- and dsDNA detected on Au and AgNPs, but ssDNA could be detected at lower concentration	A ~ C >> G ~ T	51
AgNPs	Unmodified ss- and dsDNA; thiolated ss- and dsDNA 30-mers	DNA with and w/out thiol demonstrate significant spectral similarity and high spectral quality; after thermal pretreatment, thiolated DNA is dominated by A	730 cm ⁻¹ of A peak increases significantly after thermal pretreatment, but 1330 cm ⁻¹ A peak decreases	51
Immobilized Au-nanoshells	Thiolated ssDNA and dsDNA	Thermal pre-treatment of ssDNA and dsDNA produce high quality spectra	A >> G >> C ~ T	46
Immobilized Au-nanoshells	Thiolated dsDNA with G nucleotides concentrated at 3' end opposite of thiol; 70-mer	Higher concentration of dsDNA results in higher packing density and more vertical orientation	Relative intensity of G decreases (due to increased distance from surface) as dsDNA becomes more vertical	47

Table 1. Comparison of experiments and results of selected publications of label-free SERS DNA analysis.

1.4 Silver Nanorod Array SERS Substrates Fabricated by Oblique Angle Deposition

Silver nanorod (AgNR) arrays fabricated with oblique angle deposition (OAD) have been shown to be exceptional SERS substrates [58-60]. These substrates have high sensitivity and superior reproducibility. Moreover, OAD is a versatile fabrication technique, allowing for a broad scope of AgNR substrate designs to be realized. Below gives a brief overview of the OAD AgNR fabrication process and the resulting SERS characteristics.

1.4.1 Advantages of OAD AgNRs for SERS Substrate Fabrication

OAD is special form of traditional physical vapor deposition (PVD) [61]. During PVD, atoms vaporized from a molten source material condense to solids upon interaction with low temperature surfaces. With prolonged deposition, these condensing atoms form a smooth, flat film. However, rotating the polar angle of the substrate to an oblique angle with respect to the incident source vapor causes the deposition process to become highly sensitive to the substrate surface roughness. Perturbations in the surface height become exaggerated due to the shadowing effect, i.e. vapor deposits preferentially onto higher points of the surface at the expense of the lower points immediately behind them. Initially, these points act as nucleation centers for further deposition; with prolonged deposition the nucleations begin to acquire a distinct anisotropic structure, and ultimately grow into uniform arrays of nanorods tilting towards the incident source vapor. Figure 1.7 shows a diagram of the OAD configuration and AgNR fabrication process. Various structural parameters such as tilt angle, rod length, rod width, and rod separation can be controlled by tuning the vapor deposition rate, duration, and angle [61].

Beyond providing control of the resulting nanorod structure, OAD also affords many of the fabrication benefits of traditional PVD, which in turn prove to be advantageous for SERS

applications. For example, the semi-conductor and optical coating industries have widely employed PVD as a means to generate uniform, precise thin films over a large area (i.e. wafer-scale fabrication). Moreover, because many wafers can be processed in parallel, PVD allows reproducible batch fabrication in a scalable and cost-effective manner. These same advantages have therefore been utilized to produce large-area (wafer-size) AgNR films capable of uniform SERS sensing. The batch-to-batch fabrication uniformity therefore permits more accurate quantitative comparison between experiments. In addition to these fabrication advantages, OAD offers additional technological benefits, and can in principle be integrated with other

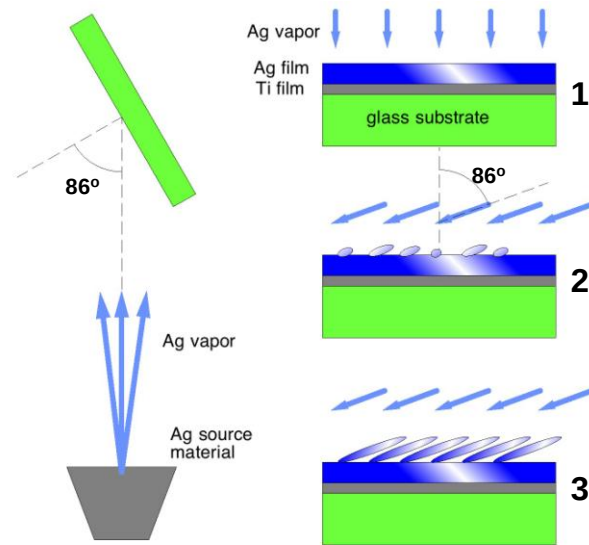


Figure 1.7. Diagram of OAD deposition configuration showing the Ag vapor originating from Ag source material and a glass substrate rotated 86° relative to the incident vapor flux; 1) before rotating, a Ti adhesion layer and then Ag film is first deposited onto the glass substrate with the substrate surface directly facing the incident vapor; 2) the substrate is rotated 86° relative to the source vapor and nucleation points begin to grow due to the shadowing effect; 3) Ag deposition is continued, allowing the nucleations to grow into a tilted nanorod array.

microfabrication techniques allowing for SERS device development. For example, OAD AgNR arrays can be deposited onto supporting substrates of virtually any size and shape assuming the surface is sufficiently smooth and flat [62]. Additionally, because OAD is a low temperature process, and does not require the use of any solvents or the application of electrical input,

AgNRs arrays can be deposited onto many different types of materials such as plastics, metals, semiconductors, etc [63]. Figure 1.8 provides SEM images of AgNR substrates deposited onto glass microscope slides.

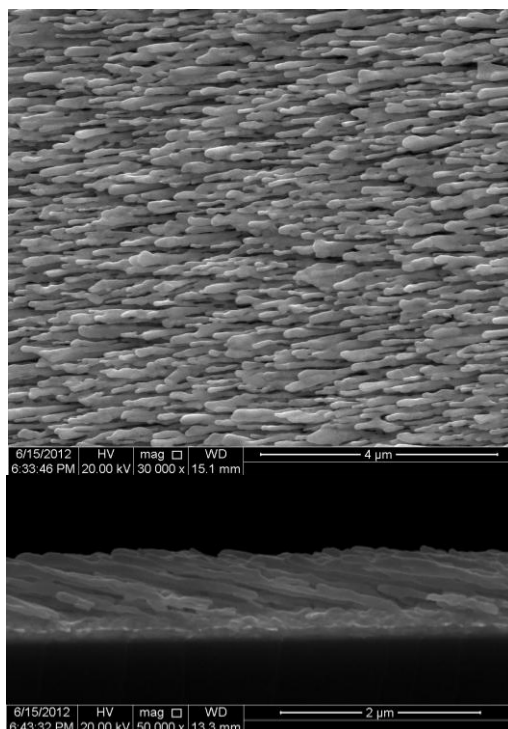


Figure 1.8. Top and side-view scanning electron micrographs of OAD-generated AgNR film.

Previous studies have shown that the OAD-generated AgNRs demonstrate superior SERS characteristics. The enhancement factor is estimated to be $\sim 10^8$ under 785 nm excitation. The point-to-point variability is estimated to be routinely $< 10\%$, whereas the substrate-to-substrate and batch-to-batch variability are $< 20\%$ [60, 64]. Therefore these substrates are promising candidates for label-free miRNA detection.

1.4.2 AgNR for Label-Free miRNA Detection

Using AgNR substrates, Driskell et al. has performed a series of studies evaluating the ability of label-free SERS analysis to detect clinically-relevant miRNA sequences [65-67]. In

the first study, solutions containing various un-modified sequences of related and un-related miRNAs families were directly applied to the AgNR substrates, allowed to dry, and the SERS spectra were acquired [66]. They found that the AgNRs produced extremely reproducible and uniform SERS spectra across different substrates. The unrelated families of miRNAs showed many spectral similarities, yet visual differences in the spectral profiles could still be discerned. The miRNA sequences from the let-7f family produced extremely similar spectra, which was expected as these sequences only differed from one another by ≤ 4 nucleotides. However, using statistical methods such as partial least squares discriminate analysis (PLS-DA), the spectra from the different miRNA sequences could be accurately assigned to the correct group with remarkable sensitivity and specificity. Moreover, the quantitative ability of the AgNRs was demonstrated as the relative intensity ratios of the ring-breathing bands of A (731 cm^{-1}) and C+U (793 cm^{-1}) was found to be directly proportional to the physical ratios of these nucleotides within each sequence.

The following study further evaluated the quantitative ability of the AgNR substrates using partial least squares (PLS) to quantify the concentration of different sequences within a mixture of miRNA sequences [65]. Two-, three-, and five-component miRNA mixtures were applied directly to the AgNRs, dried, and the spectra were measured. PLS was able to accurately predict the real concentrations of each miRNA sequence.

The third study investigated the ability of the AgNRs to capture and detect ss-miRNA (let-7f), a complementary thiolated ssDNA let-7f probe, and the let-7f miRNA pre-hybridized with a thiolated let-7f DNA probe [67]. After incubating each type of samples with the AgNRs, the residual sample solution was rinsed from the AgNRs prior to measuring the SERS signal. Only the thiolated ssDNA let-7f probe demonstrated a strong signal after rinsing. The

unthiolated ss-miRNA and the thiolated pre-hybridized ds-miRNA/DNA sequences both yielded significantly weaker signals, presumably due to the weaker adsorption interaction of these samples with the AgNR surface. In addition, the resulting signal intensity of the ssDNA probe was found to be inversely proportional to the amount of complementary target added to the probe solution.

These studies therefore have demonstrated the proof-of-principle and feasibility for AgNR substrates to be used for reproducible, quantitative, and label-free detection of clinically-relevant miRNA. However, before the AgNR substrates can be developed into a more realistic miRNA biomarker sensing device, several issues regarding the AgNR substrates must be addressed: I) SERS measurements must be optimized to maximize reproducibility and reliability; II) the substrates must be capable of efficient, high-throughput, parallel processing/analysis of multiple samples; III) AgNRs must be imbued with the necessary specificity to capture and detect a specific miRNA sequence; IV) SERS measurements in water-based *in situ* environments must be evaluated; and V) AgNRs must be demonstrated to be capable of integration into a multifunctional device such as a microfluidic electrocell. The goal of this dissertation is devise experiments, optimize processes, and design/construct an AgNR electrocell device to target these five issues.

1.5 Organization of this Dissertation

This dissertation is divided into six chapters. The first chapter has discussed how nucleic acids such as DNA or RNA can be important indicators of disease, and how a special class of molecules known as miRNA can be targeted as biomarkers. The working principles of the current nucleic acid detection technologies and some of their shortfalls, namely the requirement

for an extrinsic label, have been outlined, and the issues those strategies face when utilized with miRNA have also been highlighted. The prospect of using SERS for direct, label-free nucleic acid detection was introduced and we discussed how a special type of AgNR substrate has been successfully employed for label-free detection and quantification of miRNA. Chapter 2 will discuss the optimization of SERS measurements acquired on AgNRs using a dynamic rastering measuring technique. We will then introduce a method of fabricating a multi-well array pattern onto a large AgNR SERS substrate, i.e. a ‘SERS chip’, to allow for parallel processing and screening of a large number of samples. Chapter 3 will provide a comprehensive investigation of the SERS response of DNA probes immobilized onto a AgNR SERS chip before and after hybridization with a miRNA target sequences. The straightforward linear least squares regression method is also applied as a data analysis technique. Using this strategy, the physical ratios of the nucleotides immobilized on the surface of the AgNRs, before and after hybridization, can be quantitatively determined. Chapter 4 will discuss fundamental aspects of AgNR SERS measurements in a liquid environment. During that investigation, we discover the origin of the AgNR hot spots, and how their formation can be reversibly controlled. In Chapter 5 we introduce the prospect of voltage-assisted SERS miRNA detection, and present preliminary data demonstrating the feasibility of using the AgNRs as both a SERS substrate and an electrode. We will also show how *in situ* SERS detection of a model analyte can be improved with modulation of an applied voltage, and possible physical explanations are discussed. This chapter also highlights a novel design for AgNR SERS lab-on-a-chip device, and a completed prototype is presented. Lastly, Chapter 6 will summarize this dissertation, and provide an outlook for future developments.

Chapter 2

TOWARDS QUANTITATIVE SERS MICROARRAYS

2.1 Dynamic Rastering Measurements for Improved Detection Reproducibility

The reproducibility of a signal is clearly a vital requirement for quantitative chemical analysis. Because SERS is a quantitative technique, the signal intensity is dependent on the number of analytes adsorbed to the substrate surface. Concordantly, quantitative SERS analysis requires that the signal enhancement be reproducible so that changes in the signal intensity can be accurately correlated to the quantity of analyte on the surface. Traditionally, SERS measurements have generally suffered from poor signal reproducibility. This is primarily due to variation of enhancement factor (EF) from point to point on a substrate, or between two different substrates, (i.e. intra- and inter-substrate variation, respectively) [19]. The variation in the EF results from the intrinsic variation of surface morphology. Moreover, the distribution of analyte across the sensing area may not always be uniform, and depends on the sample preparation procedures, which will further reduce quantitative fidelity. For example, applying a microliter drop of analyte solution to a SERS substrate often results in the analyte adsorbing preferentially along the edge of the droplet, causing the ‘coffee ring’ effect. The presence of contaminants pre-adsorbed to the surface may also prevent uniform analyte adsorption.

Although AgNR substrates yield a relatively uniform signal response, reliable and accurate quantitative SERS analysis generally entails the acquisition of multiple measurements at various locations on any given substrate. This is to assure that the measured signals are accurate, and not the result of uneven analyte distribution or variations of the AgNR film morphology.

Measuring multiple points adds substantially more time to the analysis and requires more time to process larger volumes of data and calculate the necessary statistics. Clearly, elimination of these time consuming steps would be desirable.

Signal variability can also be introduced by the measurement itself. This is because SERS measurements typically require the use of a high power-density excitation. This may lead to photothermally-induced ablation, rearrangement, or decomposition of adsorbed analytes [68-71]. Such an issue may be problematic for repetitive measurements consecutively acquired at the same location (e.g. continuous, real-time analysis), or when very high laser power density or long excitation time is employed (e.g. for low analyte concentration studies). Likewise, mitigation of these issues is a desirable experimental goal.

One way to achieve this is to measure many ‘points’ across the substrates surface during a single measurement acquisition. Previous reports have shown that this strategy is feasible through quickly rastering the excitation laser beam to discrete locations on the substrate using mirrors [72], or by keeping the excitation laser fixed and rotating/translating the substrate [73, 74] so that the laser spot is continuously translating across the surface. By reducing the effective excitation dosage of any given point on the substrate, those studies focused primarily on reducing the excitation-induced changes of the spectral response. In addition to that benefit, sampling a larger area of the substrate also allows for a more accurate spectral signal to be acquired.

The use of this type of ‘dynamic’ SERS measurements was investigated in the context of AgNRs [64]. A special motorized translation stage is built. This stage consists of a small aluminum plate mechanically coupled to a small stepper motor via an offset shaft. Other points of the plate are attached to offset shafts, which are not connected to the motor but allowed to

freely rotate. Thus, the rotating motion of stepper motor is converted to an in-plane circular ($r = 1\text{ mm}$) translating motion of the sample stage. A custom LabView program was used to control the stepper motor rotation speed. Figure 2.1 presents a picture of the translation stage apparatus.

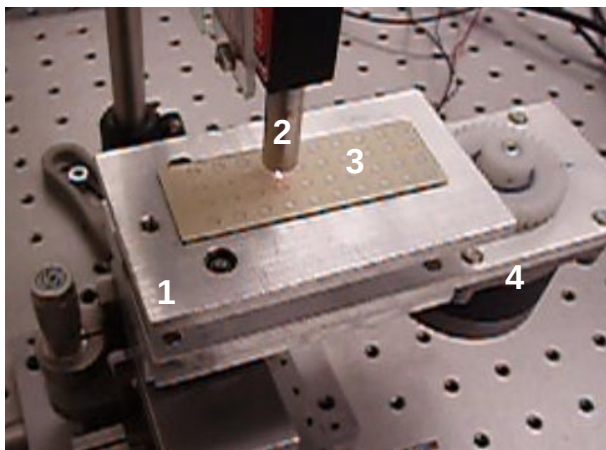


Figure 2.1. Picture of the stepper motor translation stage: 1) translating metal stage, 2) stationary Raman probe, 3) AgNR SERS substrate, and 4) stepper motor.

AgNR substrates were first incubated in 400 μM EZ-link biotin HPDP (Thermo Scientific) solution. EZ-link biotin HPDP is a biotin molecule modified with a carbon spacer arm, disulfide, and pyridyl group (see inset of Figure 2.2). The disulfide allows the modified biotin to bind strongly to the Ag surface. After prolonged incubation in the biotin solution, the substrates were soaked in methanol to remove unbound biotin, and then blow-dried. Standard static measurements were acquired at multiple locations ($n = 5$ or 9 points) in a regular array pattern on each of the 15 substrates analyzed. All measurements were performed on a Raman HRC-10HT portable Raman system (Enwave, Inc.) equipped with a 785 nm laser coupled to a Raman probe via optical fibers. The excitation laser and collected signal were both transmitted through the same lens (180° backscattering configuration). Excitation power was 30 mW, and measurement integration time was typically 10s. The excitation laser was focus to a spot size diameter $D \approx 100\text{ }\mu\text{m}$; the corresponding excitation power density $I_{\text{exc}} \approx 380\text{ W/cm}^2$.

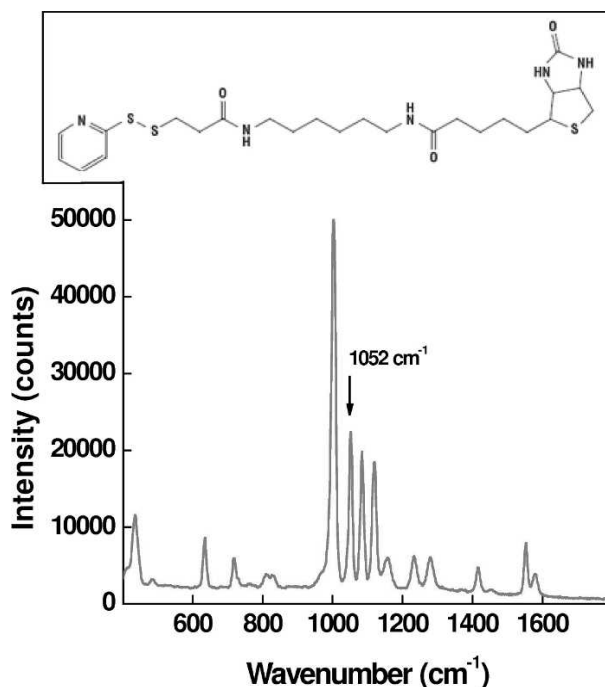


Figure 2.2. Representative SERS spectra of EZ-link Biotin HPDP. The peak near 1052 cm^{-1} is used for quantitative analysis. The structure of the molecule is also presented. (From Ref [64]).

The biotin HPDP molecule presented a very distinct and intense signal when measured with the standard, static measurements (Figure 2.2). Well-defined peaks are present at 435, 634, 717, 1002, 1052, 1084, 1155, 1230, 1280, 1417, 1553, and 1579 cm^{-1} . The 1002 cm^{-1} peak is the most intense, and corresponds to the pyridine ring-breathing mode. Because this very intense peak often times saturated the detector, the next most intense peak at 1052 cm^{-1} was used for quantitative analysis, yet the vibrational mode corresponding to this peak is unknown. The baseline corrected intensity of the peak at 1052 cm^{-1} is therefore used to quantify the SERS intensity I_{SERS} . After acquiring static measurements, the same substrates were then measured using continuous translation, and the Biotin HPDP SERS spectra remained virtually unchanged. However, the spectra acquired from the translation/dynamic measurements demonstrated more point-to-point signal uniformity: 13 of the 15 substrates demonstrated lower relative standard

deviations (RSD) when the translation technique was used, where $RSD = \frac{\sigma}{I_{SERS}} \times 100\%$, and σ and I_{SERS} is the standard deviation and average, respectively, of the SERS intensity acquired from a single substrate. Figure 2.3 shows the RSD of each substrate with rastering and static measurement. Even after removing the abnormally high and low intensity measurements occasionally acquired with the static measurements, the average point-to-point RSD (7.3%) of the static measurements was higher than the dynamic/translating techniques (3.2%).

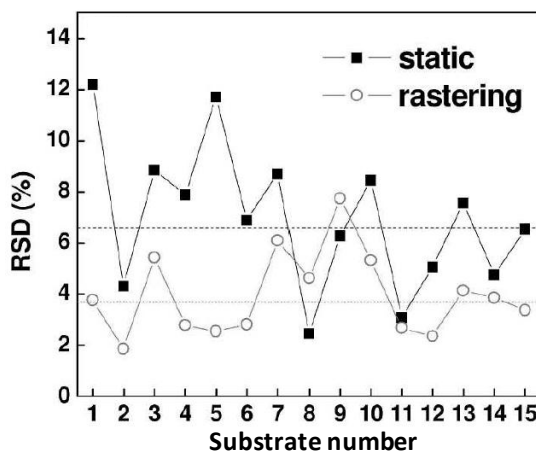


Figure 2.3. The RSD calculated from 15 substrates, each measured at multiple locations ($n = 5$ or 9) using standard static measurements or rotating rastering measurements. The rastering measurements demonstrate a strong tendency to produce a lower RSD for the substrates. The dark and light dashed lines show the average RSD for the static and rastering measurements, respectively. (From Ref [64].)

To quantify the effect of the translating speed and rotation radius r , Biotin HPDP-treated substrates were fixed to the center shaft of a chopper motor. The laser spot was fixed at varying offset distances r from the axis of rotation, allowing the laser to sweep out a solid of revolution with a radius r equal to the offset distance. The chopper motor allowed precise control of the rotation/translation frequency f between 10-400 Hz. The average point-to-point RSD for $n = 6$ substrates did show a steady yet subtle decrease from $\sim 3.5\%$ to $\sim 2.0\%$ as r increased from 0 to 1 mm (Figure 2.4A). On the other hand f did not appear to effect the average RSD (Figure 2.4 B).

These results are expected because translating the laser spot across the surface during the measurement essentially allows for the measurement to probe a larger portion of the AgNR film's surface, i.e. measuring more 'points', effectively averaging the SERS response from a larger area. From a statistical perspective, the Law of Large Numbers states that as the number of measurements increases, the average value of those measurements will approach the expected, or true, value. Thus, assuming that the point-to-point variance follows that of a normal distribution, as the sampling area A_{sampling} increases (i.e., $A_{\text{sampling}} \propto r$), the SERS signal should converge to the true value. Likewise, because f does not affect A_{sampling} , it is not expected to affect the RSD. Indeed, both of these expectations are confirmed with the experiment.

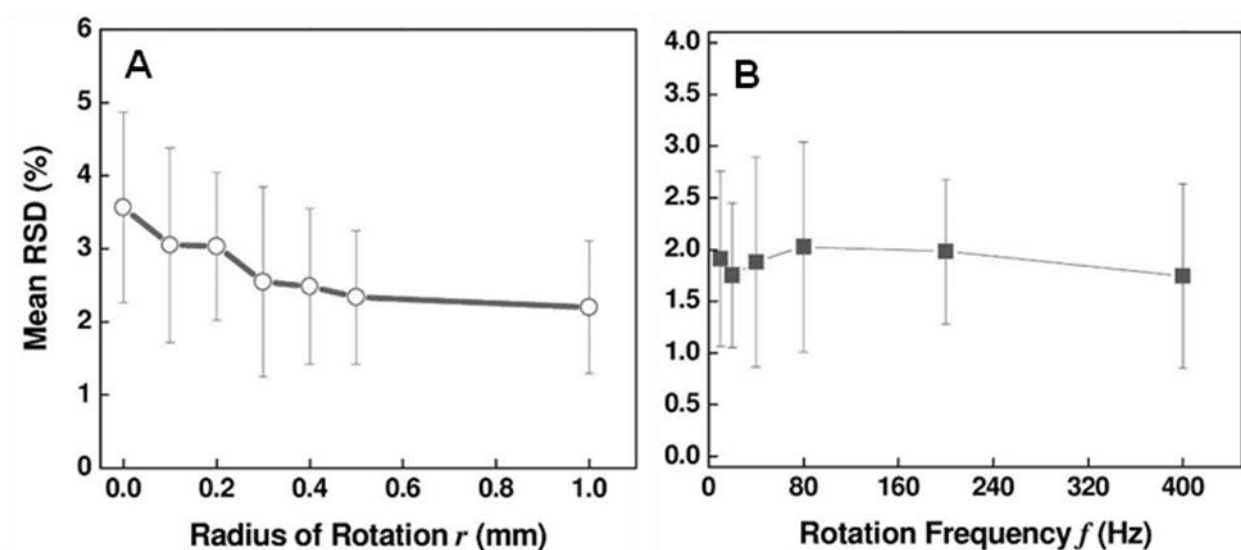


Figure 2.4. Effect of rotation A) radius r and B) frequency f on the average RSD of $n = 6$ AgNR substrates using a chopper motor to rotate the substrates. (From Ref [64].)

In addition to improving the spatial reproducibility, the dynamic/translating measurements also improve the temporal reproducibility. As mentioned above, the cumulative excitation can induce significant spectral changes. In fact, this is exactly what is observed with the Biotin HPDP. Continuously measuring a single location of the AgNR substrates over an extended period of time shows a consistent increase in the spectral intensity of the Biotin HPDP

as the cumulative excitation time t increases. Figure 2.5A shows the I_{SERS} of the Biotin HPDP (normalized to the first data point) versus cumulative exposure time; each plot was acquired from continuously measuring a single point on the AgNR substrates. The exact cause of this excitation-induced change is not completely understood, but is undesirable for quantitative measurements nonetheless. As seen in Figure 2.5B, however, continuously translating the laser spot across the AgNR surface mitigates this effect [64].

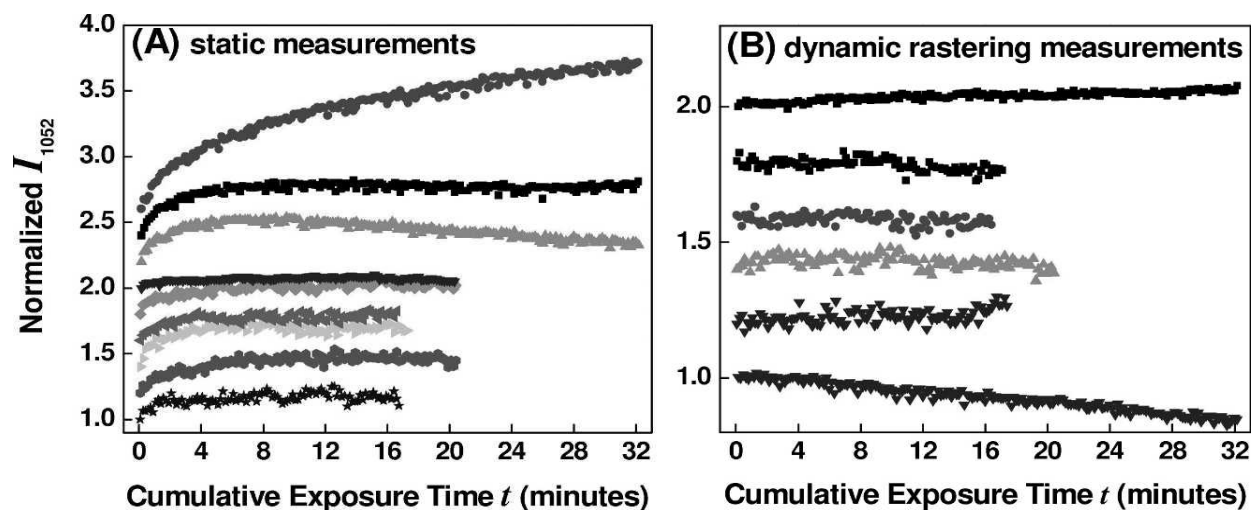


Figure 2.5. Normalized I_{SERS} versus cumulative exposure time over a period of ~15-30 minutes for A) standard static measurements and B) dynamic rastering/translating measurements. Each plot represents a different location on the substrate or a different substrate. All plots are normalized to the first data point of each data set and the plots are vertically offset for clarity. (From Ref [64].)

2.2 Design, Fabrication, and Evaluation of AgNR Array Chip Device for High Throughput SERS Screening

DNA and RNA screening assays often require the analysis of many different types of samples in addition to many replicates. This is evident with the DNA microarrays discussed in Section 1.2, where thousands of different DNA probes are spotted onto a standard microscope slide, which permits parallel screening of thousands of DNA sequences (i.e. thousands of

individual ‘experiments’) simultaneously. For such a strategy to be feasible with a SERS detection platform, the SERS enhancement must be uniform across the entire sensing area of the microarray. However, as pointed out in Section 1.3, many SERS substrate fabrication techniques cannot produce a uniform, high-enhancement surface over a large area. Fortunately, as discussed in Section 1.4, AgNRs produced by OAD can indeed yield a highly enhancing, uniform SERS signal over a large area (e.g. a microscope slide). This means that AgNR SERS analysis can in principle be integrated with a microarray technique.

To make an array-style AgNR SERS device compatible with SERS experiments, each point of the array should be capable of containing an isolated liquid sample and prevent cross contamination with other samples at adjacent points of the array. Therefore, the best approach for this is to use an array of small wells containing AgNR films on the bottom surface. Although in principle the construction of such an apparatus may seem straightforward enough, the limitations of OAD fabrication, along with the chemical, thermal, and mechanical sensitivity of the SERS-active AgNR film, means that realization of such a device requires some inventiveness.

Considering that fact that OAD is based on the shadowing effect, the nanorod films must be fabricated first, prior to patterning 3-dimensional wells. The best strategy to construct the wells is to apply a patterned array of pores/wells on top of the pre-formed AgNR film (where the AgNR films will form the bottom surface of the wells). Because the AgNRs are sensitive to chemical contamination, even from vapors, adhesives should be avoided. This means that the well pattern cannot simply be glued to the AgNR film.

Therefore we have developed a molding technique [75]. By precisely machining an array of individual ‘molding posts’ onto a metal plate one can avoid the use of adhesives. Figure 2.6A

shows the components of the mold assembly. An aluminum well-patterning plate was machined consisting of an array of 4×10 molding posts. Each post is 1 mm tall, and 4 mm in diameter. The center of each post is recessed to avoid damaging the delicate AgNR film when bringing the mold into contact with the AgNR film. Pressing the molding posts against the AgNR film therefore defines a 1 mm void space between the AgNR film and the aluminum plate. This forms a sufficient seal between the molding posts and the AgNRs, while the recessed center of each molding post does not contact the AgNRs that will be used for sensing. The substrate/patterning plate assembly can then be placed into a base plate (which holds the patterning plate and substrate assembly together, while defining the sides of the mold). Attaching a top plate to the base plate via screws holds the mold patterning plate tightly against the AgNR substrate.

Polydimethylsiloxane (PDMS) was chosen as a molding material because it is cheap, non-toxic, chemically stable, easy to work with, and can be cured at low temperature [76]. In addition, the mechanical properties (e.g. rigidity) can be tailored by changing the ratio of the PDMS polymer and the curing agent. PDMS also demonstrates enough fluidity to be poured into the 1 mm wide gap and gravimetrically seep completely into the mold cavity. At the same time PDMS is viscous enough to avoid leaking between the seal formed by the AgNR film and molding post.

Because the PDMS curing rate and the AgNR film integrity are both temperature-sensitive, meticulous attention must be paid to the heating/curing process. Pre-heating and curing are performed with a bench top resistively-heated oven. Heating at too high of a temperature and the PDMS cures before completely filling the mold cavity, leaving many wells poorly formed. Using too low of a temperature allows polymer residues enough time to seep into the AgNR sensor area before curing. Heating too long would lead to degradation of the AgNR film [77].

After careful evaluation, the best temperature to use for pre-heating and curing was $95 \pm 10^\circ \text{C}$. In addition, the pre-heating and curing times were also optimized, which entailed pre-heating the substrate/mold assembly for 20.0 ± 0.5 minutes, while pre-heating the PDMS for 5.0 ± 0.5 minutes with mixing; this is followed by pouring of the pre-heated PDMS into the mold assembly and curing 20.0 ± 2 minutes. The process flow of the well patterning is shown in Figure 2.6B.

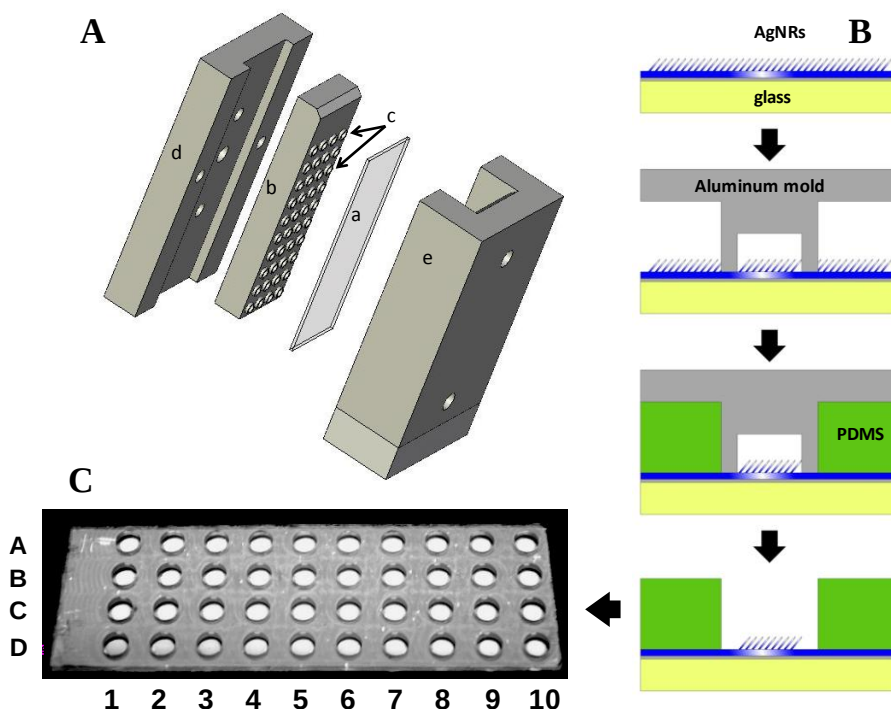


Figure 2.6. A- Components for of the well patterning mold assembly: a) $1 \times 3 \text{ in}^2$ AgNR substrate, b) well patterning plate, c) well molding posts, d) top plate, and e) base plate. B - Fabrication process of PDMS wells: i) AgNRs are first uniformly deposited onto a glass microscope slide, ii) attach the aluminum mold so that molding posts are in direct contact with the AgNRs, iii) injecting PDMS, and iv) after curing the mold is removed. C - Picture of a completed PMDS-patterned 4×10 well AgNR SERS chip.

After the newly patterned AgNR SERS chip is separated from the well-patterning plate, it is ready to use. The patterned SERS chip contains a uniform 4×10 array of 4 mm diameter, 1 mm deep cylindrical wells; each well has a cylindrical volume of $12.6 \mu\text{L}$. A picture of a

completed SERS chip is presented in Figure 2.6C. The PDMS also forms a strong liquid-proof seal with the AgNR film. Leak tests showed that a high concentration analyte test solution produced a very strong signal in the well that it was applied, but no signal could be observed in any of the surrounding wells, even after prolonged incubation [75]; therefore, the PDMS-to-AgNR seal is sufficient to completely confine liquid samples.

Various concentrations of BPE solutions (in methanol) were applied to each of the forty wells to test the sensitivity and uniformity of the SERS response. After the BPE solution dried, each well was measured at three different points using an inVia Renishaw system equipped with a 5× objective and a 785 nm laser. The entire chip was scanned in an automated fashion using the Renishaw's programmable translation stage. Prior to adding BPE solution, each well was first treated with 5µL of pure methanol (0 M BPE) and allowed to dry before measuring. Next, 5µL of 10^{-8} M BPE was applied to each well and allowed to dry before measuring. This was then repeated for 10^{-7} , 10^{-6} , and 10^{-5} M BPE.

The BPE signal is composed of several strong spectral peaks near 1000, 1200, 1610, and 1640 cm^{-1} which corresponds to the ring-breathing, in-plane ring mode, aromatic ring stretching, and ethylene stretching modes, respectively [78]. The BPE signal was quantified by the baseline-corrected 1200 cm^{-1} peak intensity I_{1200} . One particular well in the corner demonstrated noticeably lower intensity than that of other wells at all BPE treatments; therefore the data from this well is removed from the proceeding statistical analysis. Prior to MeOH or BPE treatment, the mean I_{1200} and standard deviation (SD) was determined to be 32 ± 14 counts. After adding pure methanol, I_{1200} increases to 56 ± 42 counts. For the 1×10^{-8} M BPE treatment, $I_{1200} = 909 \pm 412$ counts. The limit of detection (LOD) can be defined using the standard definition $\text{LOD} = I_0 + 3 \times \text{SD}$, where I_0 is the background intensity prior to adding analyte (MeOH only); for the

SERS chip tested here the LOD = 182 counts. The lowest concentration tested (1×10^{-8} M) therefore produces a mean signal that is 727 counts above the LOD. The higher concentration samples 1×10^{-7} , 1×10^{-6} , and 1×10^{-5} M BPE yields I_{1200} of 5500 ± 2200 , 21000 ± 7700 , 49100 ± 11700 counts, respectively [75].

Figure 2.7 shows a SERS chip I_{1200} mapping for all 40 wells after each successive BPE treatment, where each map represents a different treatment and each rectangular panel in the map represents the average I_{1200} for one well. The I_{1200} is represented as a grey scale, with black indicating 0 counts, and white representing 80,000 counts. Clearly the intensity is observed to increase as the concentration of BPE is increased.

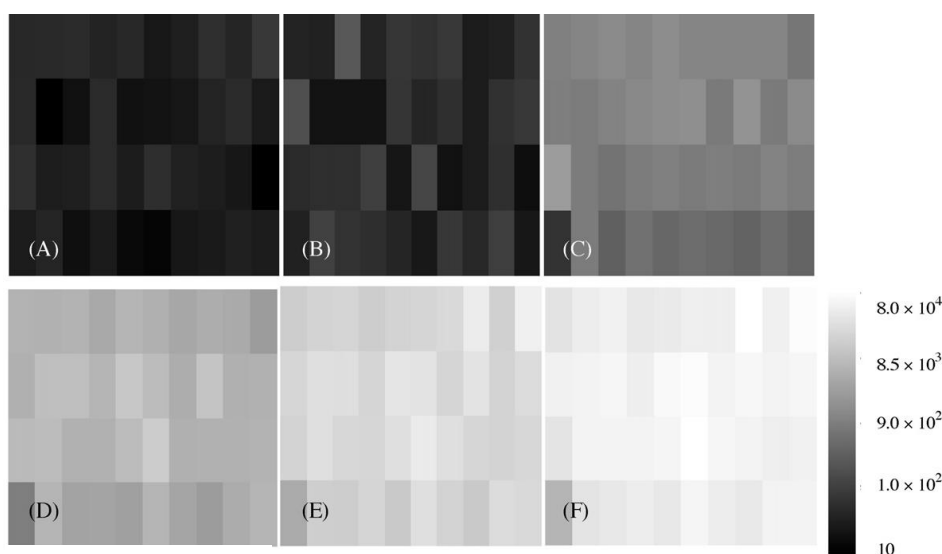


Figure 2.7. Mapping of average I_{1200} acquired from all 40 wells for each treatment A) untreated, B) MeOH only, C) 1×10^{-8} M, D) 1×10^{-7} M, E) 1×10^{-6} M, and F) 1×10^{-5} M BPE. The average I_{1200} is represented by the logarithmic gray scale with units of counts (shown at the right). Each rectangular panel represents a single well. The well in the lower left corner can be seen to be unresponsive and is excluded from the analysis. (From Ref [75].)

Figure 2.8A is a bar graph showing the mean I_{1200} calculated from the 39 active wells of the chip versus BPE concentration. The corresponding well-to-well SD is expressed by the error bars; the relative standard deviation (RSD, i.e. $SD/I_{1200} \times 100\%$) is displayed above each bar.

The lowest RSD of 24% (for 1×10^{-5} M BPE) is significantly higher than the <15% point-to-point RSD values reported elsewhere for AgNRs [60, 64]. Either the patterning process degrades the intrinsic SERS uniformity of the AgNR, or the presence of the wells affects the analyte distribution uniformity on the AgNR film in the well. Although the 5 μ L sample volume was sufficient to completely cover the AgNR film at the bottom of the well, as the MeOH dried it could visually be observed to accumulate as a small droplet at the edge of the well before completely drying. This means that more BPE could be preferentially deposited/adsorbed to this part of the AgNR film.

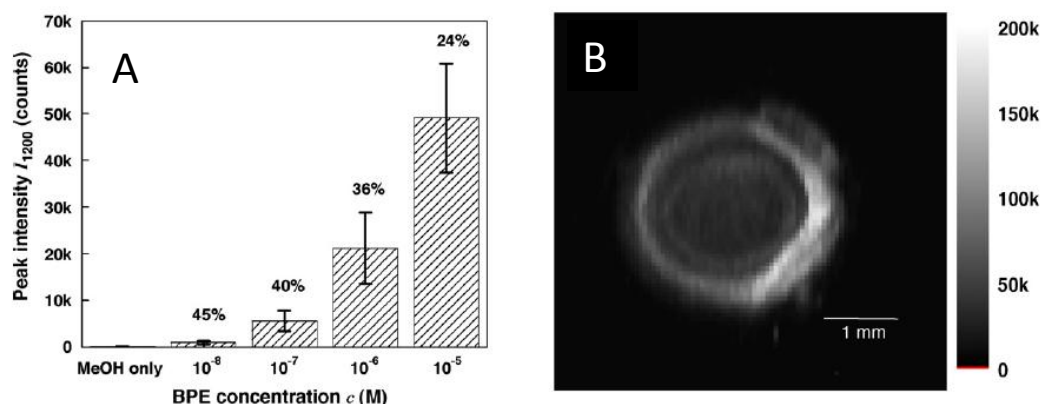


Figure 2.8. A) Bar graph showing the mean I_{1200} of 39 wells for each BPE concentration C treatment from Figure 2.7. The error bars represent the standard deviation, and the RSD is provided for each treatment. B) A high resolution spatial I_{1200} mapping after addition of drying of BPE sample in the well. The mapping is composed of ~ 5000 points measured at 50 μ m steps; the I_{1200} is expressed by the grey scale shown to the right. (From Ref [75].)

To investigate the possibility of these effects, Figure 2.8B shows a high-resolution I_{1200} mapping (i.e. 50 μ m measurement steps sizes; ~ 5000 total points measured) of a single well after BPE treatment. Again the I_{1200} is represented as a grey scale, with each ‘pixel’ in the mapping representing a single measurement. From this image we can see very clear and discernible patterns of the I_{1200} spatial distribution. First, ‘coffee-ring’ patterns are observed as the I_{1200} varies as a function of radial distance from the center of the well. Secondly, there is an obvious

high intensity region along one side of the well, presumably where the BPE solution formed an isolated droplet before drying. These observations indicate that the increased signal variability observed after patterning could be due to non-uniform analyte distribution, which could contribute to large signal variation.

The *apparent* sensitivity of the patterned AgNR SERS chip compared to previous studies using unpatterned AgNR substrates was also evaluated. We define the apparent sensitivity as the change in measured SERS intensity with respect to the change in the number N molecules of BPE adsorbed to the AgNRs, i.e. I_{1200} vs. N_{BPE} . N_{BPE} can be estimated if the BPE sample concentration C_{BPE} and the spreading area are known (assuming a roughly uniform distribution of BPE) and by approximating the probed area, i.e. the laser spot size. Figure 2.9A plots the I_{1200} versus C_{BPE} in a log-log scale for the 39 active wells with a linear regression for each well along with the averaged linear regression. For this study and the two previous studies by Driskell et al. [60] and Chu et al. [79], the change in the $\log I_{1200}$ vs. the change in $\log N_{\text{BPE}}$ demonstrates a strongly linear behavior described by $\Delta \log I_{1200} = \alpha \Delta \log N_{\text{BPE}}$, where α is the slope and can be treated as the *apparent* sensitivity. Acquiring the raw data from the previous studies by Chu et al. and Driskell et al. that used unpatterned AgNR substrate, α was calculated to be 0.38 and 0.37 respectively. Figure 2.9B plots compares the average linear regression from Figure 2.9A (with C_{BPE} converted to N_{BPE}), to those calculated from Driskell et al. and Chu et al. The patterned SERS chip gives $\alpha = 0.57$ which is higher than those of the other reports. This discrepancy could be due to the intrinsic differences in measurement sensitivity from the different instruments used for these different studies.

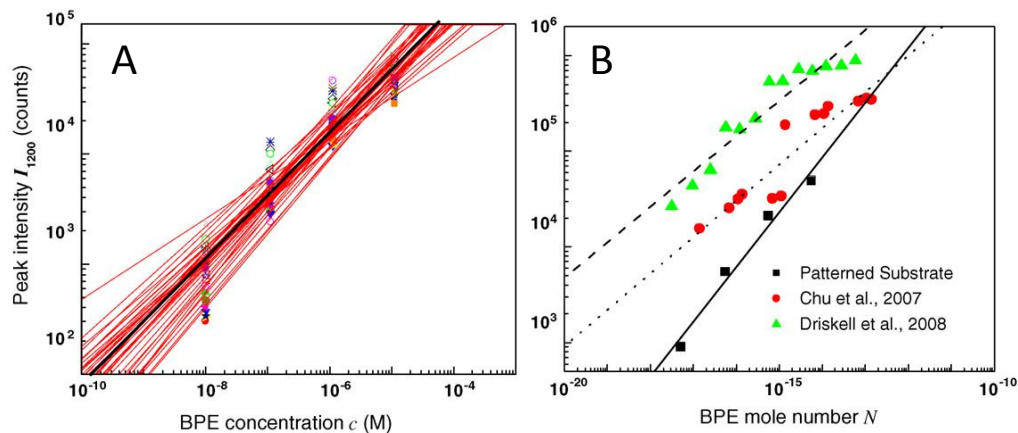


Figure 2.9. A) The I_{1200} vs C_{BPE} for the 39 active wells each fit with a linear regression (thin lines). The average linear regression is plotted as the bold line. B) The average apparent sensitivity (from part A; with respect to N_{BPE}) compared to the apparent sensitivity calculated from BPE data from Driskell et al. and Chu et al. (From Ref [75].)

Since the shape of the well is defined by the mold, the mold shape can be changed to address issues related to the sample spreading. For example, the previous discussion utilized a well-patterning plate with cylindrical molding posts that yields cylindrical wells. However, by tapering the molding posts into cones, one can yield conical/funnel-shaped wells with a reduced AgNR sensing area (Figure 2.10). The sloping walls of the well, in addition to a significantly smaller AgNR sensing area at the bottom of the well (sensing area diameter = 1.5 mm), assist the spreading of the sample solution as it dries onto the AgNR film. This allows the sample solution to stay more evenly distributed across the AgNRs as it dries, ultimately producing a more uniform deposition of the analyte onto the sensing surface. A conical well chip is fabricated in the same way as the cylindrical well chip, except a different well patterning plate is used. Preliminary data suggests that the point-to-point and well-to-well signal reproducibility is improved with the conical design [80]. In Figure 2.10, the I_{1200} is plotted for each of the 40 wells of a chip containing cylindrical wells and also a chip with conical wells. The average I_{1200} of all 40 wells is presented as a solid horizontal line, and one standard deviation above and below the

average is presented as dotted horizontal lines. The well-to-well RSD is found to be 65.5% and 28.0% for the cylindrical and conical wells, respectively.

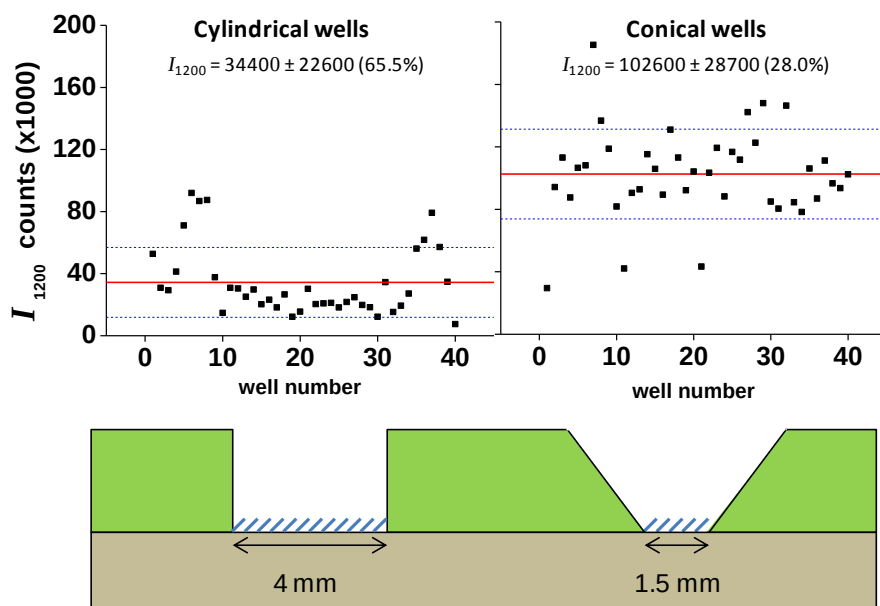


Figure 2.10. TOP: The I_{1200} of each of the 40 BPE-treated wells from a cylindrical and conical well chip. The solid horizontal lines represent the average I_{1200} acquired from the whole chip, and the dotted lines represent one standard deviation above and below the average. (From Ref [80].) BOTTOM: Cross sectional diagram showing the profile of the cylindrical and conical wells (not drawn to scale).

In addition to improving uniformity, the conical wells also show improved sensitivity compared to the cylindrical wells. For example, Figure 2.10 demonstrates the average I_{1200} from the cylindrical well chip is 34400 counts, and 102600 counts for the conical well chip. To further evaluate the improved sensitivity of the conical wells, thiolated DNA probes complementary for let-7f miRNA were used as a test analyte. 5 μ L of probes in 2 $\times$ SSC buffer was incubated in conical and cylindrical wells for $\sim$ 12 hours followed by rinsing and drying and then measured [65]. Figure 2.12 shows that with the conical wells the DNA intensity (quantified with the peak intensity of the 730 cm^{-1} adenine peak, I_{730}) shows a faster increase as a function of DNA probe concentration compared to the cylindrical counterparts. The conical wells demonstrate a

saturated DNA signal at ~ 250 nM, whereas the cylindrical wells do not demonstrate saturated signal intensity until ~ 1000 nM. With a reduced sensing area A , and therefore less total adsorption sites, the relative DNA surface coverage θ_{DNA} will be higher for a given DNA concentration. Because θ_{DNA} is proportional to the SERS intensity, a smaller A will yield a higher I_{730} .

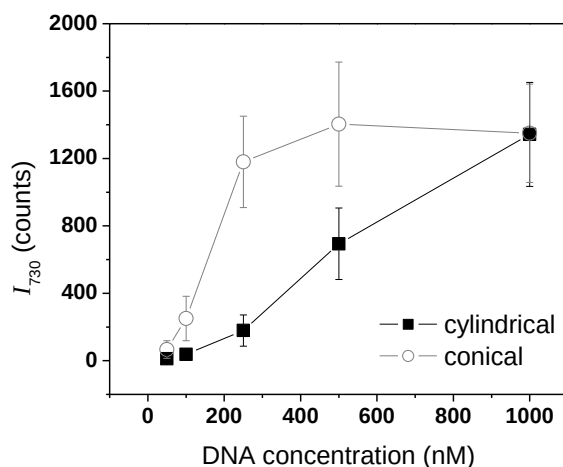


Figure 2.11. The 730 cm^{-1} adenine peak intensity (I_{730}) plotted as a function of thiolated let-7f DNA probe concentration for both the cylindrical and conical wells. (Data courtesy of Jeremy Driskell.)

2.3 Conclusions

In this chapter we have highlighted ways of optimizing the AgNR SERS measurements using dynamic rastering measurements to improve point-to-point reproducibility and reduce excitation-induced changes. We also pointed out that because dynamic measurements are more accurate, fewer SERS measurements need to be acquired, shortening the analysis time. We also described how a AgNR multi-well array SERS chip could be fabricated to improve SERS analysis for high-throughput screening applications. Methods of optimizing the SERS chip were also discussed. Now that we have devised a way to fabricate an array-style AgNR SERS substrate, we may now utilize this SERS chip for miRNA analysis.

Chapter 3

LABEL-FREE MICRO-RNA HYBRIDIZATION AND DETECTION USING A SILVER NANOROD SERS CHIP

As stated in Chapter 1, label-free nucleic hybridization detection via SERS has been a challenging endeavor in part due to the substrate-related issues such as low intensity or poor reproducibility. Such a detection strategy has also been hampered by the high degree of chemical similarity between the probe and the target. The fact that the target and probe sequences are both composed of the same chemical constituents, i.e. nucleotides, means that they produce essentially identical SERS signatures. This will ultimately impede the signal specificity. Deciphering the miRNA signal amongst the DNA probe signal is therefore a non-trivial endeavor. In addition, each of the four nucleotides produces a rather complex signal with significant overlap, which further complicates detection of miRNA hybridization.

Because of these issues, there have been only to date a very limited number of studies that have attempted to use SERS to directly detect the hybridization of DNA targets with substrate-immobilized DNA probes. One strategy is to use a chemical analogue. Barhoumi and Halas utilized substituted the adenines of the DNA probe with 2-aminopurines [48]. This analogue produces a significantly different SERS signature than adenine, yet is still complementary with T. Thus, the probe signature lacks the adenine signal, but can still hybridize with the probe. Upon hybridization with the target, which contains natural adenine, the SERS signal will definitively display the adenine signature of the target. Other studies have examined traditional probe-target hybridization. Dong et al. monitored how the spectrum of immobilized

DNA changed after treating with a complementary target sequence. The study discloses subtle spectral changes, yet the overall spectral quality was poor, no thorough quantitative analysis was given, and no negative controls were reported. However, Green et al. demonstrated a more convincing study. In that report, a DNA probe was thiol-immobilized onto Ag ‘nano-torus’ structures. The DNA probe produced spectrum with acceptable quality, and clear quantitative changes in the relative peak intensity of the nucleobase ring breathing modes is observed after hybridization with a complementary target. In addition, the report also demonstrates that no significant quantitative change occurs in the relative peak intensity when the probes are treated to single-base mismatch targets. This suggests that cross-hybridization is not interfering with the detection. Yet, that study did not discuss the use of high-throughput SERS analysis in a microarray-style platform. However, the study did not discuss quantitative analysis in context of DNA spectra demonstrating a large degree of similarity before and after hybridization.

In this chapter, we will turn our attention to the direct SERS detection of miRNA hybridization with DNA probes using a patterned AgNR multi-well array SERS chip. In addition to highly sensitive and uniform SERS measurements, this SERS platform allows for parallel processing and analysis of multiple sequences. The use of linear least squares regression analysis will also be introduced to aid in the quantitative analysis of miRNA hybridization when spectral changes due to hybridization are subtle. First, however, a careful and systematic SERS analysis of all the relevant chemical components must be performed before label-free miRNA SERS analysis can be realistically achieved.

3.1 SERS Analysis of miRNA Building Blocks: A, C, G, and U

Nucleic acids are composed of nucleotides, which are in turn composed of three general moieties: a phosphate, a ribose, and a nucleobase. The phosphate group provides the nucleic acids with an intrinsic negative charge and is bonded to the 5-carbon ribose. The ribose bonds to the phosphate of another nucleotide, ultimately generating the phosphate-sugar backbone and giving DNA its polymeric property. Moreover, the presence or absence of a hydroxyl (-OH) group on the 2' carbon of the pentose ring is one of the characteristics defining the molecule as a RNA (ribonucleic acid) or DNA (*deoxyribonucleic acid*) nucleotide, respectively. The ribose is also bonded to the nucleobase, which is either a purine or pyrimidine base. The purines, i.e. adenine and guanine, consist of a double-ring structure; the pyrimidines, i.e. cytosine, thymine, and uracil, are single-ring structures. Both DNA and RNA share the same nucleobases, except that thymine is replaced with uracil in RNA. Therefore, the nucleobases adenine, cytosine, guanine, thymine, and uracil instill each nucleotide with its specific identity as A, C, G, T, and U, respectively. Table 3.1 shows the structures of A, C, G, and U RNA nucleotides and also the T DNA nucleotide. The nucleobase moiety is ultimately responsible for the complementary nature of DNA and RNA: a sequence of nucleotides is capable of forming a stable hydrogen (H)-bonding network with the nucleotides of a different sequence. However, the molecular structure of each nucleobase only permits the efficient H-bonding with its complement, where A is complement to T (or U), and C is complement to G.

Because these five nucleotides are all capable of generating a SERS signal, understanding their individual SERS signatures will aid in the analysis of their nucleic acid polymers. To do this, AgNR films were deposited onto glass microscope slides pre-cut into 0.9×0.9 cm² pieces using the same general procedure as previously described in Section 2.1 [60]. RNA

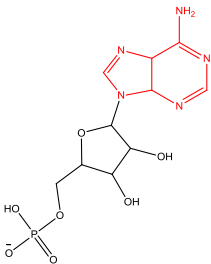
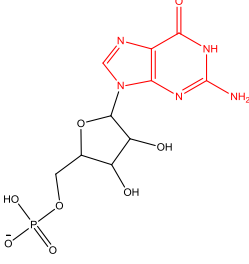
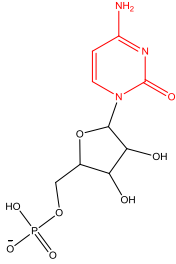
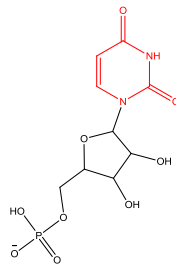
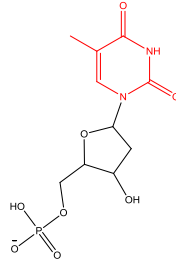
Adenosine 5' monophosphate (AMP)	Guanosine 5' monophosphate (GMP)	Cytidine 5' monophosphate (CMP)	Uridine 5' monophosphate (UMP)	Thymidine 5' monophosphate (TMP)
				

Table 3.1. Ribonucleic acid nucleotide structures of A, G, C, and U with the nucleobases highlighted in red. The deoxyribonucleic acid of thymidine (T) is also shown.

(MNTs) were purchased as X5'- monophosphate disodium salt (X = adenosine, cytosine, guanosine, or uridine, i.e. AMP, CMP GMP, and UMP) with $\geq 99\%$ purity from Sigma Aldrich. The MNTs were dissolved in high purity de-ionized (DI) 18 M Ω -cm water to a concentration of 10 mM. 1 mL of each MNT solution was added to a cleaned glass test tube. Pre-adsorbed contaminants may interfere with the adsorption of the MNTs, so an Ar⁺ plasma treatment is used to clean organic contaminants from the AgNR surface [81]. This entails briefly treating the AgNR substrates with Ar⁺ plasma using a custom-made plasma chamber (90 s treatment with 30 W power and Ar pressure = 600-700 mTorr) prior to immersing the AgNRs into the MNT solution. The AgNR substrates were allowed to incubate for ~1 hour before removing, rinsing with DI water, and blow-drying with N₂. The substrates were then measured (Enwave HRC-10HT; 30 W, 5 s integration) at $n = 9$ locations across the substrate surface. Figure 3.1 shows the average SERS spectra acquired from each AgNR substrate treated with different MNT solutions [82]. The resulting spectra showed very high intensity, exceptional point-to-point reproducibility, and unique spectra for each of the MNTs.

From Figure 3.1 we can see that each MNT produces a fairly distinct spectrum. We can also see that the four spectra can be approximately separated into two groups. CMP and UMP (i.e. pyrimidines) produce similar spectra and both have their most prominent band at 783 and 789 cm^{-1} , respectively, which corresponds to the C and U ring breathing modes [51]. They also share prominent spectral features at ~ 1020 , ~ 1290 , and ~ 1630 cm^{-1} , yet CMP produces an overall higher intensity for these peaks. AMP and GMP (i.e. purines) demonstrate some similarity between their spectral profiles, sharing only one prominent band near 1320 cm^{-1} which has been assigned to the ring stretching mode. AMP's second most prominent band is located at 732 cm^{-1} (ring-breathing) [51]; other prominent peaks are located at 550, 1463, 1560 cm^{-1} . GMP is reported to have a ring-breathing mode between 650 and 690 cm^{-1} [42, 51], and shows an overall lower intensity compared to its AMP counterpart

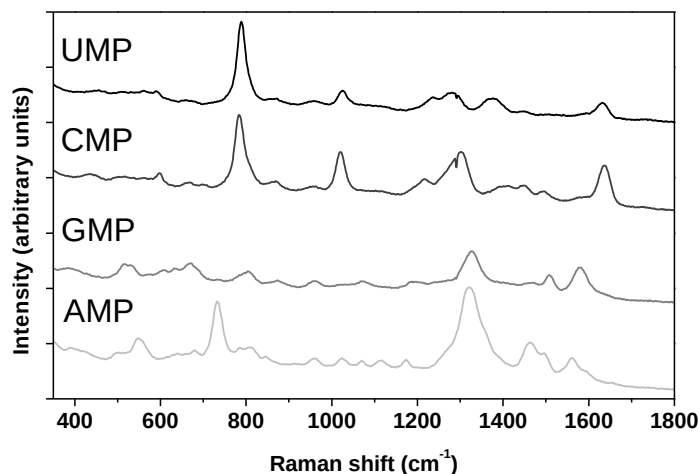


Figure 3.1. Average SERS spectra acquired for each MNT from Ar^+ plasma-cleaned AgNR substrates. The spectra are all scaled equivalently and vertically offset for clarity. (From Ref [82].)

From Section 1.3, recall that the general spectral features, peak assignments, and relative cross sections of nucleic acids have been under considerable debate in the literature, with different research groups producing different results and interpretations. However, the MNT

spectra presented here show exceptional similarity with those obtained by concomitant studies performed by Steven Bell's group [51, 55]. Despite the fact that they used different substrates (Ag colloids), different nucleotides (DNA), and different measurement conditions (in liquid) compared to the experimental conditions presented here (i.e. AgNRs, RNA, and 'dried', respectively), the spectral profiles are remarkably similar. This also indicates that the presence or absence of the hydroxyl group on the ribose ring does not change the spectral profile to any discernible extent. Moreover, the MNT spectra are also predominantly characterized by spectral features originating from the nucleobases. Bell's group has also demonstrated that the corresponding nucleosides (i.e. nucleotide without a phosphate) produce spectra that are essentially identical to their corresponding nucleotides; likewise, they showed that the phosphate signal did not contribute to the measured nucleotide signal [55]. After a close examination of the SERS signatures of the MNT components, we can now turn our attention to the SERS detection of the DNA probe and miRNA target.

3.2 Functionalizing AgNRs with DNA Probes and Spacer Molecules

Before a miRNA target can be specifically captured and detected with SERS, one must first imbue the AgNR surface with the requisite specificity by immobilizing a complementary probe. In order to do this, the immobilization technique should allow the probe sequence's nucleobases to adequately interact with the sample to allow efficient hybridization with a complementary target. Additionally, the probe layer should provide a high degree of stability so that it does not degrade during the experiment.

The most direct way to immobilize the DNA probe to the surface is to use an unmodified sequence and allow it to non-specifically adsorb to the surface. Single-stranded nucleic acids are

known to strongly bind to Au and Ag surfaces via the lone-pair nitrogens of the nucleobases [50, 51, 56, 83, 84]. In this case, the nucleobases cannot efficiently interact with a complementary miRNA target sequence in the surrounding solution. Moreover, although the nucleobase-metal interaction is fairly strong, it is not stable enough to withstand the presence of other common sample constituents such as high concentrations of Cl^- or citrate, which will out compete the nucleobases for adsorption to the surface [51].

Therefore, a more desirable strategy entails tethering one end of the DNA probe to the surface with an irreversible covalent bond, while leaving the rest of the molecule free to interact with the sample. This strategy has already been adopted for traditional (label-based) DNA microarrays. In that case, the DNA probe is modified on one of its terminal ends with various types of linker groups capable of forming stable/permanent bonds with glass surfaces [85, 86]. A similar strategy can be adopted to immobilize DNA probes onto silver surfaces with a thiol (-SH) modification. Thiols have been used in many SERS studies due to their proclivity to form strong stable bonds with Ag and Au surfaces, and readily form self-assembled monolayers (SAMs). Several reports have also demonstrated that thiol-modified ss- and dsDNA are readily capable of producing high quality nucleic acid SERS signals [44, 46, 47, 49, 67, 87].

Although thiol-modified DNA can be used to form a highly stable probe layer, additional attention must be paid to the resulting probe layer characteristics before the requisite specificity can be achieved. Intuitively, one may assume that a highly packed probe layer with a high DNA surface coverage, i.e. $\theta_{\text{DNA}} \approx 1$, would yield the best capturing efficiency. However, a high θ_{DNA} can actually impede miRNA detection. This is because efficient hybridization with a target sequence requires ample space around the DNA probe. When θ_{DNA} is too high, steric hindrance

from neighboring DNA sequences prevents the efficient intercalation of the miRNA probe into the probe layer, which will occlude the miRNA from hybridizing with the DNA [44, 83, 88].

On the other hand, a low θ_{DNA} is detrimental to the detection sensitivity as well, as a sufficient number of probes must be immobilized to allow sufficient capture of targets. A low θ_{DNA} will also leave much of the metal surface exposed. Although the DNA probe is tethered at one end to the metal surface via a thiol, DNA is a flexible molecule and the nucleobases may adsorb to the exposed metal surface. Likewise, the miRNA target may also non-specifically adsorb to the exposed Ag surface. To circumvent this issue, a spacer-molecule strategy may be employed [83]. Spacer molecules are typically short, straight-chained *n*-alkane thiols (e.g. *n* = 6). The spacer is usually added after a sub-monolayer probe layer is immobilized, and used to ‘back-fill’ the remaining exposed metal surface. When a spacer is used, the thiol-linker of the DNA probe is typically modified with an equivalently long alkyl spacer so as to elevate the bottom of the DNA probe above the spacer layer.

3.2.1 Immobilization and SERS Response of DNA Probes on AgNRs

For the following experiments, modified DNA probes were purchased from Integrated DNA Technologies, Inc. The probe sequences were designed to be complement to several different miRNA sequences referred to as let-7f, Test, reverse (rev) let-7f, and miR-224 (see Section 3.3). The 5' ends of the probes were modified with 6-carbon linker terminated with a disulfide (5ThioMC6-D); with the other half of the disulfide containing a hexanol. A patterned SERS chips (see Section 2.2) was used for all experiments. Each well was treated with 20 μL of DNA solution and allowed to incubate in a humidity chamber for 8-12 hours, followed by thorough rinsing with DI water and blow-drying with N_2 . All SERS measurements were

performed using a Raman HRC-10HT portable Raman system with the same conditions as discussed in the previous sections ($\lambda_{exc} = 785\text{nm}$, excitation power = 30 mW, measurement time = 10 s). Multiple static measurements ($n = 5$) were acquired from different locations in each well. The sequences of the DNA probes used can be found in Table 3.2.

Sequence Name	Sequence 5'→ 3'	Ratio A:C:G:T (or U)	Relative Ratio (in %)
let-7f probe	AAC TAT ACA ATC TAC TAC CTC A	9:7:0:6	41 : 32 : 0 : 27
let-7f target	UGA GGU AGU AGA UUG UAU AGU U	6:0:7:9	27 : 0 : 32 : 41
Test probe	TCT CTC TCT CTC TCT CTC	0:9:0:9	0 : 50 : 0 : 50
Test target	AGA GAG AGA GAG AGA GAG	9:0:9:0	50 : 0 : 50 : 0
miR-224 probe	GTT CAG TGA TCA CCA AGG CAA	7:5:5:4	33 : 24 : 24 : 20
rev miR-224 target	CAA GUC ACU AGU GGU UCC GUU	4:5:5:7	20 : 24 : 24 : 33
rev let-7f probe	ACT CCA TCA TCT AAC ATA TCA A	9:7:0:6	41 : 32 : 0 : 27

Table 3.2 Sequences, absolute nucleotide ratios, and relative nucleotide ratios of DNA probes and miRNA targets.

Initially, the DNA probes were suspended in pure water; based on Figure 2.11, and according to various references [44, 56], the DNA concentration was chosen to be 1 μM (dissolved in DI water) to achieve a maximum DNA signal, which presumably represents approximately one monolayer. After immobilization of the DNA probe, the AgNRs were rinsed with DI water and dried, and the SERS signal was measured. The measured SERS spectra are presented in Figure 3.2. These preliminary experiments produced relatively low-quality spectra, with significant point-to-point variation and low DNA peak intensity. Moreover, many other unidentified and exogenous spectral features appeared to be present in addition to a relatively

intense baseline signal. Based on these findings, we may anticipate that the DNA is not adsorbing uniformly to the AgNR surface (hence the spectral variability) and/or is it not adsorbing to a significant extent (hence the low intensity). This could be a result of poor adsorption efficiency or irregular adsorption configuration of the DNA probe [46].

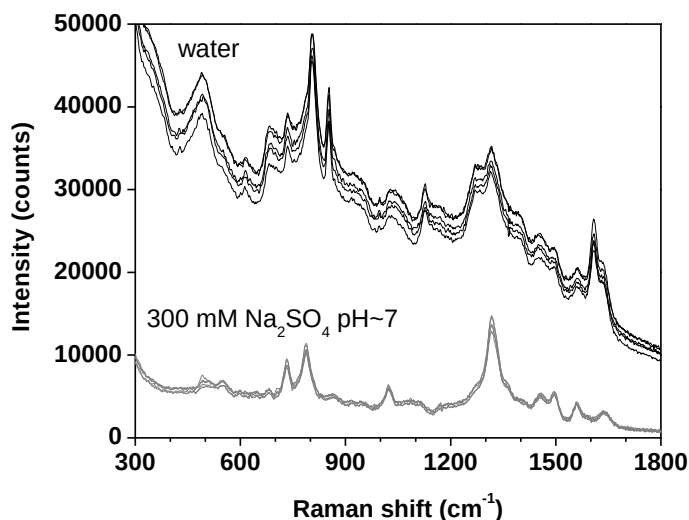


Figure 3.2. SERS spectra of thiolated let-7f DNA probe immobilized onto AgNRs using water and 300 mM Na_2SO_4 pH~7. Five measurements from the same well are shown for each sample.

To address this issue, one must first consider the physical properties of the DNA molecule. As a result of its phosphate-containing backbone, DNA has a significant negative charge, which can impede high adsorption efficiency due to the electrostatic repulsion. This means that some DNA probes may readily adsorb to the Ag surface at first, but the adsorbed DNA will begin to electrostatically repel the other DNA probes in solution; thus preventing a high density adlayer of DNA probes from forming [56]. Whereas a vertical, extended conformation is desirable for higher surface coverage, DNA sequences are known to form random irregular coils, which can impede their adsorption efficiency, take up more space on the metal surface, and ultimately degrade the SERS detection [46]. Thus, this undesirable electrostatic effect should be mitigated and requires that the charge of the DNA molecule be

electrostatically screened. Fortunately, this can be easily achieved using a salt solution. Herne and Tarlov have previously demonstrated such an effect using KH_2PO_4 as a buffer solution [56]. Cations in the solution will congregate around the negatively charged DNA and screen the negative charge. That study showed that for a 1 μM thiolated-DNA solution, the surface coverage of DNA on Au increased drastically as the KH_2PO_4 concentration increased from 0 to 0.1 M, before leveling off when $\text{K}^+ \geq 0.4 \text{ M}$.

To test this possibility with our experiment, the probes were first suspended in salt solutions of Na_2SO_4 . This particular salt was selected because Na^+ and SO_4^{2-} ions do not adsorb to or react with Ag surfaces and should not interfere with DNA adsorption. The Na_2SO_4 concentration was set to 3, 30, or 300 mM and the pH adjusted to be ~ 3.5 or ~ 7 . The DNA solutions were then added to the wells of a SERS chip and incubated as described previously. After rinsing and drying, the resulting SERS measurements showed vastly improved spectral quality. Figure 3.2 also shows the spectra from DNA immobilized with 300 mM Na_2SO_4 at pH ~ 7 . The DNA immobilized in salt solution demonstrated high intensity, good reproducibility, and very clear characteristic spectral DNA features. With 30 mM Na_2SO_4 pH ~ 7 , the spectral response comparable with that of the 300 mM Na_2SO_4 sample. The improvement in spectral quality was also observed with 3 mM salt concentration, but to a lesser extent as it demonstrated overall lower DNA peak intensities, higher background signal intensity, and also demonstrated unidentified exogenous spectral features. Na_2SO_4 and HNa_2PO_4 buffer at pH ~ 3 were also evaluated and produced similar results (data not shown).

Figure 3.3A shows the average spectra of four different probes immobilized in the presence of 30 mM Na_2SO_4 in different wells of a SERS chip. The vertical dashed lines represent the primary spectral feature observed for the AMP, CMP, GMP, or UMP pure nucleotide spectra

discussed in Section 3.1 and shown in Figure 3.1. The spectral signatures of the individual nucleotides can be readily discerned in the convoluted DNA spectra. The let-7f, miR-224, and rev let-7f all demonstrate very similar spectra. For example, they all present the 730 cm^{-1} adenine ring breathing mode, the $\sim 790\text{ cm}^{-1}$ uracil and cytosine (and thymine, see Section 3.4) adenine ring breathing mode, the $\sim 790\text{ cm}^{-1}$ uracil and cytosine (and thymine, see Section 3.4) ring breathing mode, and the $\sim 1320\text{ cm}^{-1}$ adenine ring stretching mode. For the miR-224 probe, which is the only probe that contains G, the 1580 cm^{-1} peak of G can also be seen as a shoulder on the 1560 cm^{-1} adenine peak. Likewise, the Test probe, which does not contain A or G, does not present the aforementioned signature peaks 730 , 1320 , or 1580 cm^{-1} . Figure 3.3 B shows the miR-224 probe (which contains all four nucleotides) spectra superimposed onto the SERS spectra of the individual nucleotides from Figure 3.1. We can see that virtually all of the spectral features of the DNA probe can be attributed to the spectral features of at least one type of nucleotide.

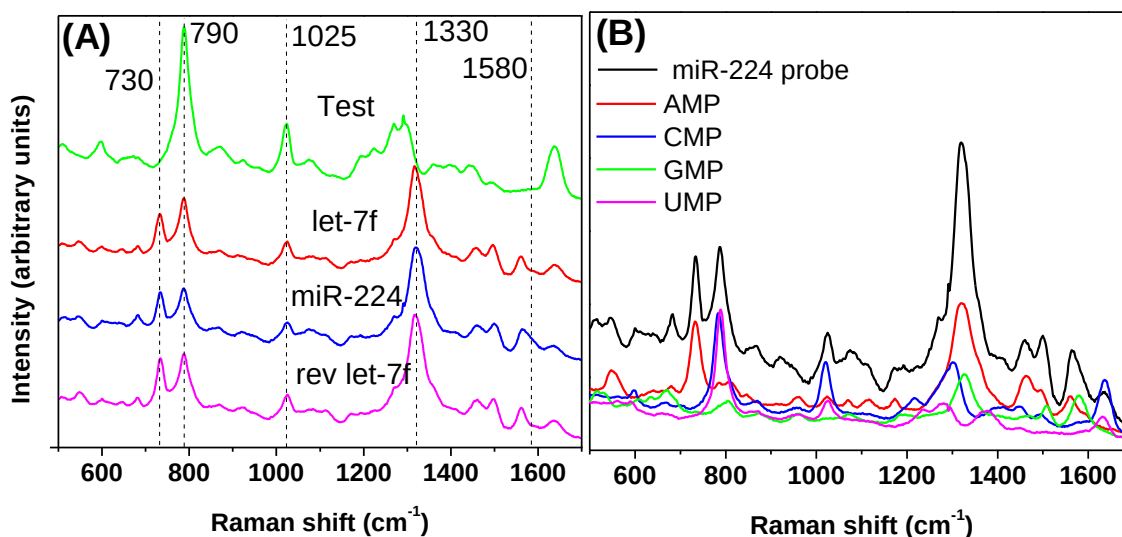


Figure 3.3. A) Raw SERS spectra of four different DNA probes. The dotted lines highlight some of the primary spectral features that indicate the presence or absence of specific nucleotides. Spectra are vertically offset for clarity. B) Spectra of miR-224 probe (black, which contains all four nucleotides) overlaid with individual nucleotide (colored, scaled for easier comparison).

3.2.2 Mercaptohexanol (MH) Spacer Immobilization

After acquiring a satisfactory DNA probe signal, a spacer molecule was immobilized. Mercaptohexanol (MH) is commonly employed as a spacer for DNA immobilization studies, and therefore utilized here. Additionally, the MH spacer can displace portions of the DNA backbone that are directly adsorbed onto the Ag surface, and help reorient the tethered DNA probe to a more vertical configuration [83]. The structure of MH is presented in Figure 3.4.

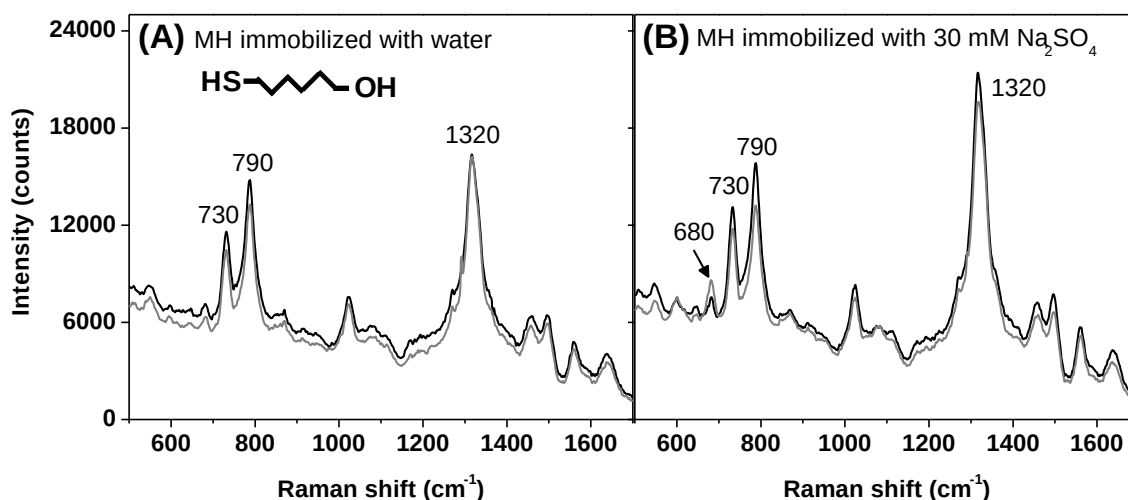


Figure 3.4. Comparison of spectra of the let-7f probe treated with MH in A) water, and B) 30 mM Na₂SO₄. The structure of MH is also included. The let-7f probe spectra are presented before MH treatment (black), and after MH treatment (gray). The structure of MH is also presented.

The MH is suspended in DI water or 30 mM Na₂SO₄ pH ~7, and 20 μ L of the 10 μ M solution is added to each DNA-treated well and allowed to incubate for ~3 hours. After rinsing and drying the MH-treated wells, the SERS response was again measured. For the MH spacer immobilized in DI water, very little change is observed in the spectra compared to the same sample prior to MH treatment. As seen in Figure 3.4A, only a slight decrease in the relative intensities of the 730 and 790 cm⁻¹ peaks are observed. On the other hand, for the MH immobilized in sulfate buffer produces slightly more noticeable changes (Figure 3.4B). The

small peak at $\sim 680\text{ cm}^{-1}$ increases by approximately 2-fold. Other changes include a slight decrease in the 730, 790 cm^{-1} , and 1320 peaks. In general, however, the DNA spectra before and after MH immobilization are highly similar.

The overall lack of spectral change could be a result of several factors. First, the MH molecule does not have a very large SERS cross-section, i.e. its relative signal intensity is rather weak. Secondly, the MH molecule may not be adsorbing to a significant extent, as the AgNR surface may already be completely covered with the DNA probe. In this case, an overly packed probe layer could be detrimental to the subsequent miRNA hybridization and detection. However, the DNA probe molecule is connected to a mercaptohexanol via the disulfide. Because disulfides can readily adsorb to the Ag surface to form two co-adsorbed thiolate molecules, each DNA probe is likely co-adsorbed with a mercaptohexanol. In principle, this will yield a surface coverage that is 50% thiolated probe and 50% mercaptohexanol. This means that an MH molecule is automatically included during the probe immobilization. In this case, additional MH may not be needed, nor capable of adsorbing to the pre-coated surface, therefore the SERS signal remains unchanged after addition of more MH.

A cursory look at Figure 3.3 suggests the majority of spectral features appear to be well accounted for by the individual nucleotide signals, and little change is observed in the spectra after treatment with MH. This suggests that the MH spacer is not contributing significantly to the spectral response of the DNA probe. We also note that the 6-alkyl carbon linker connecting the 5' end of the DNA sequence with the coordinated sulfur has a structure analogous to the mercaptohexanol, and therefore we may expect this group to produce a spectrum similar to that of MH. That being said, the presence of MH and the 6-carbon linker should effectively yield

layer of 6-alkyl carbon chains, yet we do not see any clear indication of these structures in the SERS spectra of the DNA probe.

To more specifically determine the effect of the MH spacer on the spectral response, increasing concentrations of MH were added (in Na_2SO_4) to different wells with pre-immobilized let-7f probes [82]. Figure 3.5 shows the spectra of the let-7f probe treated with 10, 25, 50, 75, and 100 μM MH. The spectra of pure 100 μM MH applied directly to a clean AgNR substrate is also presented as a reference. The most prominent bands of MH are at 706 (trans S-C stretching mode $\nu(\text{S-C})_{\text{T}}$), 870, and 1080 cm^{-1} (trans C-C trans stretching) [89, 90]. In the SERS spectra of the DNA probe, these bands can be seen to increase in intensity as the MH concentration increases from 10 to 50 μM , while the DNA peak intensities decrease. From 50-100 μM , the DNA or MH spectral features do not demonstrate a further increase in intensity.

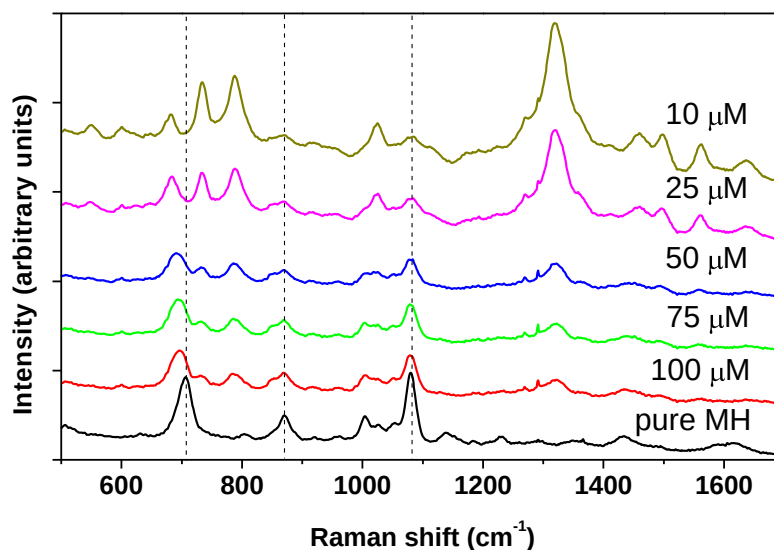


Figure 3.5. SERS spectra of let-7f probe as a function of increasing MH-treatment concentration. The bottom spectrum shows the pure MH spectra (no DNA probe); the dotted lines highlight the primary MH spectral features, which can be seen to increase in intensity with the increase in MH concentration. Spectra vertically offset for clarity. (From Ref [82].)

We can also observe that for the 10 μM MH treatment the $\nu(\text{S-C})_{\text{T}}$ appears to be at 683 cm^{-1} , but as the MH concentration increases, this peak blue shifts to 696 cm^{-1} which is much closer to the 706 cm^{-1} position for the pure MH. This behavior may be explained by examining the surface coverage-dependent SERS behavior of alkane-thiols [89]. At lower thiol surface coverage θ_{thiol} , alkanethiols adopt a more horizontal, disordered orientation and configuration. This leads to the rotation about the S-C bond producing a gauche (G) conformation, and the corresponding stretching mode $\nu(\text{S-C})_{\text{G}}$ manifests a peak at $\sim 630\text{ cm}^{-1}$. As θ_{thiol} increases, the alkanethiol (e.g. MH), are forced to adopt a more structured, vertical conformation, leading to a rotation of the S-C bond to yield a higher energy trans (T) conformation and the $\nu(\text{S-C})_{\text{T}}$ blue shifts to $\sim 700\text{ cm}^{-1}$. Thus, assuming the initial MH/probe monolayer may have a relatively high surface coverage (therefore yielding primarily a T-conformer, i.e. $\Delta\nu = 687\text{ cm}^{-1}$), addition of higher concentration MH may yield a higher packed and more dense MH surface coverage. This could further force the S-C bonds into the T-conformation, possibly manifesting as a further blue-shift of the $\nu(\text{S-C})_{\text{T}}$ to the observed 696 cm^{-1} position. However, we must not rule out that the 683 cm^{-1} peak is perhaps resulting from the alkyl spacer (i.e. the C6 chain between the sulfur and probe), in which case the observed 696 cm^{-1} peak may be a convolution of the 683 cm^{-1} DNA peak and 706 cm^{-1} MH peak. Unfortunately, the literature does not discuss the effect of the carbon linker or thiol spacer on the resulting DNA spectra. Clearly more analysis will be required to determine the source of these spectral features. Comparing Figure 3.5 to that of the pure nucleotides in Figure 3.1, we can see, however, that the 6-carbon spacer and MH appear to produce peaks that overlap somewhat with those of the nucleotides, such as the peak at $\sim 1025\text{ cm}^{-1}$ and $\sim 1075\text{ cm}^{-1}$, which implies that the MH is detected in the DNA spectra, but is convoluted with the nucleotide peaks.

Conclusively, according to the semi-quantitative analysis of the let-7f probe as a function of increasing MH treatment concentration, we can see that when using a salt solution (Na_2SO_4) more of a spectral change is induced. This includes the slight increase of MH peaks and a concomitant small decrease of many of the DNA-related peaks. Those observations suggest that the MH is able to intercalate into the probe layer, and at higher concentrations, may be able to displace some of the immobilized probes.

3.3. Hybridization and SERS Detection of miRNA on AgNRs

Let-7f miRNA is used as a model target analyte in this study [82]. Let-7f is a clinically-relevant a 22 nucleotide-long miRNA sequence and is part of the let-7 family of miRNAs which is highly conserved across many species (e.g. *C.elegans*, *Drosophila*, and humans) [91]. Numerous isoforms of let-7 exist (e.g. a, b, ... , f, etc.) and are known to be key regulators in cellular development and development of cancers. Humans are known to possess at least ten mature let-7 sequences. The sequence of let-7f is presented in Table 3.2.

The general procedure for miRNA capture via probes immobilize on AgNRs is as follows. The miRNA sequences are first mixed with the hybridization buffer sodium saline citrate (SSC) pH 7.0. SSC buffer is first prepared as a $\sim 20\times$ stock solution (1.5 M NaCl, 0.15 mM sodium citrate pH 7.0), this is the diluted 10-fold in DI water to a $2\times$ working concentration when mixing with the miRNA targets. The sample solution is applied to the MH/probe-treated AgNR substrate and allowed to incubate at room temperature for several hours. After incubation, the solution is rinsed with water to remove the SSC buffer and the un-hybridized miRNA targets, then blow-dried, and measured. For the following experiment, the same four probes discussed in Section 3.2.1 are used here.

3.3.1 Label-free SERS Analysis of miRNA Hybridization

Because a probe will not necessarily have the same nucleotide composition/ratio as the complementary target, upon hybridization the relative ratios of the nucleotides immobilized at the surface will change. Since SERS is a quantitative technique, cursory logic suggests that after hybridization, any observed spectral changes of the DNA probe would be a direct result of the captured miRNA, which has been reported previously [44, 48]. Thus, the differences in the physical nucleotide ratios should manifest as a change in the relative peak intensities of the probe spectra. Table 3.2 summarized the physical nucleotide ratios of different probes used in this study before and after hybridization.

However, because SERS is also sensitive to the orientation and configuration of adsorbed analytes, careful attention must be paid to spectral fluctuations resulting from changes of the adsorbed probe or spacer molecules. Thus, before a proper SERS analysis of the let-7f miRNA hybridization can be achieved, these effects must be accounted for, and appropriate negative controls must be included.

Prior to hybridization, weakly bound probes need to be dissociated from the probe layer so as not to interfere with hybridization process. Thus, the AgNRs in each well were treated with a washing step prior to hybridization. This entails rinsing each DNA-containing well with 2×SSC/0.5% SDS wash buffer with a squirt bottle, followed by incubation in the wash buffer for ~ 1 hour. Afterwards, the wells are rinsed thoroughly with DI water and then blow dried and measured. We note that after incubation in the wash buffer, the spectral intensity increases by ~2-fold, yet the overall spectral profile remains unaffected. However, the peak intensities at 730 and 790 cm^{-1} (A and C/T ring breathing modes, respectively) appear to have increased more than the 1320 cm^{-1} peak intensity (A ring-stretching). Figure 3.6 shows the result for let-7f probe

before washing and after washing. The same behavior was observed for the other three probes (not shown), with the exception that the Test probe did not demonstrate as much of an increase in intensity after washing. The reason for the increased intensity is currently unclear.

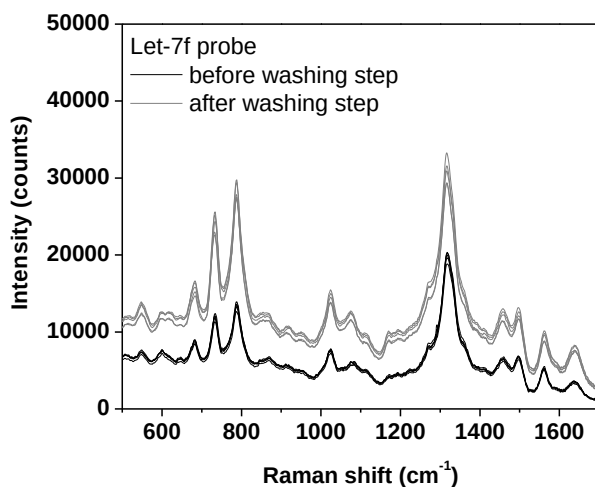


Figure 3.6. Let-7f probe spectra before (black) and after washing (gray).

3.3.1.1. Raw Spectral Analysis

In addition to let-7f target, non-complementary miRNA-targets were also used as controls, including Test and rev miR-224 (see Table 3.2 for sequences). SSC buffer was used as a negative control. The raw spectra of the let-7f probe treated with complementary target, non-complementary targets, and SSC buffer are presented in Figure 3.7. The SSC buffer and the rev miR-224 target treatments yield spectra that are identical. This is to be expected since the miR-224 is not complementary to the let-7f target, and the SSC buffer does not contain any nucleotides or miRNA sequences that can bind to the surface and change the nucleotide composition of the probe layer. We may also expect the same result for the Test target treatment since it too is not complementary to the let-7f probe, yet a definitive decrease in the overall spectral intensity is observed instead. Although the overall spectral profile remains the same (i.e. new peaks do not appear, or pre-existing peaks do not disappear), the overall spectral intensity

decreases. The 1320 cm^{-1} (A, ring-breathing) band demonstrates a larger intensity, being approximately the same as that obtained for the SSC buffer and rev miR-224 treatments. For the complementary let-7f target, the overall intensity is 30-50% lower than that of the SSC buffer and rev miR-224 controls, but at an initial glance the spectral profile appears to be unchanged. Unfortunately, the raw spectra do not show any clear indicators that the let-7f probe has selectively hybridized with the let-7f target.

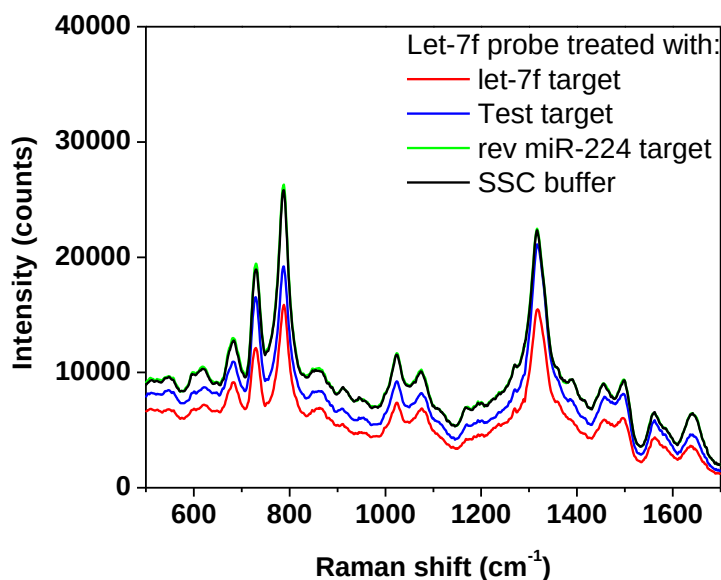


Figure 3.7. Raw hybridization spectra of let-7f probe.

3.3.1.2. Normalized Spectral Analysis

From Figure 3.7 we see that many of the spectral changes occurring under different treatments are rather subtle. In addition, the disparity of the spectral intensity makes direct visual comparison of the relative peak intensities difficult and unreliable, especially when attempting to monitor subtle changes. One way to obviate the issue of the intensity disparity is to normalize the spectra. Spectral normalization is typically used to re-scale the entire spectral intensity by an internal data value to make it more comparable with other spectra than have different overall

intensities. Most commonly the entire spectral intensity is divided by the highest intensity point of the spectrum; the result is a spectral signal scaled with all intensities between 0 and 1. Although straightforward, this approach is more appropriate when differences between spectra are primarily due to changes absolute spectral intensity, and not relative intensity. In the case of the hybridized DNA, the overall intensity changes somewhat, yet many of the peaks, and baseline, demonstrate relative changes. Thus, normalization to the most intense peak does not necessarily result in DNA spectra that allow easier visual comparison of subtle peak intensity changes.

One way to scale the such spectra is unit-length vector normalization [66]. This strategy treats the digital spectral data as a vector in high-dimension Euclidean space, i.e. each x-axis wavenumber (cm^{-1}) value is treated as a single component i of the vector $\bar{y}$; the measured intensity (in counts) at that point defined as the component's magnitude y_i . For the spectral measurements discussed here, each spectra is therefore a vector with $n = 1000\text{-}2000$ components. Despite such high dimensionality, Pythagorean's theorem may still be applied to determine the vector length $|y|$

$$|y| = \sqrt{y_1^2 + y_2^2 + \dots + y_n^2} \quad (3.1)$$

Vector normalization is accomplished by calculating $y_i/|y|$ for all i . The result is essentially a spectral re-scaling weighted by the most consistent, or average, y_i values, i.e. scaling by an intrinsic baseline. Thus, this type of normalization makes direct visual comparison easier for spectra containing many peaks with varying relative intensity.

The hybridized let-7f probe spectra from Figure 3.7, as well as the hybridization spectra from the other three probes, have been vector-normalized and are plotted in Figure 3.8. Visualization of subtle spectral fluctuation is now much easier and more reliable. In Figure 3.8A

(let-7f probe) the let-7f target treatment (red) demonstrates a 730 cm^{-1} peak slightly lower than that of the SSC, rev miR-224, or Test target treatments; the 788 cm^{-1} peak is slightly higher than that of the Test target treatment, yet slightly lower than that of the rev miR-224 and SSC buffer treatment. We also note that a small shoulder on the 1560 cm^{-1} has appeared in all the spectra around 1580 cm^{-1} , which indicates the presence of G (see Figure 3.3). This could be indicative of hybridization, as the let-7f probe does not contain G but the let-7f target does. However, the fact that this particular spectral feature is arising even in the presence of the SSC buffer indicates that it is more likely a result of reorientation of the probe.

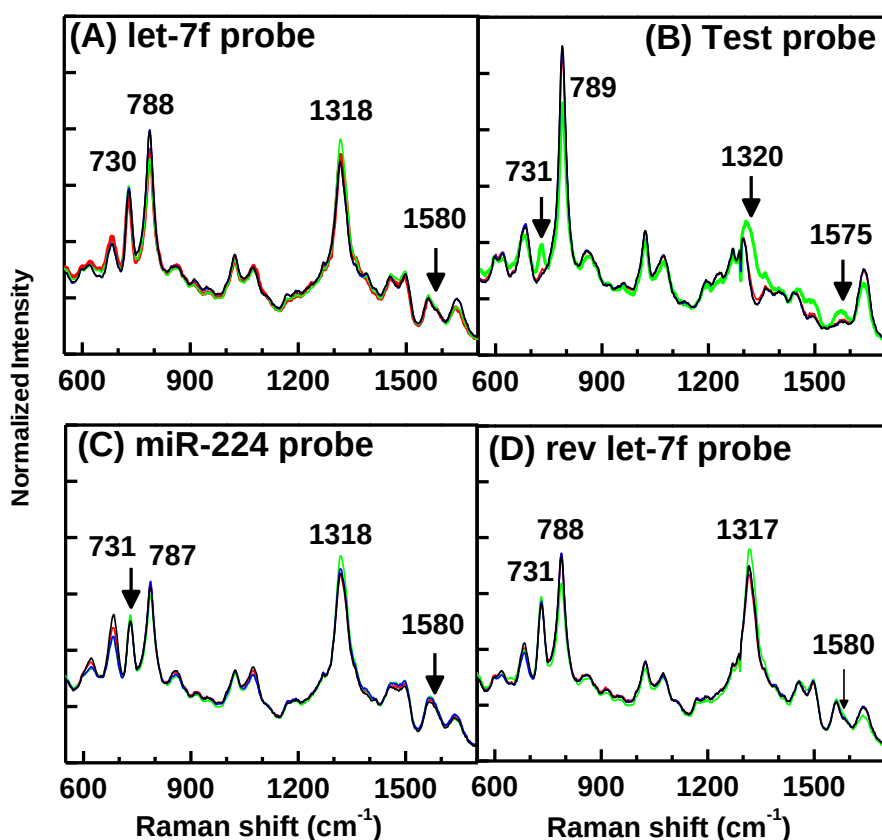


Figure 3.8. Normalized spectra of probes: A) let-7f, B) Test, C) miR-224, and D) rev let-7f after treatment with targets: let-7f (red), Test (green), rev miR-224 (blue), and SSC buffer (black).

The test probe demonstrates more obvious spectral features indicating hybridization (Figure 3.8B). For one, all three of the non-complementary/negative control treatments produce spectra that are virtually identical, which is an ideal result. Additionally, the AgNRs treated with the Test target (green) show a noticeable decrease in the C/T peak intensities at 789 cm^{-1} and C peak at 1630 cm^{-1} . This is expected considering that the Test probe contains only C and T, and hybridization with the Test target (which contains only A and G) will decrease the ratio of C and T as the spectral contributions from A and G increase. A more significant change, however, is the appearance of the signature A peak at 730 cm^{-1} and the shifting of the C peak at 1300 cm^{-1} towards the A peak at 1320 cm^{-1} . G is also present in the Test target, yet because G has a weaker signal than A, and because A has only produced a minor signal, the signature G peaks are probably undetectable under the current conditions. Yet the peak emerging at 1575 cm^{-1} could be a convolution of the A signal at 1560 cm^{-1} , and the G signal at 1580 cm^{-1} .

The miR-224 and rev let-7f probe were not treated with complementary targets (they were only treated with non-complementary let-7f, rev miR-224, and Test targets). Whereas the let-7f and rev miR-224 target treatments produce spectra virtually identical to that of the SSC buffer treatment, the Test target treatment yields a slightly different spectrum. For both the miR-224 and rev let-7f probe, the Test target treatment produces a slightly higher 731 and 1320 cm^{-1} peak, which are the primary peaks of A. Meanwhile, the C/T(or U) peak at $\sim 790\text{ cm}^{-1}$ decreases slightly, the cause of which is explained as follows. The Test target is composed of only A and G; therefore, if the Test target were to cross-hybridize with the non-complementary let-7f, miR-224, or rev let-7f probes, one would expect to observe an increase in the A (and G) signal. This is exactly what is observed. Because the vector-normalized spectra represents the *relative ratios* of each component signal, increasing the relative signal intensity of one component, will therefore

result in the decrease of the relative ratio of the other component, hence the decreased C/T signal intensity (790 cm^{-1} peak).

3.3.2 Confirmation of let-7f Target Hybridization Using an Extrinsic Label

So far we have seen that the intrinsic SERS signal of the let-7f probe treated with complementary target produces spectra very similar to that of the let-7f probe treated with non-complementary (e.g. Test or rev miR-224) targets, or SSC buffer. However, before we assert that this small signal discrepancy is in fact due to specific hybridization, analytical rigor dictates that we must first validate if the miRNA target is in fact hybridizing with the probe.

One way to confirm this is to use an extrinsic Raman reporter attached to the target sequence. Such an approach has been readily used throughout the literature for label-based SERS DNA detection (see Section 1.3.2). In the current study [82], let-7f miRNA with a covalently attached FAM label was purchased from Integrated DNA Technologies, Inc. The label was attached to the 5' end of the miRNA sequence, so that upon hybridization with the immobilized let-7f probe, the FAM label would be on the distal end of the hybridized duplex (i.e. on the end furthest from the AgNR surface). FAM is used because it allows for a very strong surface-enhanced resonant Raman scattering (SERRS) signal when excited at 532 nm.

The FAM-labeled let-7f target hybridization experiment was performed in parallel with and in exactly the same way as the label-free analysis. The let-7f-FAM target was applied at different concentrations from 30 nM to 3.3 μM to different wells containing pre-immobilized let-7f probe. The only experimental difference was that these samples were analyzed with a Bruker Senterra confocal microRaman system. The 10 $\times$ objective and 532 nm laser were used to collect

the SERRS spectra. In addition, 1 μM let-7f-FAM targets were added to wells containing non-complementary Test probe, miR-224 probe, and rev let-7f probe.

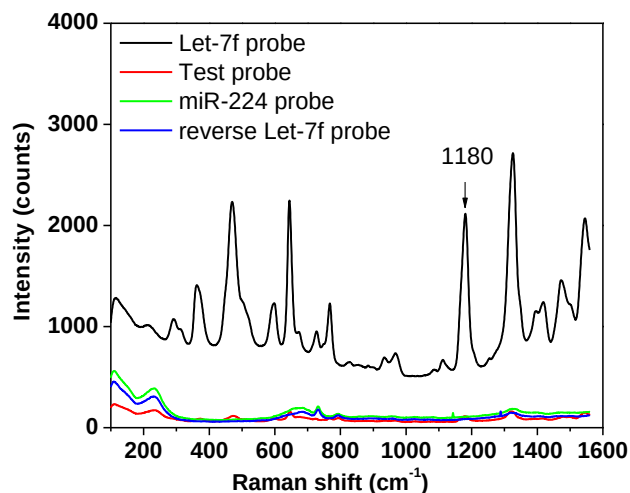


Figure 3.9. SERRS spectra of wells with different probes treated with 1 μM let-7f-FAM target. The 1180 cm^{-1} peak is highlighted.

The wells with let-7f probe produce a very strong FAM spectra, yet the wells with non-complementary probes only produce a negligible signal (Figure 3.9). The 1180 cm^{-1} peak is an intense signature peak of FAM that does not overlap with peaks of DNA, therefore the baseline-corrected intensity I_{1180} was used to quantify the SERRS response of the let-7f-FAM as shown in Figure 3.9. In Figure 3.10A, we can see that the well with let-7f probe has $I_{1180} \approx 1500$ counts. The wells containing miR-224 and rev let-7f probes do not generate a detectable signal ($I_{1180} \approx 0$ counts). On the other hand, a very small signal of $I_{1180} \approx 50$ counts is acquired from the well with Test probe, which suggests that the Test probe is capable of non-specifically hybridizing with the let-7f target to a small extent. The concentration-dependent analysis was performed to determine the limit of detection (LOD) and dynamic range of SERRS detection of let-7f-FAM. In Figure 3.10B the FAM signal is still detectable at the lowest concentration of 30 nM ($I_{1180} = 220$ counts), and increases quickly to a maximum at 0.3 μM let-7f-FAM ($I_{1180} = 1630$ counts).

Further increase in concentration leads to a slight decrease in I_{1180} , the exact cause of which is unclear.

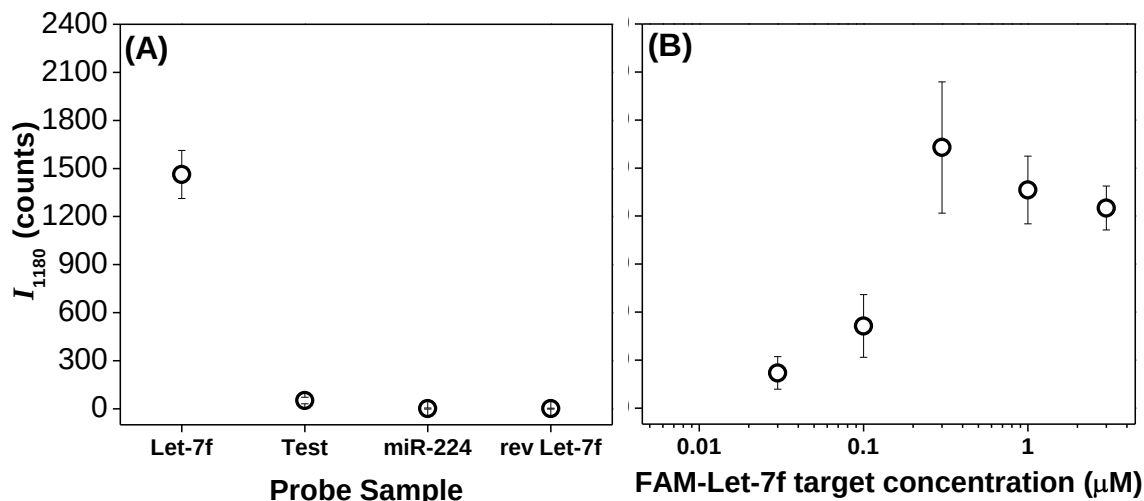


Figure 3.10. A) The intensity of the signature FAM peak at 1180 cm^{-1} (I_{1180}) measured from the spectra in Figure 3.8. B) The measured I_{1180} of let-7f probe treated with increasing concentrations of let-7f-FAM target. Both plots share the same y-axis scale.

The use of an extrinsic reporter has thus provided two critical pieces of information. First, the let-7f target is indeed hybridizing with the let-7f probe. Secondly, the let-7f target is not cross-hybridizing with the non-complementary probes to any significant extent. Yet, the aforementioned visual analysis of the label-free target treatment could not provide any clear evidence to corroborate the conclusions obtained with the labeled miRNA let-7f target. Therefore, visual analysis of label-free SERS hybridization of miRNA may not be feasible because the SERS signals of the probe and the target are qualitatively so similar. However, since SERS is intrinsically a quantitative technique, and we understand the source of the probe and target signal, i.e. the nucleotides, we could use a spectral decomposition method to quantify the subtle differences in the nucleotide ratios before and after hybridization.

3.4 Quantitative SERS Detection of miRNA Using Least Squares Regression Analysis

We have seen in the previous sections that the DNA probe signal S_{DNA} can be approximated as a *linear combination* of the SERS signals of AMP, CMP, GMP, and UMP, which we denote as *source signals* S_A , S_C , S_G , and S_U , respectively (see Figure 3.3B). Figure 3.11 presents the individual source signals plotted with the a linearly combined artificial mixture signal $S_{mix} = (S_A + S_C + S_G + S_U)/4$. By comparing this artificial signal with the measured S_{DNA} of miR-224 probe (which contains all of the nucleotides, albeit at slightly different ratios), we can see a remarkable amount similarity in the overall spectral profile between the two spectra.

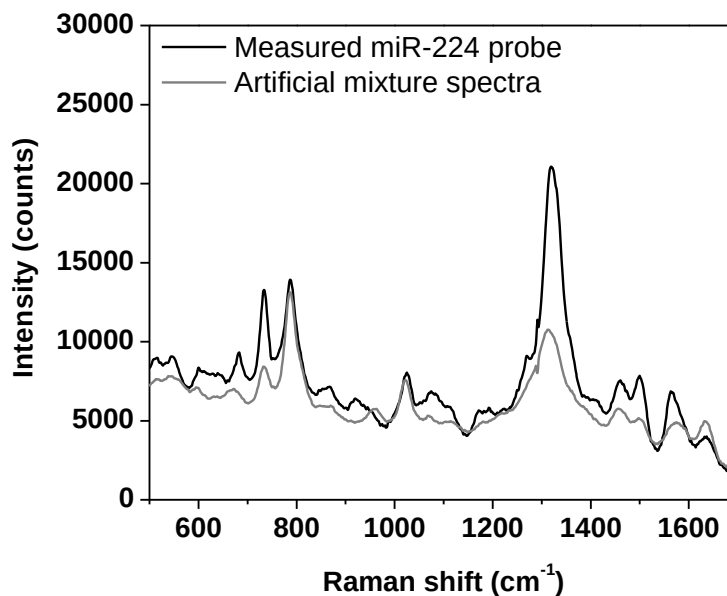


Figure 3.11. Direct, un-scaled comparison of the raw miR-224 probe spectrum with the artificial mixture spectrum $(S_A + S_C + S_G + S_U)/4$.

Based on these observations, and the fact that SERS is a quantitative technique, we assume that the relative contribution of each source signal S_A , S_C , S_G , or S_U to the total signal S_{DNA} is proportional to the physical ratio of that nucleotide with respect to the other nucleotides. We therefore postulate the following relationship,

$$S_{DNA} = a \cdot S_A + c \cdot S_C + g \cdot S_G + u \cdot S_U \quad (3.2)$$

where a , c , g , and u represent the relative numerical ratios of A, C, G, and U immobilized at the surface of the AgNRs, respectively [82]. With the exception of a probe in which all nucleotides are present at equivalent ratios (i.e., $a = c = g = u = 0.25$), hybridization with a complementary miRNA target will cause a change in a , c , g , and u . For example, the relative A:C:G:T number ratio of the let-7f probe is 0.41 : 0.32 : 0 : 0.27 (i.e. the absolute ratio = 9:7:0:6) ; however, after hybridization with the complementary target, the ratio changes to 0.34 : 0.16 : 0.16 : 0.34. Thus, detection of the hybridized miRNA may be possible by quantitatively examining these ratios. Since Equation 3.2 shows a linear combination, and therefore linear least squares (LS) regression analysis may be used to solve for the parameters a , c , g , and u if S_{DNA} and the source signals S_A , S_C , S_G , and S_U are known. A very similar method was utilized by Prado et al., but rather than attempting to quantify the relative ratios of nucleotides within a heterogeneous sequence (e.g. any of the ssDNA probes discussed so far), they focused on determining the physical ratios of homogenous sequences (i.e. polyA, polyG, etc.) in a mixture of homogenous sequences [52].

We should point out that the source spectrum of TMP was not measured, yet T is indeed present in the DNA probes. Rather, its RNA analogue UMP, has been measured. From Equation 3.2 we have implicitly assumed that S_U can be used in lieu of S_T ; clearly, such an assumption must be justified. Unfortunately, we are unable to make a direct spectral comparison because TMP was not measured. Fortunately, however, Bell et al. has reported the SERS spectra of UMP and TMP using Ag colloids [55]. That study also produced spectra for the AMP, CMP, GMP, and UMP highly similar to those presented in Figure 3.1. That study shows TMP to have peaks at 578, 795, 1206, 1238, ~1300, 1356, 1398, 1450, 160, and 1648 cm^{-1} ; UMP on the other hand demonstrated peaks at 654, 792, 1034, 1085, 1238, 1292, 1384, and 1628 cm^{-1} . Some

discrepancy definitely exists between these two spectra, yet the most prominent bands at 792 and 794 cm^{-1} , i.e. the ring breathing modes of UMP and TMP, respectively, are very similar. Likewise, the UMP spectra shown in Figure 3.1 presents a band at 789 cm^{-1} . Accordingly, based on the slight blue-shift of the TMP ring-breathing mode relative to UMP observed by Bell and Sirimuthu, we may assume the ring-breathing mode of T in our spectra to appear at 792-795 cm^{-1} . In addition, if we look at the spectra of the miR-224 probe which contains T but not U, we can see a large ring-breathing band at 786 cm^{-1} , which is likely a convolution of the 783 cm^{-1} band, and another band probably arising from T around $\sim 790 \text{ cm}^{-1}$ band. Thus, we conclude that U can be used in lieu of T.

LS analysis has been extensively used in all fields of research to fit data with linear mathematical models. More specifically, the LS regression determines the ‘best’ parameters d and b for a function $f(x) = d + bx$ to fit a given set of (x_i, y_i) data values where i is the number of data points measured. The sum of the vertical difference between each measured valued y_i and the model is referred to as the residual R . Squaring this value gives the general sum of squares definition [92]

$$R^2(d,b) \equiv \sum_{i=1}^n [y_i - (d + bx_i)]^2 \quad (3.3)$$

See Appendix A for the solution to the linear least squares derivation using Equation (3.3).

In our case, multiple values of “ b ” (i.e. a , c , g , and u , from Eq. 3.2) are incorporated into the model, and x_i is replaced by S_{Ai} , S_{Ci} , S_{Gi} , and S_{Ui} , while y_i is replaced by S_{DNAi} . In this case, i does not represent measurement number, but rather *spectral position*, thus $n = 1247$. Equation 3.3 therefore becomes

$$R^2(d,a,c,g,u) \equiv \sum_{i=1}^n [S_{DNAi} - (d + aS_{Ai} + cS_{Ci} + gS_{Gi} + uS_{Ui})]^2 \quad (3.4)$$

where d is an intercept term. Because we are solving for 5 parameters, the matrix in Equation A.7 becomes a 5 by 5 matrix.

A custom *Mathematica* program (see Appendix A) was used to calculate the LS regression coefficients a , c , g , and u using the form of Equation A.7. The results of this code were checked against the results generated with the built-in regression analysis function of Statistical Analysis Software (SAS) and the results were found to match exactly. The multiple probe spectra (S_{DNA}) from each well were averaged together and truncated below 350 cm^{-1} and above 1800 cm^{-1} , but otherwise unprocessed (i.e. not normalized) prior to input into the LS program. The same conditions are also true for the source signals S_A , S_C , S_G , and S_U , and the exact same spectra from Figure 3.1 were used for all LS fittings. After output of the LS fitting coefficients a , c , g , and u , any coefficients with a negative value were fixed to be zero, and the LS algorithm was re-run. This procedure was repeated until no negative values were generated [82].

The feasibility of the LS-SERS technique was first evaluated using unhybridized probe spectra discussed in Section 3.2. Figure 3.12 presents the estimated LS coefficient ratios for each of those probes, using the entire spectral region from $350\text{-}1800\text{ cm}^{-1}$. In addition, the theoretical ratios of each nucleotide for each probe are also plotted (pink stars). According to Equation 3.2, we may expect the theoretical ratios of A:C:G:T for a given probe to show a large degree of correlation with the LS coefficients estimated using the nucleotide SERS source signals. Figure 3.12A also shows the LS estimates for several different identical experiments performed on different days with different AgNR SERS chip substrates. We can see that for the Test and let-7f probes (Figures 3.12A and 3.12B, respectively), the LS estimated values match well with the theoretical nucleotide ratios for Experiments 2 and 3. For example, for Experiment 3, the let-7f

probe yields LS estimated values are 0.51, 0.26, 0.04, and 0.19, respectively, compared to the corresponding theoretical values of 0.41, 0.32, 0, and 0.27. Experiment 2 yields nearly identical values. Likewise, for the Test probe (for Experiment 2) the LS values are estimated at 0.02, 0.42, 0.06, and 0.51, which closely concur with the theoretical values of 0, 0.5, 0, 0.5.

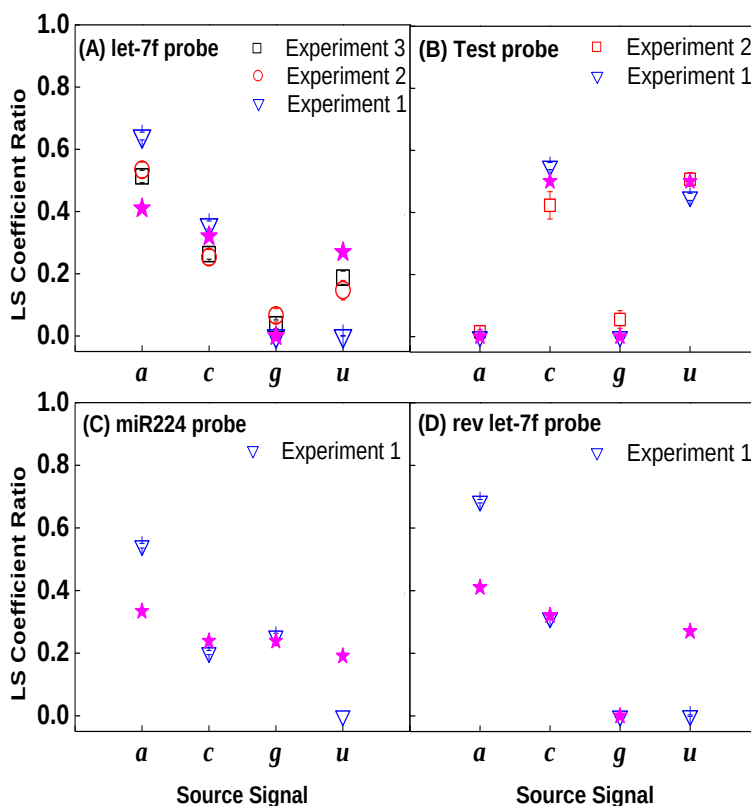


Figure 3.12. Estimated LS fitting coefficient ratios for four different DNA probes using the entire spectral region from 350-1800 cm^{-1} . The results of additional experiments are presented for let-7f and Test probe. The pink stars represent the theoretical values based on the number ratio of each nucleotide in the sequence. (From Ref [82].)

However, from Figure 3.12 we can see that for Experiment 1 (with the exception of the Test probe) only the *c* and *g* LS estimates appear to show a consistently close match to the theoretical ratios, whereas *a* and *u* are significantly over- and under-estimated, respectively, with *a* estimated to be > 0.5 and *u* being estimated as zero. Clearly, the LS coefficients estimated

from Experiment 1 differ from those of Experiments 2 and 3, as the LS estimated coefficients are significantly different (and worse) than those acquired from the other two experiments.

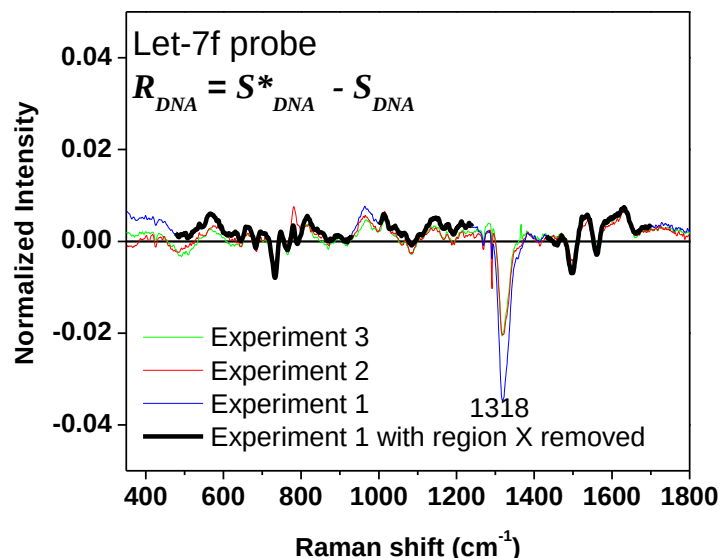


Figure 3.13. The residual spectrum R_{DNA} of let-7f probe for three different experiments showing a large residual at 1318 cm^{-1} . The bold trace is the portion of the spectrum used after region X is removed. (From Ref [82].)

To better understand the cause for this result, we should re-examine how the measured spectra are processed by the LS algorithm. As discussed previously, the LS algorithm is based upon the residual R (see Equation (3.3)), which is essentially defined as the sum of the differences between the measured spectral data and the ‘theoretical’ spectra. This theoretical spectrum assumes that the relative SERS contribution of the source signals is directly and *exactly* proportional to the physical ratio of each source component A, C, G, or U in the DNA sequence. Thus, for any given probe sequence, using Equation 3.4 we may construct theoretical spectrum S^*_{DNA} by setting a , c , g , and u equal to the known physical nucleotide ratios and solving for S_{DNA} . In this case there is no extraneous experimental variation in the data; therefore, a LS fitting of this theoretical spectra produces a residual $R = 0$, and the estimated output values a , c , g , and u are *identical* to the values we put into the equation. This is a rather obvious postulation, but

highlights the fact that the LS estimates become more accurate when the measured signal S_{DNA} more closely resembles S^*_{DNA} .

Clearly, if the measured DNA probe spectra S_{DNA} deviates in some way from S^*_{DNA} , this will manifest as errors in the estimated LS coefficients a , c , g , and u . Obviously, we may expect at least some deviation from S^*_{DNA} , but one possible way to avoid introducing unnecessary error is to determine if there exists *regions* of S_{DNA} that deviate more significantly from the S^*_{DNA} than other regions. Such regions would be culpable sources of error that could lead to poor LS estimates, and could in theory be removed from S_{DNA} , therefore permitting a more accurate fitting. To demonstrate how the measured DNA signal S_{DNA} deviated from S^*_{DNA} , a ‘residual’ spectrum is R_{DNA} calculated. This simply entails arithmetically subtracting the measured DNA spectrum from the theoretical spectrum, i.e. $R_{DNA} = S^*_{DNA} - S_{DNA}$.

Figure 3.13 shows R_{DNA} for all three experiments discussed previously. Both the measured and theoretical spectra were vector normalized before subtraction. As expected, most of the spectral differences fluctuate about 0 but no more than ± 0.01 . However, there is indeed a large (negative) residual value observed at 1318 cm^{-1} , which corresponds to the large spectral peak observed at $\sim 1320 \text{ cm}^{-1}$ (ring-stretching mode for A). In addition, this residual feature is present in all three experiments, but it is approximately twice as large for Experiment 1; accordingly, this is the same experiment that produced the worst LS coefficients estimates. Hence, the spectral peak at 1320 cm^{-1} is the likely culprit for the poor LS estimates for Experiment 1. This spectral discrepancy between the different experiments could be due to subtle differences in the orientation of the probe relative to the AgNR surface, but a more detailed analysis is required to elucidate a more thorough physical explanation. Regardless, now that we have identified portions of the measured spectra that yield detrimental deviations from the

theoretical spectra, we can attempt to improve the LS analysis by removing these spectral regions, which are collectively referred to as X . The dark trace in Figure 3.13 shows the spectral regions used in the following LS analysis.

Figure 3.14 plots the LS coefficients calculated for the DNA probe spectra after region X has been removed. Indeed, the LS coefficients for Experiment 1 have improved greatly. For example, a was estimated at 0.64 using the entire let-7f probe spectra. After removing region X ,

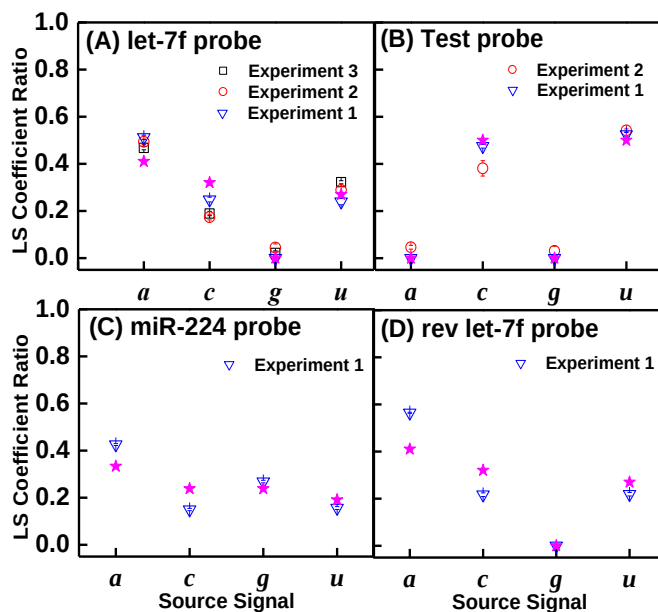


Figure 3.14. Estimated LS fitting coefficient ratios for four different DNA probes after using the same spectral data from Figure 3.11, but removing region X from the spectra. The pink stars represent theoretical nucleotide ratios for each probe. (From Ref [82].)

this value decreased to 0.51 which is much closer to the theoretical value of 0.41. Additionally, for the same spectrum u was initially estimated at 0, but after removing X , u increased to 0.24, which is very close to the theoretical value of 0.27. The LS coefficients for the other two let-7f experiments have changed slightly, but maintain the same overall accuracy prior to removing X from the spectra. The same general behavior was observed with the miR-224 and rev let-7f probes. The Test probe LS coefficient ratios do not demonstrate any significant changes after

removing X . With a proper analysis established for the DNA probe, we may now discuss the prospect of applying LS analysis for quantitative label-free detection of the miRNA hybridization.

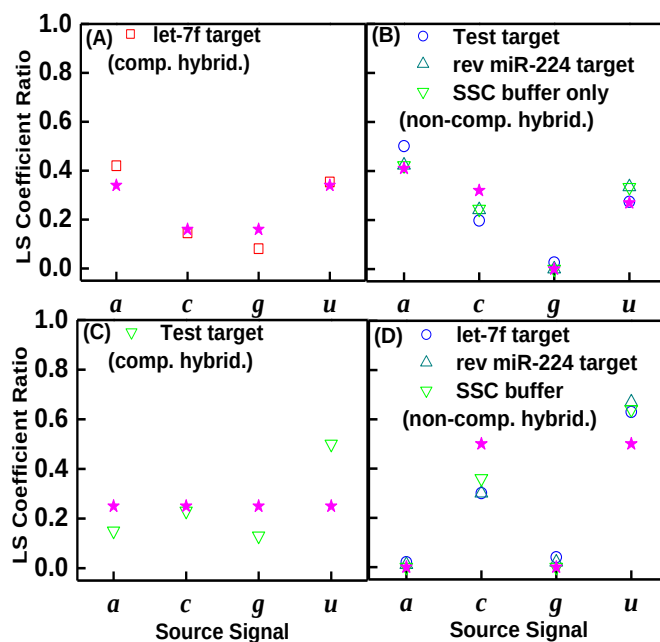


Figure 3.15. Estimated LS fitting coefficient ratios for let-7f or Test probes from Experiment 1 after treatment with complementary target, non-complementary target, or SSC buffer. The pink stars represent theoretical nucleotide ratios for probes after complementary hybridization (A and C) or non-complementary hybridization/negative control treatment (B and D). (From Ref [82].)

The spectra acquired after treatment with the miRNA targets and the SSC control were processed in the same manner as discussed above (i.e with region X removed). Figure 3.15 shows the LS coefficients estimated for both the Test and let-7f probe treated with both complementary and non-complementary miRNA sequences in addition to the SSC negative control.

Based on the expected nucleotide ratio, after complementary hybridization the let-7f probe is expected to have a , c , g , and u values of 0.34, 0.16, 0.16, and 0.34, respectively. That is to say, a and c decrease while g and u increase after complementary hybridization with the

miRNA target. Indeed, Figure 3.15A (let-7f probe treated with let-7f target) shows the g and u , estimated as 0 and 0.24 before hybridization, increase to 0.08 and 0.35, respectively, after hybridization. These values are *therefore converging towards their respective theoretical values* of 0.16 and 0.34. Meanwhile, before hybridization a and c are estimated as 0.51 and 0.24, and after hybridization decrease to 0.42 and 0.15, respectively, and thus converge towards their respective theoretical values of 0.34 and 0.16.

So far, the LS approach has demonstrated the ability to extract quantitative differences in the nucleotide ratio of the hybridized spectra compared to the pre-hybridized data. This demonstrates that LS method has the requisite *sensitivity* to be used for miRNA hybridized detection. However, this tells us nothing about the method's *specificity*. To examine this, we can look at Figure 3.15B. This figure shows LS estimates for let-7f probe treated with the negative controls. Theoretically, the a , c , g , and u values should be unchanged after treatment with SSC buffer or a non-complementary sequence (assuming no cross-hybridization). Indeed, from Figure 3.15B we can see that experimentally estimated ratios are very similar to the theoretical ratios for both the non-complementary Test and rev miR-224 treatments. Additionally, we can see that the SSC buffer treated sample produces LS-estimates virtually identical to those of the non-complementary rev miR target, which is exactly what is expected. For the non-complementary rev miR-224 and SSC treatments, a and g are estimated to be nearly an exact match to the theoretical values expected for the un-hybridized let-7f probe. The c and u values are under- and over-compensated, by ~ 0.07 , respectively. The non-complementary Test target treatment yields acceptable results, yet the estimated ratios deviate (within ± 0.09) from the theoretical values, which is more than that observed for the SSC buffer or rev miR-224 target treatments. However, referring back to the spectral results obtained in the previous sections, we

can see that the Test target appears to have a higher proclivity to cross-hybridize with non-complementary probes. For example, from Figure 3.7 we can see that the probes treated with non-complementary Test target miRNA produced spectral differences not seen with the other negative controls. Therefore, the slightly worse LS estimates observed are likely due to physical limitation such as cross-hybridization rather than intrinsic limitations of the LS fitting technique.

The same analysis was performed using the Test probe. After complete hybridization, the Test probe should yield a , c , g , and u values of 0.25. In Figure 3.15C, however, we can see the estimated values of both a and g (0.15 and 0.13, respectively) are noticeably less than the theoretical value of 0.25, while $u = 0.5$ is significantly higher. These discrepancies could be a result of incomplete hybridization. This idea is corroborated by the spectrum shown in Figure 3.7, where we can see only weak A and G signals relative to the more intense C and U signals. Yet, with complete hybridization of the Test probe, we may expect the resulting spectrum to resemble that of the miR-224 probe (which contains all four nucleotides) in Figure 3.3, and show a significant contribution from A (i.e. prominent 730 and 1320 cm^{-1} peaks). Regardless, for the Test probe treated with Test target, all of the LS coefficients, with the exception of u , indicate hybridization as they are converging towards the theoretical value of 0.25. The odd behavior of u is not clearly understood at this point.

For the three negative controls (Figure 3.15D), a and g are very close to the theoretical values of zero, but the c and u values appear to be under- and overcompensated, respectively, by approximately 0.2. This deviation may be in part a result of incomplete hybridization. For example, the Test probe treated with the FAM-labeled let-7f target produced a noticeably higher FAM intensity than either of the other two non-complementary probes (Figure 3.9). Regardless, compared with the negative controls, the estimated a , c , g , and u values for complementary Test

hybridization are indeed much closer to the theoretical value of 0.25, which unambiguously demonstrates hybridization.

Up to this point, the theoretical LS-coefficients expected after hybridization of the Test or let-7f probes have been based on the assumption that each and every probe completely hybridized with a complementary target sequence. In reality, however, this is not likely a reasonable assumption as the hybridization efficiency is likely well below 100% [88]. We may therefore safely assume that incomplete hybridization is also occurring. In this case, one is justified in assuming that the estimated LS coefficients should actually have values intermediate that of the pure probe and the completely hybridized sequences.

The extent of hybridization is dependent on the concentration of the miRNA target present in the sample, thus the estimated LS coefficient should also depend on the miRNA target concentration. As the miRNA concentration is increased, the number of probes hybridized to miRNA targets will also increase. Likewise, as the number of miRNA targets captured by the let-7f probe increases, one would reasonably expect that the theoretical ratios of a , c , g , and u to change from their initial (i.e. probe-only) values, and begin to converge towards the complete 100% hybridization values. To examine this effect, increasing concentrations of let-7f target from 33 nM to 3.3 μ M were applied to a series of wells with pre-immobilized let-7f probe. After allowing time to hybridize, the SERS spectra were acquired and the LS coefficients were estimated as a function of target concentration. Figure 3.16 presents the LS coefficients estimated for each concentration. The negative control (0 μ M, i.e. SSC buffer only) is plotted at 0.001 μ M for comparison. From Figure 3.16 we can see a very clear trend as the let-7f target concentration is increased, i.e. g and u demonstrate a definitive increase while a and c clearly exhibit a decrease. Such a trend is to be expected considering that the initial theoretical a , c , g ,

and u of the let-7f probe are 0.41, 0.32, 0, and 0.27, respectively, which should converge towards 0.34, 0.16, 0.16, and 0.34, respectively, as more probes become hybridized. These theoretical convergence values are plotted in Figure 3.16 as horizontal dotted lines. However, a and u appear to be converging to a value greater than 0.34, and c and g appear to converge to a value less than the expected 0.16. The *apparent* convergence values are plotted as solid horizontal lines at approximately 0.41 and 0.09. The discrepancy between the theoretical and the apparent convergence values is unclear at this point. In addition, we also see that for the a and u coefficients, the 0 μM sample demonstrates lower and higher values, respectively, compared to next highest concentration of 33 nM, and appears to disrupt the otherwise monotonic nature of the calibration curve. The exact reason for this is unclear but could be due to un-optimized experimental processing, e.g. insufficient rinsing stringency.

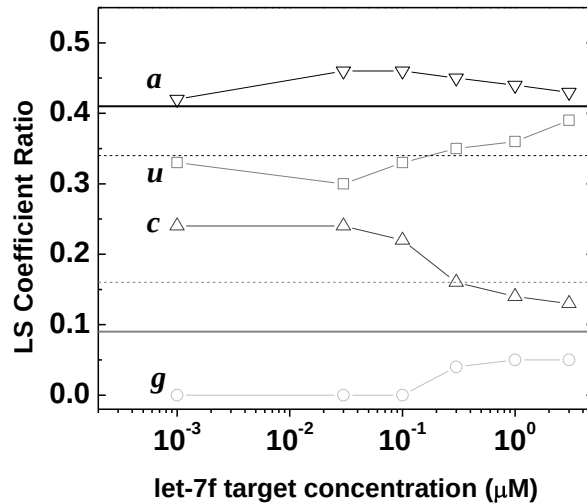


Figure 3.16. Estimated LS fitting coefficients for let-7f probe versus increasing concentration of let-7f miRNA target. The solid horizontal lines represent the approximate values to which the LS estimates appear to be converging to, and the dotted lines represent the theoretical values to which the nucleotide ratios should converge. (From ref [82].)

3.5 Conclusions

This chapter has discussed the use of the SERS chip for label-free detection of miRNA hybridization with probes immobilized to AgNRs. Visual analysis is found to be sufficient to discern the spectral difference arising from large disparity of the nucleotide compositions, e.g. differentiating between the let-7f and Test probes. Yet, visual discrimination between probes with more compositional similarity is challenging, as spectral differences are only very subtle. Moreover, the let-7f probe only demonstrates very minor spectral changes after hybridization. However, we have shown that LS can be employed to differentiate between different probes based on their composition. Such a technique was possible because of the large enhancement and good signal reproducibility of the AgNR substrates. It was also possible because we are able to directly acquire the DNA/RNA source signals, i.e. the spectra of pure nucleotides, and have showed that the DNA probe spectra is essentially a linear combination of the individual nucleotide spectra. LS regression analysis was found to provide encouraging qualitative results after proper data treatment (e.g. removing spectral regions), and demonstrated a semi-quantitative capability to determine the extent of hybridization with let-7f target concentrations of ~ 0.1 - $1\ \mu\text{M}$.

Chapter 4

***IN SITU* SILVER NANOROD SERS MEASUREMENTS IN SOLVENT**

The previous chapter showed the proof-of-concept demonstration that a AgNR SERS chip could be used to detect miRNA hybridization, but those experiments entail drying the DNA-coated AgNRs prior to measurement. Although ‘dry’ measurements are a feasible strategy for AgNR SERS miRNA detection, this requisite impedes the versatility and scope of the AgNRs SERS sensing platform. Because Raman detection is inherently insensitive to the presence of water, SERS analysis is thus possible within a water-based environment. Such a prospect opens the door to acquire measurements during the experimental processing, i.e. within a controllable, dynamic environment, which we will refer to as *in situ* analysis. In that case, the SERS response of AgNRs could be monitored in real-time as the DNA probe is immobilized, followed by spacer immobilization, then miRNA hybridization, etc., without ever experiencing an interruptive drying step.

In situ measurements may be acquired where specific control of the chemical nature, ionic strength, pH, viscosity, polarity, and electric potential of the experimental environment is desirable. Likewise, a great deal of useful assay information can be ascertained, such as kinetics, while providing more dynamic control over the DNA’s conformation and orientation, which is a non-trivial aspect of SERS analysis [43, 46, 47]. In addition, such an approach will mitigate any (and potentially detrimental) physical/chemical changes induced by the drying process. Moreover, many detection platforms have been miniaturized into highly portable lab-on-a-chip (LOC) devices. These miniaturized systems incorporate many experimental processes

traditionally used on the ‘laboratory-scale’, i.e. sample mixing, transport, separation, and detection. The reduced size (e.g. sub-wafer scale) and integrated functionalities provide rapid, efficient, cost-effective, automated, and deployable detection and greatly improve the scope and practicality of sensing systems for a variety of applications. Indeed, LOC platforms often contain microfluidic systems in which the sample processing is carried out; the use of aqueous systems is especially important for LOC technology geared towards biosensing applications. Thus, before AgNR SERS detection can be incorporated into a LOC miRNA sensing element, understanding the fundamental effects of liquid solvents on the AgNR SERS detection capability must first be investigated.

4.1 Effects of Water on the SERS Spectrum of Thiol-Based Analytes

The SERS response of AgNR substrates has been well characterized in dry, static environments and the results are published in numerous reports (for a comprehensive review see ref [93]). However, measurements in a liquid environment have been overlooked. Therefore, one may be interested to compare the SERS response of an analyte adsorbed to the AgNRs in liquid and dry environment. For such an analysis, the analyte should be able to form a strong adsorption bond with the Ag surface, so that the analyte surface coverage remains the same in both environments. For this reason a thiol molecule is an optimal choice as it can form a strong covalent bond with the AgNR surface as well as generate a sufficiently strong SERS signal.

Mercaptophenol (MPh) and mercaptohexanol (MH) are used for this study. These specific thiols are selected for several reasons. MPh is used because it provides a very strong Raman signal, allowing analysis and detection well below monolayer coverage, and its Raman and SERS spectral features have been previously investigated in detail and many of its

vibrational modes have been assigned [94]. MH is used because it is widely employed in SERS and non-SERS applications as a spacer molecule for DNA probe immobilization (see Section 3.2.2). Additionally, both MH and MPh have a terminal hydroxyl group, which is, at least for a highly packed monolayer, assumed to be oriented away from the metal surface providing, a highly hydrophilic interface. Finally, unlike the aforementioned DNA probes, these types of molecules are relatively simple and their behavior upon adsorption to a Ag surface has been more thoroughly characterized [89, 95, 96]. Appendix B presents the spectra and molecular structures of MPh and MH.

The AgNR substrates were fabricated and plasma cleaned in a way similar to that described previously (see Sections 2.1 & 3.1). MPh and MH were dissolved into 18 M Ω ·cm water and prepared at various concentrations. The 0.9×0.9 cm² AgNR substrates were immersed into 1-4 mL of the thiol solution in clean glass test tubes or petri dishes for various immersion times. All measurements entailed manually rastering the beam across the AgNR film by moving the sample translation stage continuously in the x- and y-directions. This allowed for a single measurement to sample a large portion of the AgNR film so as to provide a more accurate signal without having to measure multiple points individually, while mitigating any laser excitation-induced effects [64] (see Section 2.1).

The first set of experiments entailed immobilizing MPh onto the AgNRs at varying surface coverage θ_{MPh} , which was controlled by either changing the MPh concentration C_{MPh} , or the by varying the immersion time t . Although we do not attempt to measure or determine the θ_{MPh} directly, we can intuitively make a safe assumption that θ_{MPh} will increase for higher C_{MPh} or longer t . After immersion, the AgNR substrates are rinsed with DI water and blow dried prior to measuring.

Figure 4.1 shows the baseline-corrected SERS intensity of two different spectral bands (400 and 1070 cm^{-1}) after the MPh has been immobilized onto the AgNRs with different θ_{MPh} . These two bands are chosen because they are the most intense peaks of the MPh spectrum, and also demonstrate obvious surface coverage-dependent behavior. The 1070 cm^{-1} peak represents the in-plane ring-breathing mode (denoted as “1” in Wilson’s notation). The 400 cm^{-1} peak is also an in-plane mode (denoted as “7a” in Wilson’s notation) [94]. Each time point represents a different AgNR substrate immersed in the thiol solution. The solid symbols show the I_{400} and I_{1070} while the AgNR film is dried (I_{dry}), while the hollow symbols represent the intensity after water is re-applied back onto the AgNR film (I_{wet}).

Figure 4.1A shows the t -dependent SERS I_{dry} signal for 10 μM MPh treatment (solid blue circles and solid red squares). As one might expect, a steady and sharp increase in the I_{1070} and I_{400} is observed as the immersion time increases from 10 to ~ 1300 s. This is interpreted to be a result of an increasing θ_{MPh} . However, rather than continuing to show an increase in intensity with further increase in t , both I_{1070} and I_{400} show a notable decrease as θ_{MPh} is assumed to continue increasing. This type of behavior has been observed in previous studies for different analytes on different types of substrates [97, 98]. Zeman et al. proposes that at low θ , the dielectric value of the surrounding environment is effectively the same as water (refractive index $n_{\text{water}} = 1.33$). As the surface coverage of the organic analyte increases, the dielectric environment begins to shift towards the value of the organic (refractive index of organic molecules $n_{\text{organic}} \approx 1.5$). Such a concept is essentially an effective medium theory approximation (EMA). The plasmonic/SERS response is indeed sensitive to the dielectric environment surrounding the metal nanostructures; such an effect could explain the change in SERS intensity as a function of increasing organic molecule surface coverage [97].

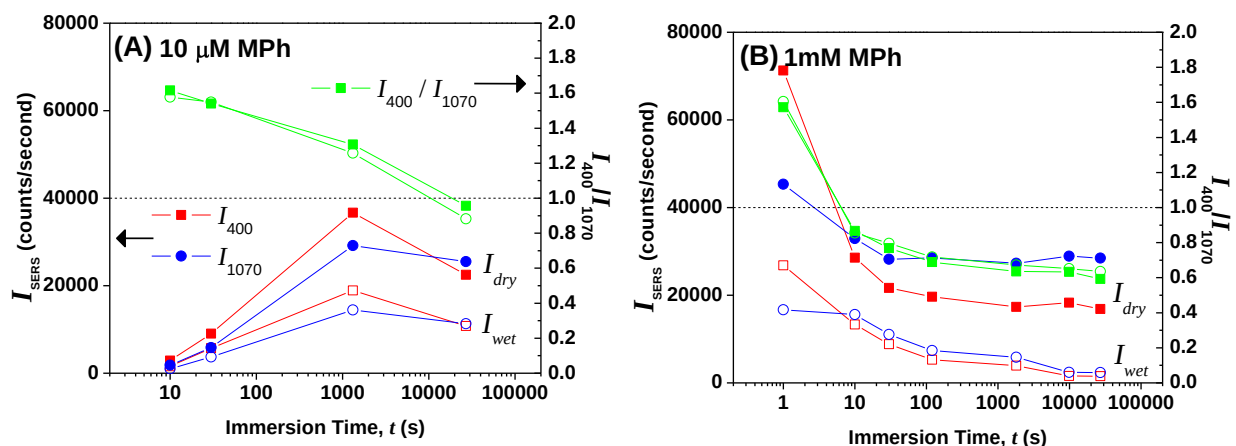


Figure 4.1. I_{wet} and I_{dry} vs immersion time t in MPH solution for concentrations of A) 10 μM and B) 1 mM. Each time point represents one substrate. The red and blue plots represent the I_{400} and I_{1070} (scaled by the left y-axis), respectively, while the green plots represent the I_{400}/I_{1070} ratio (scaled by the right y-axis). The solid symbols represent the I_{dry} , while the hollow symbols represent the measurements acquired when water was applied back onto the AgNR film, I_{wet} . The dashed line is a reference for the $I_{400}/I_{1070} = 1.0$.

On the other hand, the solid green squares in Figure 4.1A represent the I_{400}/I_{1070} ratio of the dry measurements for each time point. A steady decrease of this value from 1.62 at the shortest immersion time to 0.95 at the longest immersion time is evident. As the surface concentration of MPH increases, the analyte will be forced to change its orientation in order to accommodate a higher packing density. The changing adsorption configuration will cause a shift in the relative peak intensities as a likely a result of the SERS surface selection rules, whereby the vibrational modes with components oriented perpendicular to the surface are preferentially enhanced over vibrational modes containing components parallel to the surface [99-101]. A detailed physical description to explain the observed changes of the MPH, however, is beyond the scope of this discussion. Regardless, the I_{400}/I_{1070} ratio can be used as a simple metric to evaluate the surface coverage θ_{MPH} .

Figure 4.1A also shows the I_{1070} and I_{400} obtained after applying a few μL of water to form a thin (<1 mm thick) layer of water on the AgNR substrates (plotted as hollow blue circles and red squares symbols). The SERS intensity of the measurements acquired in water (I_{wet}) follows the same surface coverage-dependent trend as the preceding dry measurements (I_{dry} , solid symbols), and produces the exact same I_{400}/I_{1070} ratio (solid green squares). The only observable difference is that the water-based measurements yield an intensity approximately half that of the I_{dry} measurements, regardless of the surface coverage. Figure 4.2 plots the $I_{\text{wet}} / I_{\text{dry}}$ ratio (of the 1070 cm^{-1} peak) obtained for each surface coverage, and we can see that $I_{\text{wet}} / I_{\text{dry}} = 0.45\text{-}0.65$.

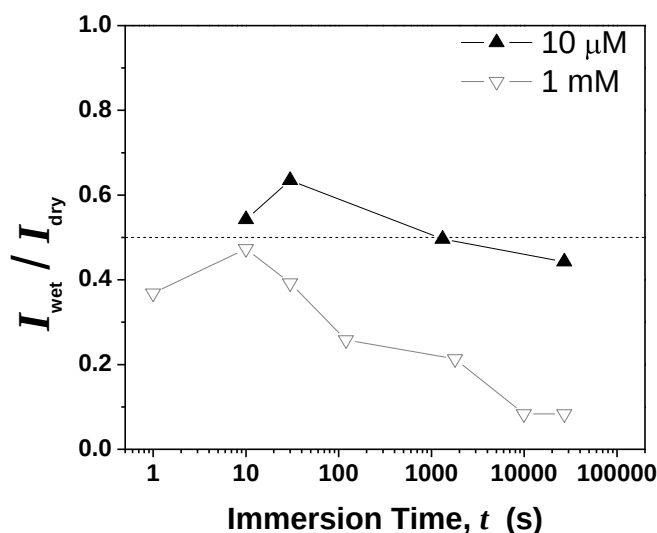


Figure 4.2. $I_{\text{wet}}/I_{\text{dry}}$ ratio (of the 1070 cm^{-1} peak) vs immersion time t in $10\text{ }\mu\text{M}$ and 1 mM MPh solutions.

This decrease in intensity could be due to three possibilities: 1) The analyte is desorbing from the AgNRs when treated with water and therefore the surface coverage is decreasing. This is not the case, however, because we do not see a change in the I_{400}/I_{1070} ratio, i.e. I_{400}/I_{1070} should increase if the θ_{MPh} decreases. Moreover, the I_{dry} signal returns back to its previous value

when the substrate is dried, i.e. the change is completely *reversible*; 2) The water causes a solvent-induced re-orientation of the immobilized MPh. A small molecule like MPh can be considered to act as a rigid body, thus during re-orientation, the rotation of one vibrational mode into a more surface-enhancing (i.e. vertical) orientation may cause another vibration mode to rotate out of a vertical orientation. This behavior would therefore manifest as changes in relative peak intensities. When water is added, however, the entire spectral intensity decreases uniformly, suggesting less overall enhancement, and not surface-selection enhancement. This is reflected in Figure 4.1A, where the I_{400}/I_{1070} is only sensitive to θ_{MPh} , and not to the dry or wet environment. 3) The third and most likely possibility is the effect of the dielectric medium (i.e. the refractive index n) surrounding the nanostructures [11]. Because water has a significantly higher dielectric value than air (1.00 vs. 1.33, respectively), this can cause a red-shift of the AgNR plasmonic frequency, which could result in less efficient SERS excitation of the AgNRS if the plasmonic frequency is shifted away from the 785 nm excitation.

Figure 4.1B plots the MPh signal intensity for another set of substrates treated in an identical manner but immersed in a higher concentration of MPh (1 mM). For the dry measurements (solid symbols), we see the highest I_{1070} and I_{400} acquired for the shortest immersion time of $t = 1$ s, followed by a steady decrease in I_{SERS} as the surface coverage continues to increase until $t = 30$ s. After this time both the I_{1070} and I_{400} stabilize with further increase of t , presumably as the θ_{MPh} becomes nearly saturated. We also see the I_{400}/I_{1070} ratio is initially large (i.e. θ_{MPh} is relatively low), but quickly decreases over the first 10 s and then starts to level off at a value of ~ 0.7 when $t > 100$ s. This observation is similar to that observed for the 10 μM MPh treatment shown in Figure 4.1A, except that we do not see an initial increasing

phase for the I_{400} or I_{1070} . This is probably because the increasing intensity phase would have been achieved very quickly (< 1 s) in the 1 mM solution.

The same explanations given for Figure 4.1A may be used to understand the observed phenomena of Figure 4.1B. However, one key difference arises regarding the I_{wet} measurements which is evident from Figure 4.2: as θ_{MPh} becomes relatively high (at long t and high C_{MPh}), the I_{wet}/I_{dry} ratio decays steadily from ~ 0.5 to ~ 0.08 as θ_{MPh} increases. Yet, for the lower concentration MPh (and presumably lower θ_{MPh}), the I_{wet}/I_{dry} always remains at ~ 0.5 . This observation is indeed peculiar and naturally leads to the question *why does a higher θ_{MPh} cause I_{wet} to be so low when I_{dry} remains unchanged?*

4.2 The Affect of Solvent Polarity on the SERS Intensity

We have already shown that the water does not induce a change in θ_{MPh} because the I_{dry} signal is the same before and after the water treatment. There is also a large ‘global’ decrease in the spectral intensity, and not a change in relative peak intensities; therefore, analyte re-orientation is not likely a contributing factor. Thus, we have already determined that adding water to the AgNRs causes a change in the dielectric environment which could explain, at least for low θ_{MPh} , the observed $\sim 50\%$ decrease in intensity when measuring in water. Yet, this last factor does not directly explain why the I_{wet}/I_{dry} ratio continues to decrease upon further increase of θ_{MPh} , such as that observed in Figure 4.2.

In an attempt to shed some light on this perplexing phenomenon, another experiment was performed but this time the I_{wet} was measured in several with varying polarity and dielectric values. AgNR substrates were first immersed in a high concentration of MPh (1 mM; aqueous) for an extended time (>24 hours). According to the aforementioned experiments, such a

treatment will ensure a virtual monolayer of the thiol, which is where we expect to see the lowest $I_{\text{wet}}/I_{\text{dry}}$ ratio. After immersion in MPh solution, the substrates were rinsed with DI water and blow dried, and then measured. First the substrate is measured dry (I_{dry}). Then the substrate is immersed into water and measured ($I_{\text{wet}}^{\text{water}}$). Then removed from water, dried, and I_{dry} is measured again. This same procedure is then repeated on the same substrate using isopropanol (IPA), toluene, then hexane. While the AgNRs are immersed in each solvent we measure $I_{\text{wet}}^{\text{IPA}}$, $I_{\text{wet}}^{\text{toluene}}$, and $I_{\text{wet}}^{\text{hexane}}$, respectively. We note that the AgNRs are always rinsed thoroughly with water last (to remove residual solvent from the AgNR film) before drying; in the case of toluene, or hexane, an IPA rinsing step is included before rinsing with water. This entire process is then repeated for a total of 3 cycles. The same procedure was also used for mercaptohexanol (MH) on a separate substrate.

The measured I_{dry} and I_{wet} values (connected gray squares) and corresponding $I_{\text{wet}}/I_{\text{dry}}$ ratios (disconnected black circles) obtained for both MPh and MH are plotted in Figure 4.3. A definitive pattern is emerges for all three cycles. For both MPh and MH we see that I_{wet} is highly dependent on the solvent $I_{\text{wet}}^{\text{water}} < I_{\text{wet}}^{\text{IPA}} < I_{\text{wet}}^{\text{toluene}} < I_{\text{wet}}^{\text{hexane}}$ (with the exception of the first $I_{\text{wet}}^{\text{IPA}}$ measurement of MH, which is higher than $I_{\text{wet}}^{\text{toluene}}$). In contrast, I_{dry} demonstrates very consistent behavior regardless of the prior solvent treatment, and only shows a very slight decrease for the MPh-treated AgNRs as they are consecutively immersed in solvents and dried. Because I_{dry} is so consistent, we also see that $I_{\text{wet}}^{\text{water}}/I_{\text{dry}} < I_{\text{wet}}^{\text{IPA}}/I_{\text{dry}} < I_{\text{wet}}^{\text{toluene}}/I_{\text{dry}} < I_{\text{wet}}^{\text{hexane}}/I_{\text{dry}}$. Repeating this process multiple times on the same substrate yields very reproducible results, as shown in Figure 4.3. This indicates that the thiol is not desorbing when exposed to different solvents. We also note that MPh produces an overall higher $I_{\text{wet}}/I_{\text{dry}}$ ratio for all solvents compared to MH. For

example, the I_{wet}^{water}/I_{dry} ratio for MPh and MH is ~ 0.07 and 0.03 , respectively, and I_{wet}^{hexane}/I_{dry} is ~ 0.50 and ~ 0.35 , respectively.

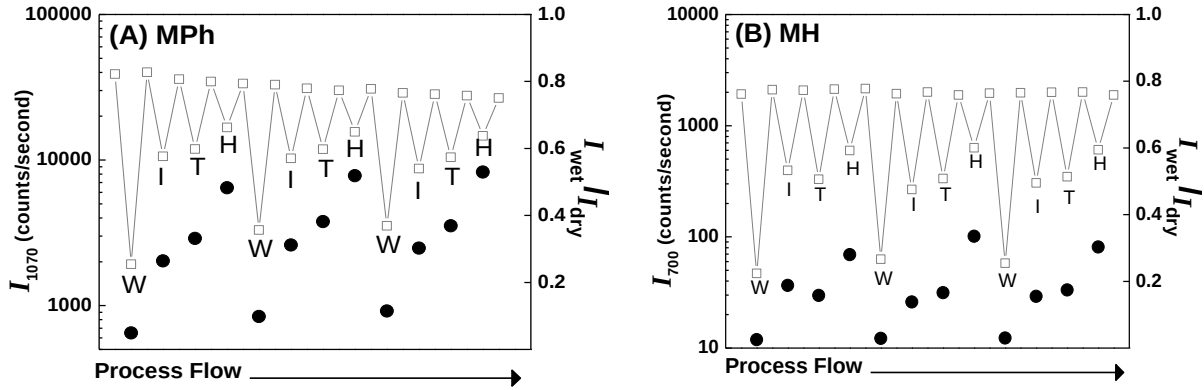


Figure 4.3. I_{wet} or I_{dry} of AgNR substrates treated with A) MPh and B) MH under different conditions. The x-axis represents the process flow of the processing/measurement conditions; W = water, I = IPA, T = toluene, and H=hexane; the values in between are the I_{dry} measurements. The connected gray squares represent the I_{wet} and I_{dry} (plotted with respect to the log scale of the left y-axis). The black circles represent the I_{wet}/I_{dry} ratios for each liquid measurement and its preceding dry measurement (plotted with respect to the right y-axis).

From the previous discussion, we know that the dielectric value (i.e. the index of refraction, n) can affect the plasmonic response, and therefore alter the SERS intensity. Thus, one may initially postulate that the observed disparity between the I_{wet} measurements is a result of the different dielectric properties of each solvent. However, comparing the refractive index n of the different solvents, we can quickly see there is no strong correlation to support this supposition. For example, water has the lowest refractive index of all the solvents ($n_{water} = 1.33$) and also has the lowest I_{wet}^{water}/I_{dry} ratio. Toluene has the largest refractive index ($n_{toluene} = 1.50$) yet it produces an intermediate $I_{wet}^{toluene}/I_{dry}$ ratio that is actually slightly lower than I_{wet}^{IPA}/I_{dry} , but IPA has an intermediate refractive index ($n_{IPA} = 1.38$). Hexane has a refractive index almost identical to that of IPA ($n_{hexane} = 1.37$), but produces the largest I_{wet}/I_{dry} ratio.

However, another physical characteristic that varies greatly between these solvents is their polarity. This quality can be quantified with a polarity index P which is an empirically determined value useful for applications involving solvent interaction with solutes and surfaces, such as chromatography. For example, water is a very polar compound capable of strongly interacting with other polar moieties, e.g. hydroxyl groups (such as that found MPh and MH), and therefore has a high polarity index $P_{\text{water}} = 10.2$. In contrast, hexane is a very non-polar substance that interacts very strongly with other non-polar compounds, yet demonstrates a negligible interaction with polar compounds; likewise, $P_{\text{hexane}} = 0.001$. IPA and toluene have intermediate values 3.9 and 2.4, respectively, and are expected to interact with polar compounds accordingly. Therefore, $P_{\text{water}} > P_{\text{IPA}} > P_{\text{toluene}} > P_{\text{hexane}}$ thus we indeed find a trend that correlates strongly with the trend $I_{\text{wet}}^{\text{water}} < I_{\text{wet}}^{\text{IPA}} < I_{\text{wet}}^{\text{toluene}} < I_{\text{wet}}^{\text{hexane}}$ observed in Figure 4.3. We can therefore postulate that *as the P increases, the $I_{\text{wet}}/I_{\text{dry}}$ decreases*. Upon discovering this correlation, a causal explanation must be provided. To address this, we now turn to structural and mechanical properties of the AgNR film.

4.3 Solvent-Induced Bundling of AgNRs

Recent studies have demonstrated that free-standing nanopillar arrays can be used as highly sensitive SERS substrates [102, 103]. These nanopillar substrates are fabricated using methods such as EBL or reactive ion etching (RIE) which yield an array of vertically aligned nanopillars/nanorods with dimensions similar to that of the AgNRs. The nanopillars can be composed of various materials, e.g. Si,, but can be made SERS-active by depositing Au onto their tips. However, simply depositing Au onto the tips does not produce a significant amount of

SERS enhancement; to achieve high enhancement, those studies show that a requisite wetting/drying step is needed.

As liquid on the nanopillar array dries, capillary forces induced by the receding solvent front cause the tips of the free-standing nanopillars to bundle together. Meanwhile, the base of the nanopillar remains fixed to the underlying substrate. These bundled Au tips are ideal configurations to generate nano-gap hot spots between the metal surfaces, greatly amplify the SERS signal.

The ‘nano-bundling’ effect could be clearly resolved from standard top-view SEM images, because the nanopillar tips bundled in a *lateral* direction. Fan et al. has previously observed the same type of behavior with arrays of vertical Si nanorods (SiNR) fabricated with glancing angle deposition (GLAD) [104-109]. As with the Au-tipped nanopillars, the lateral nano-bundling, or nano-carpet effect, was evident from top-view SEM images. Tilted SiNR arrays fabricated with OAD also underwent structural changes upon drying of a solvent, yet the effect was not evident from a top-view SEM image. However, the nano-bundling effect could be observed with a cross sectional SEM image [109]. From this perspective, the tilted SiNR film demonstrated a definitive decrease in the tilting angle ϕ (relative to the surface) and therefore a corresponding decrease in film thickness T , i.e. the SiNRs were bundling in a *vertical* manner, and the nanorod film becomes more compact. Meanwhile, the liquid also introduced more disorder in an otherwise highly ordered and uniform array of tilted Si nanorods. Those studies provide critical insight into how OAD structures are affected by liquid solvents. In light of this evidence, one may want to determine if such an effect is manifesting with the AgNRs. This could answer the relevant question: *Are solvent-induced changes in the AgNR film morphology*

causing a change in the SERS signal intensity? To address this issue we will first carry out a detailed SEM analysis.

4.3.1 SEM Analysis of Water-Induced Nano-Bundling of AgNRs

SEM images were acquired from as-deposited AgNRs and also those treated with water. For better SEM resolution, AgNRs (on Ag thin film underlayer) were fabricated onto pieces of a Si wafer. Two groups of substrates were used in this SEM analysis, with each group of substrates placed at different points on the sample holder during AgNR deposition. Both groups contained two substrates placed immediately next to one another. After deposition of the AgNRs, the substrates were plasma cleaned, and then one substrate from each group was briefly immersed in water and then blow-dried. The remaining as-deposited substrate was used as a reference.

Both top-view (Figure 4.4) and cross-sectional images (Figure 4.5) of the AgNR films were acquired. We first note that the as-deposited AgNRs fabricated on the bottom of the holder (i.e. closer to the Ag source material during the deposition) produce a thicker AgNR film than those at the top of the holder, yet both groups have approximately the same tilting angle ϕ , which is to be expected. Comparing the as-deposited and water-treated AgNRs, there is no clear distinction obvious from the top-view SEM images, as expected [109]. The cross sectional SEM images, as shown in Figure. 4.5, however, do demonstrate some differences between the as-deposited and the water-treated AgNR films, the latter showing a debatable decrease in film thickness and nanorod tilting angle, and perhaps more overall disorder of the AgNR array (Figure 4.5).

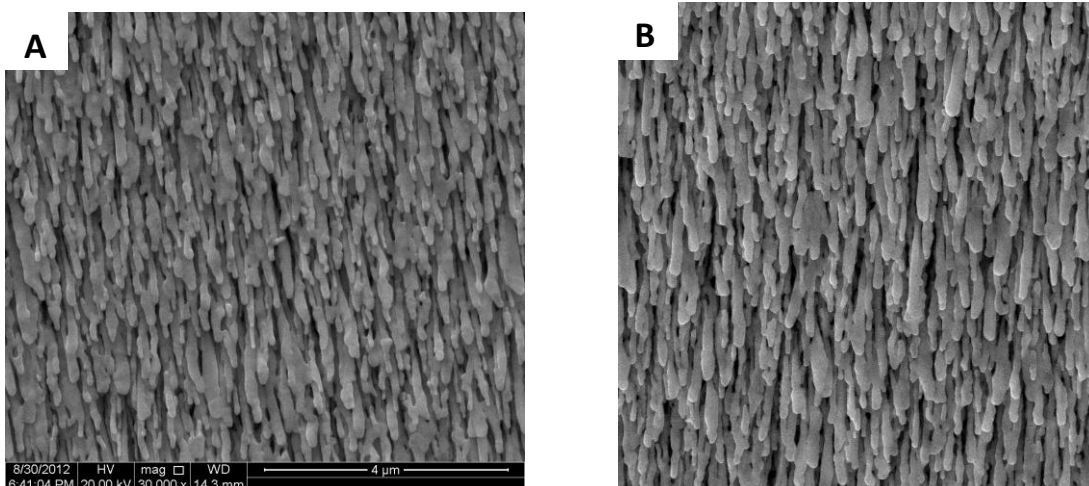


Figure 4.4. Overhead SEM images of AgNR film A) before and B) after treatment with water. Both images have the same magnification. These substrates were from the bottom of the sample holder in the evaporation chamber.

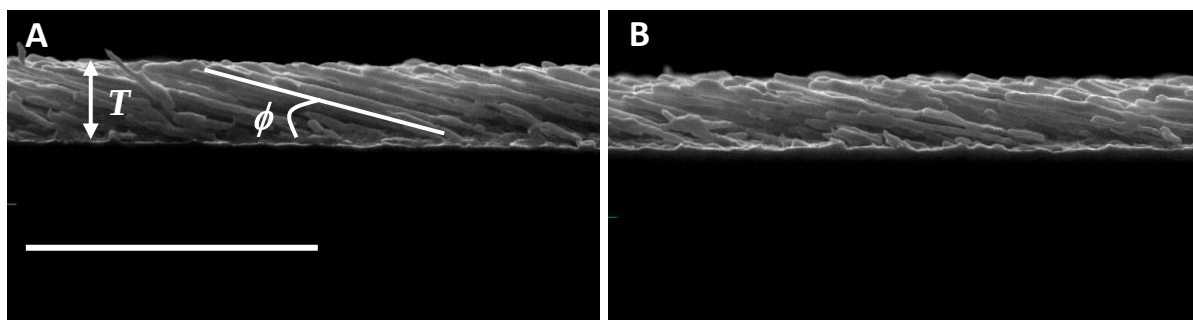


Figure 4.5. Cross sectional SEM image of AgNR film A) before and B) after treatment with water. The thickness T and tilting angle ϕ parameters used for quantitative analysis are also illustrated. The scale bar is 3 μm .

Because the observed differences between the two treatments are minor, and to avoid subjective bias, a more rigorous and objective statistical analysis was performed. For each of the four substrates, five high-magnification cross-sectional images were acquired at different locations along the AgNR substrate. For each image, the tilting angle ϕ of $n = 10$ OAD nanostructures was determined, and the AgNR film thickness T was also estimated for each image. Thus, for each substrate $n = 50$ angles and $n = 5$ thicknesses were measured.

Furthermore, the identities of each of the SEM images was concealed and randomized during the angle and thickness estimations to mitigate subjective bias.

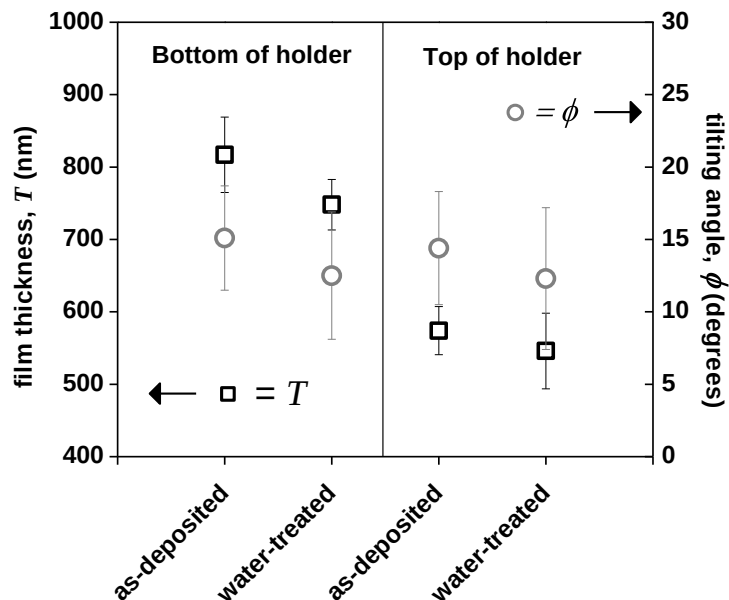


Figure 4.6. Morphology Statistics of AgNR film thickness T (squares) and tilting angle ϕ (circles) acquired from SEM images for two groups of substrates (bottom and top of sample holder) for as-deposited and water-treated.

According to the report by Fan et al. [109], we expect the water-treated AgNRs to demonstrate a decrease in ϕ , this also yields a reduced T ; meanwhile, the increased disorder of water-treated OAD films would be expected to manifest as an increase in the variability of ϕ . Figure 4.6 shows the average AgNR T and ϕ measured for all four substrates, where the error bars represent one standard deviation, i.e. a metric of the variability and disorder.

The quantitative SEM analysis in Figure 4.6 provides evidence that the expected liquid-induced structural changes are indeed occurring. For the thicker AgNR film, after the water-treatment, T changes from 820 ± 50 nm to 750 ± 40 nm and ϕ decreases from 15 ± 4 to 13 ± 5 degrees. For the thinner AgNR film, a similar trend is observed as T decreases from 580 ± 30 nm to 550 ± 50 nm, and ϕ decreases from 14 ± 4 to 12 ± 5 degrees after water treatment. In both

cases we observe the predicted results: 1) decrease in T , 2) decrease in ϕ , and 3) increase in the standard deviation of ϕ . Clearly, however, these changes are subtle, and based on the error bars from Figure 4.6, there is significant statistical overlap between structural parameters for the as-deposited and water-treated samples. To determine if the differences of T or ϕ between the water-treated and as-deposited samples are statistically significant, a paired Student's T-test was performed. Accordingly, for both groups the differences of T between the as-deposited and water-treated are determined to be *not statistically significant*; however, ϕ is *statistically significant* for both groups. The fact that T did not yield a statistically significant difference could be a result of the relatively small sampling size ($n = 5$) and also the difficulty in visually determining precisely where the underlying Ag film ends and the AgNR film begins. Whereas the SEM analysis appears to confirm the AgNR nano-bundling phenomenon, we decided to further substantiate this possibility using other methods such as film AgNR film conductance measurements.

4.3.2 Conductance Analysis of Water-Induced AgNR Nano-Bundling

Although Ag is a highly conductive material, the AgNRs fabricated by OAD are isolated and discrete structures; thus, the as-deposited AgNR films could demonstrate a large resistance (assuming that there is no pre-deposited continuous Ag film underlayer). However, after water-induced bundling, the AgNRs could cluster together, and the conductance of the AgNR film could increase significantly [110]. If nano-bundling is indeed occurring, the AgNR film is becoming more compact (via vertical bundling) and potentially forming conductive path(s) through the film. A four-point probe was therefore employed to measure the conductance of the AgNR film. AgNR were fabricated in the same manner as described in the previous chapters,

the only difference was that the AgNR films were deposited directly onto $1.8 \times 1.8 \text{ cm}^2$ glass pieces without a continuous Ag film underlayer.

A custom made four-point probe was used to apply current to the AgNR film via the outer pins while measuring the voltage across the two inner pins. Each pin has a individual spring-loaded holder to assure the pin makes complete and continuous contact with the sample; the pins also have sharp tips so as to prevent significant damage to the AgNR film during contact. The pin-to-pin separation was 2.3 mm. The current was applied via a source meter that was controlled with a custom Lab View program. The initial current was 1 mA and incrementally increased by 1 mA/step until a final current of 20 mA; the voltage was recorded at each interval [110]. The resistance R was calculated from the slope of the voltage vs. current measurements, and the conductance was determined as $C = 1/R$.

For all measurements, the direction defined by the four pins was aligned with the AgNR growth direction (i.e. measuring the resistance along the nanorod growth/tilt direction), and the four pins were placed in the center of the substrate (to avoid potential edge effects). First, the conductance was measured on as-deposited AgNRs (treated only with a brief Ar^+ plasma cleaning). Afterwards, the pins were removed and the substrate is briefly immersed into a liquid solvent and then immediately blow-dried with Ar, and the conductance was again measured.

Figure 4.7 shows the conductance for the as-deposited and solvent-treated samples. The as-deposited AgNRs demonstrated very low conductance as expected. However, after briefly wetting the AgNRs with water and then drying, a remarkable increase in conductance is observed. In addition, AgNR substrates were also treated with isopropanol (IPA), toluene, and hexane. The hexane treatment likewise produced a large increase in conductance, but not as

much of an increase as water. Toluene produced a slightly higher conductance than hexane, and IPA produced an intermediate conductance, between that of toluene and water.

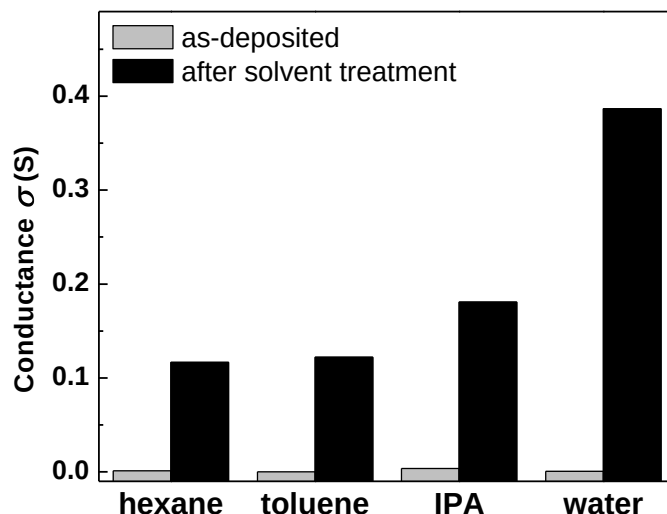


Figure 4.7. Conductance measured on AgNR films before (gray) and after (black) treatments with different solvents.

The difference in the induced-conductance observed for different solvent treatments may be a result of varying mechanical properties of the solvent. Because the solvent's surface tension γ is directly responsible for the nanobundling effect, intuitively one may expect that a change in γ could change the extent to which the AgNRs bundle. We may anticipate that lower surface tension solvents will exert a smaller capillary force on the AgNRs, ultimately yielding less nanobundling and therefore lower conductance.

IPA, toluene, and hexane have different surface tensions of 23.00, 28.40, 18.43 mN/m, respectively; and water has an exceptionally high surface tension of 72.80 mN/m. Thus, we may expect the highest conductance for water-treated AgNRs. Indeed, Figure 4.7 shows that after water dries, the AgNRs yield a conductance of $\sigma = 0.385$ S. Hexane, with the lowest surface tension tested, yields a AgNR film resistance of 0.117 S. Toluene has a higher surface tension than hexane but only demonstrates a slightly higher conductance of $\sigma = 0.122$ S. On the other

hand, despite the fact that IPA has a surface tension intermediate to that of hexane and toluene, IPA yielded a higher conductance of $\sigma = 0.181$ S, higher than that of toluene. To make sure this value was real and not an anomaly, a second AgNR substrate was treated with IPA which yielded $\sigma = 0.259$ S, which is somewhat higher than the first IPA-treatment conductance measurement, but still lower than water. The water treatment was also replicated on another substrate and produced nearly an identical conductance of $\sigma = 0.392$ S. The reason that IPA has a lower surface tension than toluene, yet produces a higher conductance (presumably because it induces more nano-bundling) is unclear. This observation could be a result of the interfacial surface energy of the solvents. This energy represents how strongly the solvent interacts with the Ag surface. Although IPA has a lower surface tension, it may interact more strongly with the AgNR surface. In that case the capillary forces may ultimately couple more efficiently to the AgNR.

4.3.3 Increasing SERS enhancement of AgNRs with Solvent-Induced Nano-Bundling

The previous SEM and conductance analysis provide strong evidence that a drying solvent induces nanobundling of the AgNRs. However, those experiments do not determine if the nanobundling creates hot spots that increase the enhancement of the nanorod arrays. The study by Schmidt et al., however, conclusively demonstrates that the nano-bundling generates hot spots that greatly increased enhancement for the Au-tipped Si nanopillar arrays [102]. This was demonstrated by first vapor-depositing the analyte onto the nanopillars. Vapor does not produce any capillary forces, and thereby avoids inducing the formation of nanobundles. This means the analyte's SERS signal could be measured prior to nanobundle formation. After the 'un-bundled' SERS response was measured, the nanopillar array was treated with water and then

dried, at which point the SERS enhancement was greatly augmented due to the formation of hot spots between the nanopillars' Au tips.

This proof-of-concept analysis can likewise be performed with the AgNR substrates. This was accomplished by first plasma cleaning the as-deposited AgNRs to remove organic contaminants that could possibly interfere with the adsorption of analyte from the vapor phase. Then the AgNR substrates were placed into a MPh vapor chamber. This chamber was simply a glass bowl with several milliliters of high concentration MPh solution (100 mM, in IPA). A small pedestal was placed into the reservoir to hold the substrates well above the MPh solution to assure that liquid did not contact them. After placing the substrates in the chamber, the top was sealed with foil to allow the chamber to saturate with MPh vapor. The substrates were incubated in the MPh vapor for approximately 10 minutes, then removed, and their SERS response was measured while the substrates were still dry. Then water was added to the AgNR film, and the SERS response was measured while the film is wet. Then the substrates are blow-dried and measured again. Figure 4.8 shows the spectra measured at each step. After depositing MPh but before adding water, the MPh response is rather low compared to the large I_{SERS} observed in Figure 4.1. We can also see that the I_{400}/I_{1070} is ≈ 1 , which indicates a relatively low θ_{MPh} . Upon adding water, the signal decreases by only $\sim 50\%$, i.e. $I_{wet}/I_{dry} \approx 0.5$. This ratio is similar to the ratios measured for the substrates from Figure 4.1 with low θ_{MPh} . The 50% drop in intensity upon immersion in water is presumed to be a result of the changing dielectric environment. However, when the same substrate is dried, and enormous increase in the signal enhancement is observed, whereby the I_{SERS} increases 5-10 fold. This behavior is concomitant with the results reported by Schmidt et al [102].

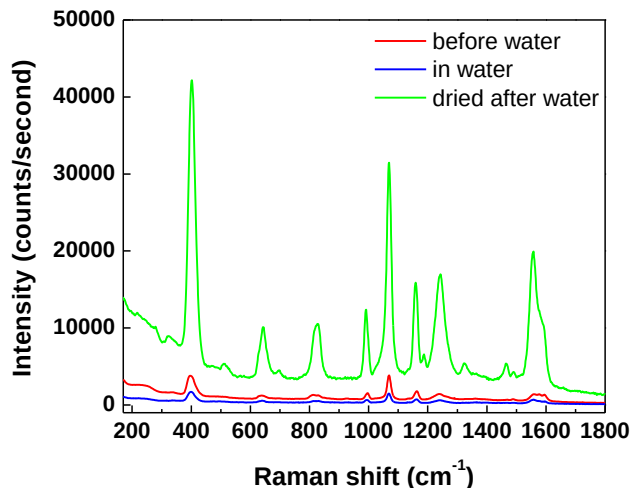


Figure 4.8. SERS spectra of MPh vapour-deposited onto AgNRs before adding water (red), while immersed in water (blue), and after drying (green).

Conclusively, these results, together with the SEM and conductance findings, indicate that the AgNRs undergo solvent-induced nano-bundling, which creates hot spots at the nanorod-nanorod junctions. Equipped with this understanding, we now turn our attention back to where we left off at the end of Section 4.3.2, and propose a novel yet reasonable explanation as to why the solvent polarity induces changes in the AgNR SERS signal.

4.4 Reversible AgNR Nano-Bundling: The Effect of Solvent Polarity

Recall from Section 4.3.2 that the $I_{\text{wet}}/I_{\text{dry}}$ ratio was strongly correlated with the solvent polarity P ; as P increased, $I_{\text{wet}}/I_{\text{dry}}$ decreased. Because I_{dry} remains relatively stable throughout the experimental processing, the decrease in $I_{\text{wet}}/I_{\text{dry}}$ directly reflects a decrease in I_{wet} ; therefore, a more polar substance (e.g. water), causes I_{wet} to be significantly lower than that of a nonpolar solvent (e.g. hexane). We also now know that a drying solvent induces a nanobundling effect within the AgNR film, which increases the effective number of hot spots, thereby increasing the

SERS intensity. With these observations in mind, we now describe a possible physical explanation for the reversible behavior of the I_{wet} and I_{dry} demonstrated in Figure 4.3.

After a MPh (or MH) monolayer has been immobilized ($\theta_{\text{thiol}} \approx 1$), the AgNRs are then dried and therefore become bundled. Upon bundling, portions of the thiol-coated AgNRs come into close proximity with each other (presumably into direct contact, as deduced from the conductance measurements) forming hot spots and producing a large signal. These regions of contact are expected to yield a very hydrophilic environment at the Ag-thiol-OH...HO-thiol-Ag interface. A highly polar solvent such as water will strongly interact with the polar -OH groups, readily intercalating into and wetting this interface. This thereby relieves intermolecular bundling forces present at the interface. Without counter-acting bundling forces, the residual stresses in the body of the AgNRs allow the AgNRs to relax (perhaps only slightly) to a less bundled conformation, which will increase the inter-rod distance, thereby disassembling the hot spots and greatly attenuating I_{wet} (i.e. $I_{\text{wet}}/I_{\text{dry}} \approx 0.05$).

On the other hand, a highly non-polar solvent will not produce a strong interaction with the polar -OH surface, and will therefore be occluded from the highly polar Ag-thiol-OH...HO-thiol-Ag interface. Because hexane cannot readily intercalate into and wet the interface, the nanobundles and hot spots stay intact, and I_{wet} remains high, only demonstrating a dielectric-induced decrease in intensity (i.e. $I_{\text{wet}}/I_{\text{dry}} \approx 0.5$). We may also anticipate that solvents with intermediate polarities (e.g toluene and IPA) produce an intermediate degree of de-bundling, and therefore an intermediate change in $I_{\text{wet}}/I_{\text{dry}}$, which is indeed observed from Figure 4.3.

This explanation implies that polar solvents can *relieve* bundling forces (for hydrophilic nanorods), but does not imply that non-polar solvents can *induce* bundling. Accordingly, if the AgNRs (with $\theta_{\text{MH/MPh}} \approx 1$) are initially de-bundled with a water treatment, the AgNRs should

remain de-bundled even after successive treatments of solvents with low polarity as long as the AgNRs are not allowed to dry during the processing. That is to say, we should observe the $I_{\text{wet}}/I_{\text{dry}} \approx 0.05$ for all solvent treatments.

The following experiment was therefore devised to test this theory and the results are shown in Figure 4.9. First the AgNRs are treated to a prolonged immersion in a high concentration of MPh (or MH) to assure $\theta_{\text{MPh/MH}} \approx 1$. Next the AgNRs are rinsed, dried, and I_{dry} is measured and a strong signal intensity is obtained as expected (data not shown). This is followed by immersion in water and $I_{\text{wet}}^{\text{water}}$ measured and shows a drastic drop in intensity to $\sim 5\%$ that of the preceding dry measurement, i.e. $I_{\text{wet}}^{\text{water}} / I_{\text{dry}} \approx 0.05$. Without letting the AgNR film dry, the AgNRs are rinsed with IPA (to displace residual water) and then immediately immersed in IPA and measured, where we find $I_{\text{wet}}^{\text{IPA}} \approx I_{\text{wet}}^{\text{water}}$. This is expected according to the aforementioned theory, as the initial water treatment de-bundles the AgNRs, and they remain de-bundled when treated with IPA. This is followed by rinsing/immersion in toluene and $I_{\text{wet}}^{\text{toluene}}$ is measured. Again, we see that $I_{\text{wet}}^{\text{toluene}} \approx I_{\text{wet}}^{\text{water}}$, as the AgNRs are still de-bundled. Next, the AgNRs are rinsed/immersed in hexane, measured, and $I_{\text{wet}}^{\text{hexane}}$ is found to be slightly higher than the previous solvent measurements (for both MPh and MH), but still much smaller than I_{dry} . The reason for this slight increase with hexane is not clear. Then the AgNRs are rinsed with IPA and water (to displace residual hexane), and immersed back into water where $I_{\text{wet}}^{\text{water}}$ is measured and it is found to be equal to the first $I_{\text{wet}}^{\text{water}}$ from the beginning of the process. So far we can see that the I_{wet} signal is relatively constant and reproducible with each solvent. Based on the prescribed theory, the AgNRs have remained de-bundled regardless of the solvent treatment: all

of the $I_{\text{wet}}/I_{\text{dry}}$ ratios correspond approximately to the $I_{\text{wet}}^{\text{water}}/I_{\text{dry}}$ ratio for water as seen in Figure 4.3.

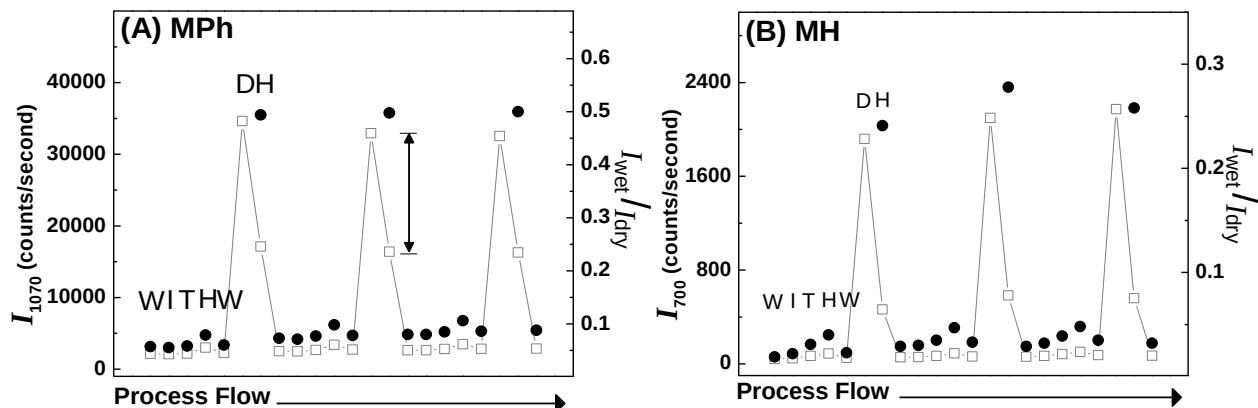


Figure 4.9. I_{wet} and I_{dry} of AgNR substrates treated with A) MPH and B) MH under different conditions. The x-axis represents the process flow of the processing/measurement conditions; W = water, I = IPA, T = toluene, and H=hexane, D = dry. The gray squares represent the I_{wet} or I_{dry} (plotted with respect to the left y-axis). The arrow in (A) indicates the change in SERS intensity expected to result strictly from the dielectric effect. The black circles represent the $I_{\text{wet}}/I_{\text{dry}}$ ratios for each liquid measurement and its preceding dry measurement (plotted with respect to the right y-axis).

The same substrate is then dried and I_{dry} demonstrates a large, ~ 10 -fold increase in SERS intensity (for both MPH and MH) due to the AgNR bundling and returns to the same level as the first dry measurement (not shown), as expected. Afterwards, the AgNRs are immersed into hexane and $I_{\text{wet}}^{\text{hexane}}$ is measured again. Rather than seeing $I_{\text{wet}}^{\text{hexane}}/I_{\text{dry}} \approx 0.05$, which is what we observed with previous hexane measurement, now $I_{\text{wet}}^{\text{hexane}}/I_{\text{dry}} \approx 0.5$ or ~ 0.25 , for MPH and MH, respectively, which correspond exactly to the ratios observed in Figure 4.3A and B, respectively. The 50% drop in intensity (marked with the vertical arrow in Figure 4.9A) is again attributed to the changing dielectric environment; the additional 25% drop observed with MH could be a result of slight de-bundling that hexane can induce with this particular analyte, but a detailed explanation is not obvious.

Finally, the substrate is removed from hexane, rinsed with IPA and water, and immersed back into water where the final I_{wet} measurement is acquired. Due to the de-bundling effect of water, $I_{\text{wet}}^{\text{water}} / I_{\text{dry}}$ decreases significantly to its original value of ~ 0.05 . This entire process was then repeated two more times on the same AgNR substrate (excluding the initial MPh immobilization step) for a total of three cycles to test the repeatability. Indeed, we observe exceptional reproducibility between each cycle, for both MPh and MH, with minimal hysteresis.

4.5 Conclusions

This chapter highlights the unexpected low intensity SERS signal that are acquired from AgNRs containing a high surface coverage of MPh or MH while immersed in solvents such as water. This observation has led to the serendipitous discovery that hot spot formation is caused by solvent-induced nanobundling. A detailed analysis was performed using SEM and film conductance measurements to confirm that the AgNR film changes its structural conformation upon treatment with a solvent. Finally, the hot spot/nano-bundling formation is demonstrated to be a highly reversible process, where the de-bundling is sensitive to the polarity of the solvent. This reversible de-bundling therefore explains why the MPh or MH signal is weak when measured in water. Considering that thiolated DNA also forms a very hydrophilic interface on the AgNRs, similar to MPh or MH, such behavior is critical to consider when devising *in situ* DNA-based SERS experiments. In general, understanding the origin of the OAD-AgNR hot spots will aid in the further development of these types of SERS substrates as feasible sensing platforms.

Chapter 5

SILVER NANOROD LAB-ON-A-CHIP ELECTROCELL FOR ELECTRIC FIELD- ASSISTED SERS ANALYSIS

Up to this point in this dissertation, we have examined how AgNRs substrates could be fabricated into a patterned multi-well array SERS chip, and how this novel type of substrate could be used for parallel label-free analysis of multiple miRNA samples. Because *in situ* measurements will be important for SERS analysis of miRNA, we then discussed how AgNR substrates behaved in a liquid environment. In addition to the capability for *in situ* analysis, the ability to induce external control over the sampling and detection conditions is also a desirable aspect. This goal has been a driving motivator for the development of lab-on-a-chip (LOC) devices. In an LOC system multiple functionalities/components are miniaturized and integrated onto a single ‘chip’ for rapid, efficient, cost-effective, and automated processing and detection [111, 112].

Electric-field assisted detection (EFAD) has been one particular strategy widely used to augment the detection capability of various sensing techniques, including nucleic acids. The intrinsic negative charge of nucleic acids allows control over transport, hybridization, and de-hybridization via an external E-field. Additionally, the well-established and facile fabrication of microelectrodes has also permitted the straightforward integration of EFAD-processing into LOC devices. Despite the analytical advantages afforded by EFAD, the SERS literature is practically devoid of studies using EFAD for label-free DNA detection (see Section 1.3.2).

Therefore, in this chapter we will first briefly review how EFAD-based techniques have been used to aid in the detection of nucleic acids for non-SERS (e.g. fluorescence) analysis. We will then turn our discussion towards a simple design to incorporate the AgNRs as an electrocell capable of *in situ* SERS measurements. Such a device allows external control of the cell voltage/E-field, and the preliminary *in situ* SERS results are discussed. Finally, we will look at a new AgNR electrofluidic LOC device that may open the doors for future label-free miRNA SERS-sensing.

5.1 E-field Assisted Detection for Label-Based Nucleic Acid Analysis

The EFAD technique has been used to improve fluorescence-based detection of DNA hybridization. Studies by Edman et al. and Heller et al. evaluated the use of 5×5 and 10×10 array of $80 \mu\text{m}$ circular gold electrodes for specific capture and detection of fluorescently-labeled DNA oligomers [113, 114]. In those studies, the Au electrodes were coated with a thin agarose/streptavidin layer, which allowed subsequent immobilization of biotin-modified DNA capture probes directly over the Au electrodes. A constant electrical current (200-500 nA) was applied to the microelectrodes in the presence of the DNA target sample solution. A counter current of negatively charged species (e.g. anions and DNA) therefore developed in the sample solution. The electrodes could be /individually addressed allowing for selective transport of the negatively charged species directly to specific electrodes. The results show a 30- to 40-fold increase in the hybridization kinetic rate when an E-field was applied, compared to passive (no E-field) hybridization. The E-field directed transport was also demonstrated to be precise enough to confine the DNA target to a single microelectrode without bleeding over to adjacent microelectrode. In addition, these studies utilized E-fields to improve the stringency of the

assay. Because DNA probes have a propensity to hybridize with target sequences that are not completely complementary, a detection assay must assure enough stringency to reduce the possibility of non-specific hybridization. The melting temperature T_m is a measure of the energy needed to overcome the strand-strand interaction of hybridized DNA. Double-stranded sequences with high complement will have higher T_m values than those with less complement. An E-field can thus be used to overcome the T_m of sequences with single mismatches, while keeping the energy below that necessary to de-hybridize completely complement sequences. Therefore, in those studies oligonucleotides target sequences with a large degree of mismatch could be readily removed from each probe-functionalized microelectrode by reversing the electronic bias (i.e. applying *negative* potential) of the microelectrode for 12 s. Single-base mismatched target sequences could be removed from the electrode by applying a negative bias for 20s, whereas completely complementary sequences remained sufficiently hybridized to maintain a strong signal despite the electronic stringency.

The preceding studies highlighted the advantage of using a DC-bias to improve hybridization time and stringency via electrostatics to augment analyte transport. However, a different set of studies by Joao Conde's group have shown that electrodynamics can vastly improve hybridization/detection speed via use of a short E-field pulse [115-118]. These studies investigated DNA oligomer probe immobilization and target hybridization using pairs of counter- and working- microelectrodes fabricated onto flexible polyimide substrates. The electrodes were fabricated by vapor deposition of aluminum onto the polyimide substrate, followed by the sputtering deposition of 200 nm Al_2O_3 electrical passivation/insulation film, and chemical vapor deposition of a SiO_2 200 nm film for surface functionalization. The DNA probes were covalently bonded to the SiO_2 layer. After applying the DNA target in buffer to the

electrodes, the effects of varying pulse parameters such as pulse width (i.e. duration) t_p and voltage (amplitude) V were tested. These studies found that the fluorescent hybridization signal could be detected in a significantly shorter time with appropriate pulse parameters compared to the passive (no pulse) hybridization. Without a pulse, the fluorescent hybridization signal slowly increased over several hours before saturating. In contrast, when $V = +0.1$ V, $t_r = 4.5$ ns, and using pulse widths $t_p = 10$ μ s to 100 ms immediately produced the same hybridization signal as that of 3 hours of passive hybridization. The maximum hybridization was found for $t_p = 1$ ms, yet detectable hybridization occurred at $t_p = 10$ ns. The highest hybridization signal is also achieved when $V = 1-5$ V. Another interesting observation is that the fluorescent hybridization signal is found evenly distributed across both the WE and CE; likewise, the signal does not appear to be dependent on the bias applied to the working electrode during the pulse, as identical results are obtained when $V = +1$ or -1 V. The authors attribute the pulse-effect to the polarization experienced by the DNA sequences near the electrode. Unlike the aforementioned DC-based E-field studies, the phenomenon observed in these studies is not due to increased transport of DNA to the electrode surface. Because the pulse durations are extremely short (i.e. the effect is observed for $t_p = 10$ ns), the duration of the applied E-field is well below the time scale required for electrophoretic transport. In addition, the effect is independent of the applied bias, unlike that of the previous studies that repelled or attracted the DNA when the working electrode was negatively or positively biased, respectively. The DNA hybridization signal is also observed everywhere the field is present, i.e. in the inter-electrode gap, and therefore not being ‘directed’ to the points of highest field density directly on the electrodes. Thus, the authors propose that the observed effect is likely due to the polarization of the DNA and solvating water molecules. Fourier analysis of a square pulse (with rise/fall time $t_r = 4.5$ ns) shows that 10-100 MHz

frequency components are present within the pulse. These high frequencies may allow for the re-orientation of the DNA and the disruption of the solvation shell, allowing for faster hybridization kinetics.

5.2 Integration of AgNR Substrates into Electrocell Device

The previous section highlighted label-based (e.g. fluorescence) detection schemes that utilized E-fields to improve the hybridization rate and stringency/selectivity of DNA oligomer targets on microelectrodes. Before we can adapt these strategies with a AgNR SERS device, we first need to devise a simple and straightforward way of implementing the AgNR substrates into a functioning electrocell. However, in addition to construction of the device, we must also evaluate the feasibility of AgNRs to be used for E-field assisted SERS detection. Because SERS is highly dependent upon the delicate nanoscale morphology of the substrate surface, one must be careful that the application of a voltage does not induce destructive electrochemical processes on the AgNR surface. Therefore, we must determine a means to properly apply an E-field in the electrocell without compromising the integrity of the AgNRs.

5.2.1 Using a Multi-Well Array SERS Chip as Electrocell

Recall from Section 2.2 that the SERS chip has a uniform AgNR film across the entire surface of a $1 \times 3 \text{ in}^2$ glass microscope slide. In addition, a 200 nm Ag thin film is first deposited as a continuous underlayer prior to the deposition of the AgNRs. This underlying film is incorporated because it provides the AgNR substrate greater SERS sensitivity [60, 119]. However, this continuous film also instills the entire AgNR substrate with good electrical conductivity. Thus, an electrical potential can be applied to all wells by connecting any other

part of the AgNR film (i.e. along the edge of the substrate) to a voltage source. Therefore the AgNR film at the bottom of each well can be utilized as a working electrode (WE).

In addition to using the AgNRs as the WE, a counter electrode (CE) must be included. Because the PDMS is electrically insulating, conductive elements can be placed directly on top of the PDMS. One way to incorporate a CE is to fabricate an array of metal electrodes (e.g. planar microelectrodes) onto a glass microscope slide that can then be attached to the top side of the PDMS layer to form the top surface of the cell. However, fabrication of an array of individual microelectrodes requires extra processing steps, and the metal electrodes may impede signal acquisition if they interfere with the optical path between the AgNRs and the excitation/collection optics. Additionally, generation of a uniform E-field within the electrocell may be complicated due to the intrinsic E-field gradient arising from non-symmetrical WE and CE configuration. One way to circumvent these issues is to use a pre-fabricated ITO-coated glass microscope slide. In this case, a continuous, conductive, transparent ITO film is present on one of the surfaces of the microscope slide; attaching the ITO side of the slide onto the PDMS film allows for a uniform and conductive surface to contact the sample solution inside the well. Figure 5.1A shows a diagram of a single electrocell from the SERS chip/ITO assembly. This configuration is essentially a parallel-plate capacitor, which can provide a more uniform E-field gradient within the cell. Because the ITO-coated glass is transparent, it is also capable of acting as an optical window through which *in situ* measurements can be obtained.

5.2.2 Preliminary Results of *In Situ* SERS Measurements of BPE with External E-field Control in Pure Water

The AgNR electrocell described in the previous section was evaluated using BPE as a test analyte. PDMS-patterned SERS chips were prepared as described previously in Section 2.2. ITO-coated glass slides were purchased from Delta Technologies, Inc. and cleaned in an ultrasonication bath of IPA and/or acetone for ~10 minutes. BPE was prepared in de-ionized water at concentrations from 10^{-5} M; for any given experiment 11 μ L of BPE solution was applied to a well and the ITO-glass coverslip was attached. This sample volume allowed for most of the 12.6 μ L well to be filled and assured adequate liquid contact with both the AgNR and the ITO films. The AgNR film was then connected to an Agilent 20210A Arbitrary Waveform Generator; the ITO film was then connected to ground. The DC-offset function on the waveform generator was used to supply a constant DC potential to the AgNR film.

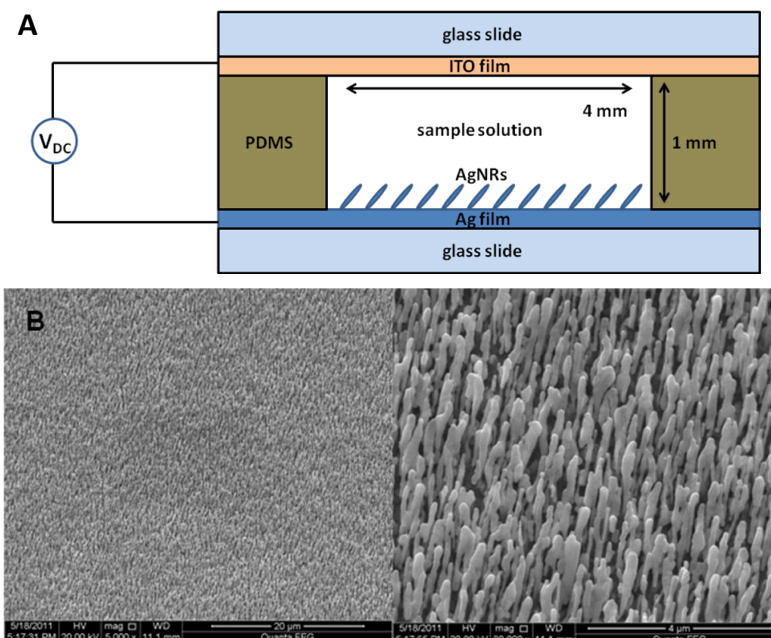


Figure 5.1. A) Schematic diagram showing a single cell of the SERS chip electrocell. B) Low and high magnification SEM images of AgNRs after treatment with $V = -2.8$ V in DI water with 10^{-5} M BPE. Both SEM images are from the same cell.

The primary purpose of the ITO-SERS chip electrocell experiments was i) evaluate the stability of the AgNRs and ITO electrodes when applying a DC potential, and ii) determine how the SERS sensitivity and detection time of BPE could be improved via application of a DC E-field.

Preliminary tests showed that the AgNR film and the ITO film undergo oxidation and reduction reactions, respectively. When V was positive, i.e. when the ITO is the cathode, the ITO film went from transparent to a dark grayish-brown color, further increasing the positive voltage applied to the AgNRs (i.e. anode) would lead to discoloration of the AgNRs to a brownish-black color. A reference electrode was not used in these experiments, nor were electrolytes, but we know from the literature that Ag undergoes oxidation/dissolution beginning at $\sim +0.2$ V (vs. Ag/AgCl) in a standard electrochemical cell [120-122]. However, a rather large negative voltage could be applied to the cell without any noticeable degradation of the ITO (anode) or Ag film (cathode). Figure 5.1B shows SEM images of an AgNR film from a cell treated with $V = -2.8$ V. From these images we can see that the AgNR film remains intact without any obvious degradation. More negative than ~ -5 V, however, bubbles can be seen in the cell. This is likely due to electrolysis of water, i.e. formation of H_2 and O_2 at the AgNR or ITO electrode, respectively.

With the above observations, we restrain our applied voltage to $0 \leq V \leq -4$ V to determine how the *in situ* SERS signal of BPE changed as a function of both the magnitude and duration of a stepped voltage V_{step} . (Note: Throughout this chapter, when referring to an applied negative voltage V , the $<$, $\leq$, $>$, $\geq$ signs are in context to the absolute value of V ; therefore, $V > -1V$ implies that the voltage is more negative than -1 V). The SERS signal was measured

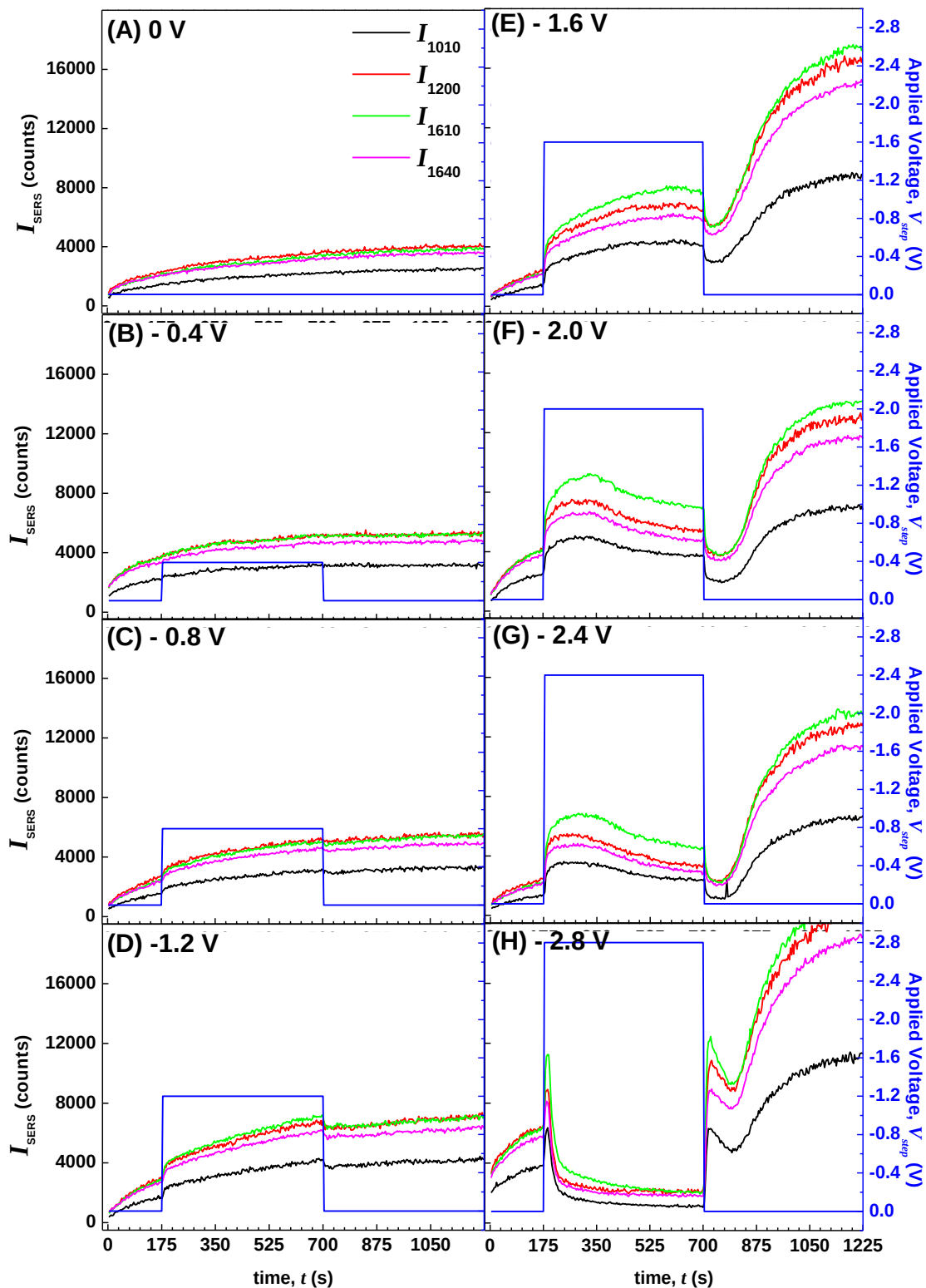


Figure 5.2. I_{SERS} of four different peaks (1010, 1200, 1610, and 1640 cm^{-1}) vs time t and with different applied step voltage V_{step} . The I_{SERS} is scaled by the left y-axis, and the V_{step} (blue) is scaled by the right y-axis. Each panel represents a different cell treated to a different V_{step} .

continuously from a single point at the center of AgNR film at the bottom of the cell. During V_{step} , however, one must pay attention to possible electrochemical processes that induce hysteresis. For example, previous SERS studies speculated the SERS-active sites have a tendency to irreversibly degrade, i.e. ‘reduce’, when potentials (e.g., vs. Ag/AgCl) become more negative than ~ -1 V [123, 124]. Yet other reports demonstrate that a large negative voltage ($V_{step} \geq -1.2$ V (vs. Ag/AgCl)) is required to desorb analytes such as pyridine or adenosine) [125, 126], but did not cause permanent degradation. Regardless, as a conservative approach, each V_{step} experiment was performed in an unused cell.

To quantitatively analyze the results, the baseline peak intensity of several of the major BPE peaks (1010, 1200, 1610, and 1640 cm^{-1} ; see Section 2.2 for assignments) are quantified by fitting Gaussian and/or Lorentzian functions. The peak positions are allowed to float during the fitting, therefore the peak intensity I_{SERS} is representative of the peak center, even if the peak position moves. I_{SERS} is plotted with respect to the measurement time t and overlaid with V versus t . (Note that each measurement ‘cycle’ lasts ~ 3.5 seconds; therefore, the experimental time scale is $t = 3.5 * N$).

The SERS response of the AgNRs is continuously measured at a single location prior to applying V_{step} . This allows BPE in the solution to diffuse and adsorb to the surface, and also allows any laser-induced changes of the I_{SERS} to stabilize and equilibrate. Figure 5.2A shows how the SERS signal of the four primary BPE peaks near 1010, 1200, 1610, and 1640 cm^{-1} (black, red, green, and pink, respectively) increase over time when $V_{step} = 0\text{V}$ (V_0). In that figure, the I_{SERS} is scaled by the left y-axis, and the applied voltage magnitude is scaled by the right y-axis (but here is just a horizontal line at $V_{step} = V_0$). Under these passive conditions, a uniform,

monotonic increase is observed for all four peaks, and they stabilize and achieve a maximum value when $t > 500$ s.

Figure 5.2B shows a similar plot using a different cell. After an initial equilibration for $t = 175$ s at V_0 the voltage is immediately stepped to $V_{step} = -0.4$ V and held for several minutes while continuously acquiring the SERS signal. From this figure we can see that the I_{SERS} remains unperturbed when V_{step} is applied. The increase in I_{SERS} for all peaks maintains the same slow steady increase without interruption by V_{step} . Upon reduction of V_{step} back to 0 V when $t = 700$ s, I_{SERS} still remains unperturbed.

However, when $V_{step} = -0.8$ V (Figure 5.2C) a slight increase in I_{SERS} occurs over a few measurements immediately after applying V_{step} . This slight increase is the same for all peaks. A slight decrease in I_{SERS} can be seen when V_{step} changes back to V_0 . This same trend is observed for $V_{step} = -1.2$ V applied to another cell, except that there is a notably greater increase/decrease in I_{SERS} when applying/removing V_{step} (Figure 5.2D).

When V_{step} becomes still more negative, the SERS response changes more drastically and demonstrates some rather peculiar behavior. For example, when $V_{step} \geq 1.6$ V (Figure 5.2E), the I_{SERS} of all the peaks rapidly increases ~ 2 -fold. However, I_{SERS} then begins to level off and begins to decrease slightly during V_{step} . When stepped back to V_0 , I_{SERS} very rapidly decreases by ~ 25 -50% (within a few measurements) then continues to decrease slowly over the next ten or so measurements. Interestingly, while still at 0V, the I_{SERS} begins to increase significantly at $t \approx 800$ s before leveling off at an intensity 2- to 3-fold greater than that observed for the smaller V_{step} treatments. This same general behavior is observed at more negative voltages ($V_{step} = -2.0$ or -2.4 V, see Figures 5.2F and 5.2G, respectively). As V_{step} becomes more negative, however, the I_{SERS} demonstrates a noticeable decreasing phase after the initial increase immediately following

the voltage application. When $V_{step} = -2.8$ V the I_{SERS} very rapidly increases ~ 2 -fold (within ~ 10 s) immediately after applying V_{step} , which is similar to the lower V_{step} treatments. However, rather than demonstrating a subsequent slow increase followed by slow decay, the I_{SERS} demonstrates a precipitous drop, whereby the intensity decreases over ~ 30 s to a value lower than that prior to the voltage treatment. The intensity continues to slowly decrease over the next 400 seconds until the BPE signal is barely detected. Interestingly, when V_{step} changes to V_0 , the I_{SERS} immediately jumps back to a value ~ 2 -fold higher than the intensity prior to applying V_{step} . As with Figures 5.2E-G, there is a small dip in I_{SERS} before a large surge in intensity starting at $t \approx 800$ s.

We should also note that many of these V_{step} experiments were repeated on different days and using different SERS chips. The same basic behavior described above is generally observed for the replicate experiments, but the I_{SERS} behavior did show some significant variation between experiments. For example, when $V_{step} \geq -2.0$ V, the slow decay following the initial increase in I_{SERS} happened sooner and/or at a faster rate than that shown in Figure 5.2. Other replicate experiments showed I_{SERS} does not produce a surge in intensity after stepping back down to V_0 , rather, the I_{SERS} decreases after removing voltage and then remains stable for the remaining measurements (see Figure 5.3A for example). This experimental variation could be due to organic contaminants adsorbed to either the AgNRs or ITO prior to starting the experiments.

5.2.3 *In Situ* SERS V_{step} Measurements Using Electrolyte

The previous experiments discussed the SERS response of AgNRs during application of V_{step} when BPE is present at a relatively low concentration (1×10^{-5} M) and dissolved in DI water, i.e. without electrolytes and therefore with a very high solution resistance R (resistivity of DI

water is $\sim 5 \text{ M}\Omega\text{-cm}$). Under these conditions, the AgNR/ITO electrodes can be thought of as *polarizable*, i.e. a potential can be maintained at the electrode without significant loss due to current i . However, R can greatly influence E-field effects. For one, electrolytes are mobile charge carriers and will greatly reduce R , permitting i to flow between the working and counter electrodes. As i increases through the electrode, the potential E of the electrode will decrease, and therefore the E-field between the electrodes will decrease. In addition, electrolytes will also screen charged surfaces, such as the electrode, via the formation of an electric double layer (EDL) [127, 128]. Thus, the E-field ‘felt’ by the sample solution will be greatly attenuated. Electrolytes are therefore generally undesirable when a large E-field and low current is needed in the sample solution.

Another experiment was performed identical to that of Figure 5.2F (i.e. $V_{step} = -2.0 \text{ V}$ in DI water) and the results are shown in Figure 5.3A. We note that the experiments from Figure 5.2F and 5.3A are identical but there is some differing I_{SERS} behavior between these two experiments. For example, in Figure 5.3A, we do not see the large intensity surge after stepping the voltage back down to 0 V. To test the effect of electrolytes for the *in situ* AgNR SERS analysis, the same experiment is repeated using a new cell with BPE dissolved in 100 mM HNa_2PO_4 pH 7.4 buffer. Figure 5.3B shows that when the electrolyte is included we see somewhat different kinetic behavior. For example, I_{SERS} increases instantaneously (i.e. within about one measurement) upon application of V_{step} and maintains a very stable value for the entire V_{step} treatment. However, I_{SERS} in the electrolyte only achieves about half of that for the corresponding DI water sample. Stepping back to 0V, I_{SERS} shows a slight yet instantaneous increase, and then behaves almost identically to the DI water sample.

From Figure 5.3B we can see that the SERS signal demonstrates an apparent-instantaneous change when V_{step} is applied (or removed). The observed change in I_{SERS} appears to modulate exactly with the application of V_{step} . On the other hand, the DI water samples have consistently demonstrated a slower, lagging response.

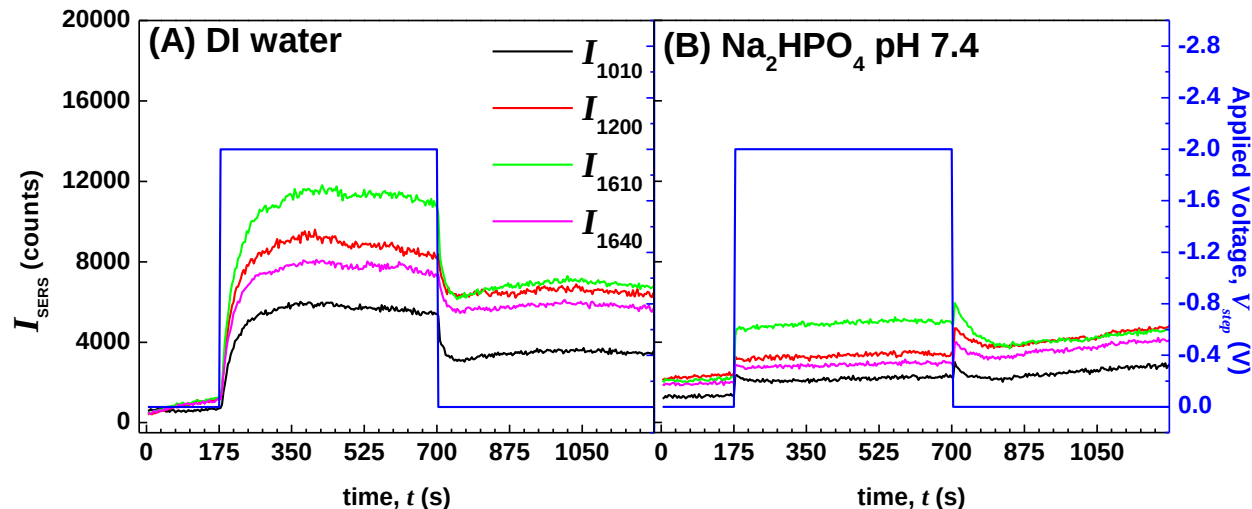


Figure 5.3. I_{SERS} of four different peaks (1010, 1200, 1610, and 1640 cm^{-1}) vs time t and with $V_{step} = -2.0$ V for BPE dissolved A) DI water and B) Na_2HPO_4 . The I_{SERS} is scaled by the left y-axis, and the V_{step} is scaled by right y-axis.

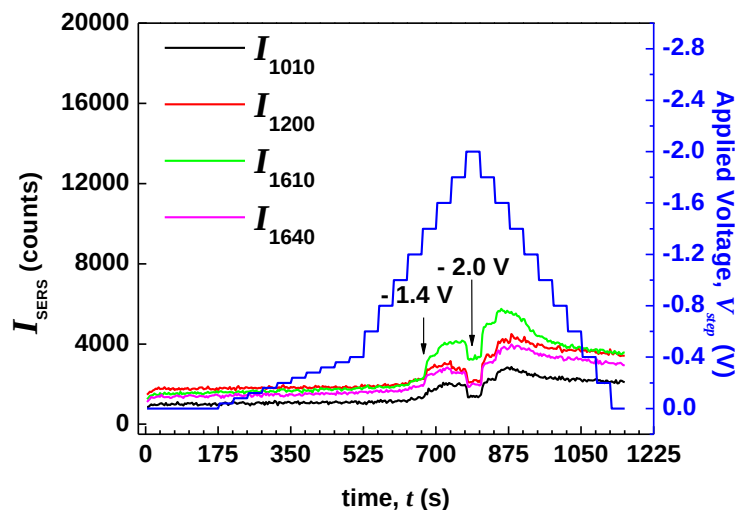


Figure 5.4. I_{SERS} of four different peaks (1010, 1200, 1610, and 1640 cm^{-1}) vs time with incremental increases of V_{step} for BPE dissolved Na_2HPO_4 . The I_{SERS} is scaled by the left y-axis, and the V_{step} is scaled by right y-axis.

To further illustrate the electrolyte effect, a second set of experiments were performed. The I_{SERS} of BPE in phosphate buffer was measured by incrementally increasing V from 0 V to -2.0 V, and then back to 0 V. In Figure 5.4, the I_{SERS} shows no modulation when V is ≤ -0.8 V. At -1.2 V we can see a very subtle increase of I_{SERS} . However, at -1.4 V, a drastic increase in I_{SERS} is observed for all peaks. The intensity of all peaks continues to increase at -1.6 V. At -1.8 V, the I_{1010} and I_{1610} remain unchanged, but the I_{1200} and I_{1640} decrease slightly. Upon further increase to -2.0 V, all the peaks decrease drastically. This same basic behavior (in reverse) is observed as the applied voltage is incrementally dropped back down to 0 V. Hence we can see that the highest I_{SERS} is achieved at -1.6 to -1.8 V, while the lowest I_{SERS} is at -2.0 V.

Based on the observations from Figure 5.4, another cell containing BPE and electrolyte was treated to short alternating V_{step} cycles between -1.8 and -2.2 V (as these voltages should allow for a large discrepancy in I_{SERS}). In Figure 5.5A, the I_{SERS} modulates in response to the changing voltage with remarkable fidelity and demonstrates exceptionally reproducible behavior for all 10 cycles. In contrast, with BPE in DI water the I_{SERS} remains completely unresponsive to the modulation of V_{step} between -1.8 and -2.2 V. After the initial increase (stepping from 0 to -1.8 V) no further change in I_{SERS} is detectable until the voltage is stepped back to 0V. In fact, the I_{SERS} profile in Figure 5.5B looks remarkably similar to that from Figure 5.3A.

5.2.4 Experimental Limitations Affecting E-field Assisted SERS Analysis

So far we have observed how an external voltage can greatly improve the detection sensitivity of BPE in a AgNR microcell. We have also seen how the use of an electrolyte

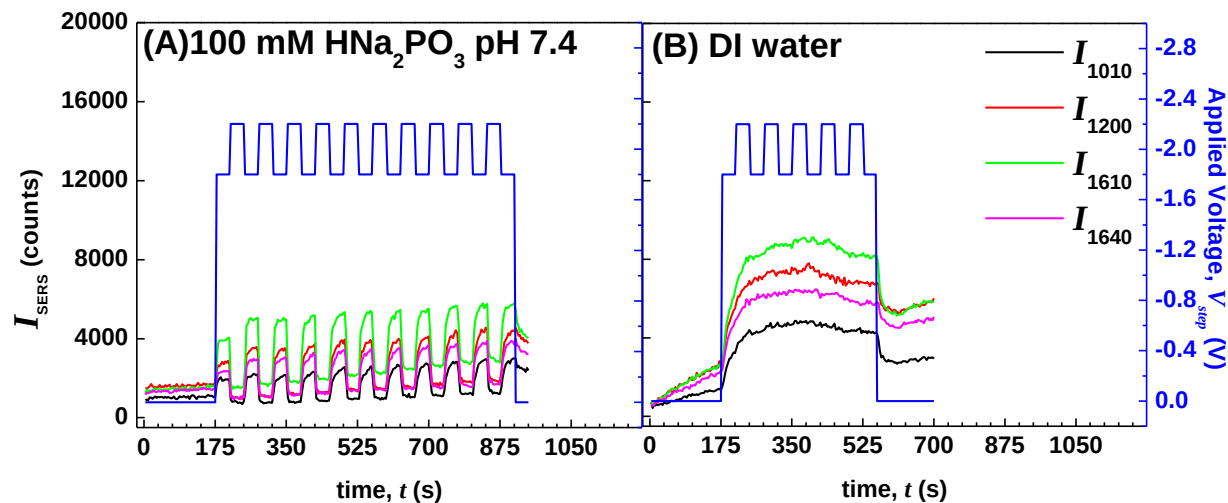


Figure 5.5. I_{SERS} as a function of multiple short voltage cycles between -1.8 and -2.2 V for BPE in A) electrolyte and B) DI water.

induces faster kinetics and more response sensitivity of I_{SERS} with respect to the applied voltage.

Yet, up to this point we have not offered an explanation for the observed phenomena.

Unfortunately, several experimental issues make interpretation of the data difficult. We therefore will outline some of the limitations that preclude an in depth quantitative analysis of the aforementioned preliminary data. After establishing these limitations, we will discuss possible physical causes that may qualitatively explain the data.

For one, no reference electrode was used in these studies. The primary reason is that standard reference electrode (even a miniature version) will not fit into the 12.6 μl cell. To incorporate a micro-reference electrocell, intricate cell designs and microfabrication techniques must be employed [129, 130]. Even if a reference electrode were used, the reported electrode potentials may not be accurate for the samples solutions that did not utilize electrolytes. The cell current was also not measured; therefore we do not know how polarized the AgNR electrode is during the experiment, and consequently the surface potential cannot be accurately ascertained.

Another issue is electrolysis. Although DI water has a very high resistance, sufficient electrode potential can still induce electrolysis of water, but we may expect such a reaction to occur at a rather large overpotential. The AgNRs, i.e. the cathode, were always treated with a negative voltage, where electrolysis is possible according to the following equation:



This reaction will generate a Faradic current. However, if such a reaction occurred during the aforementioned experiments, the reaction was likely slow and corresponding current relatively small as no obvious bubbles were observed during the experiments. Unfortunately, the current through the cell was not measured, so we cannot know for sure if, or to what extent, this reaction was proceeding during the experiment.

On the other hand if electrolysis is indeed occurring, even to a relatively small extent, it will increase the local concentration of OH^- at the surface of the AgNRs, and therefore increase the local pH. Moreover, SERS detection is known to be sensitive to pH [131-135]. This is primarily a result of the protonation/de-protonation of chemical constituents responsible for adsorption to the metal surface. In our case, the lone-pair nitrogens on BPE are the atoms responsible for coordinating with the Ag surface [78], as well as associating with H^+ at low pH; protonated BPE may therefore be less likely to bind to the Ag surface. The pK_a of pyridine is ~ 5.3 ; thus, if the pH is below this value, we may expect that a majority of the BPE molecules to be in a protonated state and therefore less likely to adsorb to the Ag surface. On the other hand, if the pH is above 5.3 we may expect the BPE to be more likely to adsorb as it is primarily de-protonated.

We should note that DI water exposed to the atmosphere has a pH of ~ 5.5 (due to absorbed CO_2) which is close to the pK_a of pyridine (pyridine is the primary constituent of

BPE). Therefore BPE may not be fully de-protonated, restricting its ability to adsorb to the AgNRs. On the other hand, under the electrolysis reaction described by Equation (5.1), the local alkaline pH near the AgNRs would bias the BPE to de-protonate, therefore increase its proclivity to adsorb, and ultimately enhance the resulting SERS signal intensity. Therefore, electrolysis of water near the surface could explain the increase in the I_{SERS} observed when V_{step} is more negative.

However, an applied voltage may still alter the local pH in pure water even if electrolysis of water is not occurring. This is because water molecules naturally dissociate to form H_3O^+ and OH^- , which themselves act as electrolytes, and at pH 7 the concentrations of these species are equal at 1.0×10^{-7} M. One recent study provides simulation results that demonstrate the effect of an external E-field on the local pH of a two-electrode setup [136], similar to the one used here with the AgNRs. In that study, a simulation was performed, where two polarizable metal plate electrodes were separated by 500 μm gap filled with pure water at pH 7.0. Upon applying $V < 1$ V across the plates, an E-field is established in the water layer, by which H_3O^+ is electrokinetically drawn to the negatively charged cathode, and OH^- is drawn to the positively charged anode, thus forming electric double layers at each electrode. The formation of the double layer has two primary effects. First, the ions drawn to the electrode surface screen the E-field from penetrating into the bulk solution. This is a time-dependent process, and when $V \leq 0.4$ V, the process achieves equilibrium within a few seconds. For example, when $V = 0.4$ V, after 3 seconds the E-field magnitude is completely mitigated beyond 3 μm of the anode's or cathode's surface. Secondly, the increased concentration of the ions in the double layer will change the local pH near the electrode. At the surface of the positively charged anode, the pH = 10.5, which decreases to 7.0 as the distance from the electrode increases to 5 μm . Symmetrical behavior was

observed at the negatively charged cathode (which would be analogous to the AgNRs), where the pH at the electrode surface is calculated to be 3.5.

Unfortunately that report does not corroborate the findings with any experimental evidence, however, the findings may lend some insight into our analysis of the AgNRs SERS response during V_{step} . Applying the same logic, we can assume that in pure water and without electrolysis, an applied voltage should induce migration of H_3O^+ ions to the AgNR (cathode) surface. This could increase the local pH substantially, perhaps below the pKa of pyridine. Yet, such an effect would bias the BPE to adopt a protonated form, which may impede adsorption, and in turn *reduce* the measured I_{SERS} . However, this logic does not hold in light of the aforementioned *increase* in I_{SERS} upon application of V_{step} .

Based on the analysis of the pH-effects, there does not appear to be any obvious correlation between the pH and the observed I_{SERS} behavior. Moreover, currently there is no feasible method to directly and accurately measure local pH in the vicinity of the electrode (i.e within microns) during V_{step} . Unfortunately, a quantitative analysis of the pH effect appears to be currently out of reach. Yet, such pH-effects are rarely discussed in the literature regarding SERS analysis at an electrode, and therefore may not be an important factor. The inclusion of the phosphate buffer for some of the experiments also helps assure that the pH remains relatively stable. Ultimately, such a dilemma should not prohibit us from investigating other, more culpable causes for the observed phenomena.

5.3 E-field and Electrical Potential Effects on SERS

Many processes could also potentially affect the observed SERS signal while applying a voltage to a SERS substrate [137]. We will therefore examine the results in context to other

SERS studies that utilized an applied potential and/or external E-field. Although direct, quantitative comparison with findings in the literature may not be possible due to the aforementioned experimental limitations (e.g. a lack of a reference electrode), we may still ascertain critical insight about the nature of relevant physical processes that may affect the SERS behavior during our experiments.

Because SERS substrates are traditionally composed of Ag or Au surfaces, these substrates can be readily implemented as polarizable electrodes for electric potential/field-based SERS analysis. Many of the fundamental studies regarding this type of analysis have been carried out in ‘spectroelectrochemical cells’, which is simply a standard 3-electrode electrochemical cell modified to provide a transparent optical path (e.g. an optical window) through which *in situ* SERS measurements are obtained [137]. The working electrode is usually a sheathed polycrystalline Ag rod that is mechanically polished and cleaned before introduction to the electrocell. (Although Au, Ag, and even Cu electrodes with various crystal faces are frequently used, when possible our discussion will only focus on polycrystalline Ag electrodes as they are most relevant to the AgNR substrates on which this dissertation focuses). Prior to SERS analysis, the polished electrode is roughened via oxidation-reduction cycling (ORC) so as to provide a surface capable of supporting SERS. Reported potentials are typically given in reference to a Ag/AgCl or saturated calomel electrode (SCE) and the counter electrode is most often made of Pt; the supporting electrolytes are typically Cl^- or ClO_4^- .

5.3.1 Electrokinetic Migration of BPE

Inducing an electrical potential on polarizable electrodes establishes an electric field between the electrodes that propagates throughout the intervening sample solution. This E-field

can induce electromotive forces onto a charged analyte in the sample solution, ultimately drawing or repelling it from the electrode/substrate surface. One study demonstrated that by imparting a slight positive charge onto a Ag substrate (+0.1 V), analytes with a negative charge (e.g. phenol) could be rapidly detected with SERS at rather low concentrations (10 nM). Likewise, positively charged analytes (e.g. aniline) could be detected when the substrate was negatively biased [138]. This observation is described as electrokinetic ‘preconcentration’ of the analyte at the substrate surface, and demonstrates the advantage selective sampling, whereby positively or negatively charged analytes in a mixture could be selectively detected when the substrate was negatively or positively biased, respectively. Another study showed that electrokinetic preconcentration could be used to greatly improve the detection sensitivity [139]. In that report, adenine (induced with a positive charge at pH 7.4) migrated along the E-field within the electrocell to adsorb on the negatively charged gold substrate. The SERS intensity was shown to be proportional to the applied E-field; after 25 minutes of a -0.6 V/cm magnitude E-field (E) the spectral intensity could be improved 51-fold compared to the intensity achieved without an E-field. The large increase in the sensitivity allowed for the detection of very low adenine concentrations of 10 fM. This study also posits a mathematical model to quantify this preconcentration effect, which we will briefly highlight in an attempt to better illustrate the BPE data obtained with the AgNRs. The analyte, e.g. adenine, is assumed to have a positive charge at the given pH; therefore, it is imparted with an electrokinetic mobility μ_{ek} . This parameter (with units of $\text{m}^2 \text{V}^{-1} \text{s}^{-1}$) describes the electromotive velocity u_{ek} of the particle within a E , where $u_{ek} = \mu_{ek} E$. Upon application of E , the analyte is brought *towards* the oppositely charged surface at a rate of u_{ek} . Therefore the electromotive-induced flux is $\mu_{ek}C$, where C is the analyte concentration. However, the development of a concentration gradient (with respect to distance z

from the surface) $\frac{\partial C}{\partial z}$ will result in a diffusive flux *away* from the surface described by $-D\frac{\partial C}{\partial z}$,

where D is the molecular diffusivity of the analyte. Assuming E is constant, after some amount of time these two processes will achieve equilibrium and the net molecular flux J will zero

$$J = D\frac{\partial C}{\partial z} + u_{ek}C = 0 \quad (5.2)$$

The solution to this differential equation produces a concentration gradient as a function of distance z from the surface ,

$$C(z) = C_i \frac{\gamma}{1 - e^{-\gamma}} e^{-\frac{\gamma}{d}z} \quad (5.3)$$

where $\gamma = \frac{\mu_{ek}}{D}V$, C_i is the initial concentration at the surface prior to preconcentration, and d is a

distance from the surface. Thus, $\frac{\gamma}{1 - e^{-\gamma}}$ describes the ‘preconcentration factor’ (PF), which is

a ratio of the number of analyte molecules at the surface with and without the E-field. From this ratio, we can conclude that as V is increased, PF will increase. Because I_{SERS} is proportional to the concentration of analytes at/on the surface of the substrate, a larger V will yield a larger I_{SERS} .

A cursory examination of this theory appears to describe the voltage-dependent results acquired in Figure 5.2, i.e. as V_{step} increases, I_{SERS} likewise increases, at least when $V_{step} \leq -2.0V$. More negative than ~ -2.0 V and the I_{SERS} begins to decrease. Equation (5.3), however, only predicts a monotonic, unlimited increase in PF as V_{step} increases. This theory also assumes an electrokinetic mobility μ_{ek} for the analyte, i.e., the analyte molecules have a charge opposite that of the substrate. However, we have assumed that BPE is likely neutral because the $pK_a = 5.3$ for pyridine, which is less than the pH of DI water or phosphate buffer, i.e. ~ 5.5 and 7.4 ,

respectively, and therefore BPE is primarily de-protonated under these conditions. Under this preliminary assumption, we may anticipate that preconcentration is not possible, or the effect is negligible.

Yet we should also point out that the pK_a determines when half of the BPE molecules are de-protonated and half are protonated. Thus, a significant number of BPE molecules may still be protonated when the pH is slightly above that of the pK_a , such as the situation with DI water. The relationship between pK_a , pH, and concentrations of protonated and de-protonated BPE is described by the well-known Henderson-Hasselbalch equation

$$pH = pK_a + \log \frac{[BPE]}{[H \cdot BPE]} \quad (5.4)$$

where [] represent the molar concentrations of the protonated and de-protonated forms of BPE. According to Equation (5.4), when the pH is 5.5, such as that with DI water, approximately 40% of the BPE is still protonated (and therefore positively charged). In that case, electrokinetic pre-concentration of BPE may definitely be possible under those conditions. However, when the pH is 7.4, such as that for phosphate buffer, only ~1% of BPE is protonated. In this latter, case, we may expect electrokinetic pre-concentration to have significantly less effect on the PF of BPE. Although the above theory supports the possibility that such an effect is due to electrokinetic preconcentration, further experiments will need to be performed to corroborate this possibility.

5.3.2 Potential of Zero Charge (PZC)

The potential of zero charge (PZC) may be one possible explanation for the increase of I_{SERS} observed when $V_{step} \geq -1.4$ V. The PZC is the potential applied to the working electrode/substrate that yields zero charge on the electrode surface. Such a potential can be determined by varying the electrode potential during differential capacitance measurements; the

potential that yields the capacitance minimum is the PZC [128]. The PZC is dependent on the specific electrolyte species present in the sample solution, in addition to the microscopic surface structure/crystallinity. Ag electrodes typically demonstrate a PZC of -0.7 to -1.0 V (vs. Ag/AgCl) in the presence of various electrolyte. Likewise, neutral molecules, such as de-protonated BPE, will demonstrate their highest adsorption efficiency at the PZC, which is where we may expect to find the highest SERS intensity. For example, an applied potential of -0.6V (vs. SCE), which is very close to the PZC, has been used to quickly generate a strong SERS signal of pyridine (in KCl buffer) [126].

For measurements obtained in pure water (Figures 5.2 and 5.3A), the PZC may not exist, or it may exist at a much different potential than the PZC measured in electrolyte solution. The literature is not explicit on how pure water affects the PZC. Therefore, we are hesitant to assume the PZC is responsible for the observed increase in I_{SERS} during application of V_{step} in the DI water samples. On the other hand, the PZC of polycrystalline Ag electrodes in phosphate buffer has not been explicitly determined. Therefore, we will assume that the PZC for AgNRs in our experiment is similar to that obtained for a Ag electrode in Cl^- solution. However, because we have not used a reference electrode, we cannot draw a direct, quantitative comparison anyways.

Nevertheless, we may use the PZC concept in an effort to explain the SERS data acquired with the AgNRs in phosphate buffer. For the AgNRs, as the potential is made more negative and approaches the PZC, we would expect to find that the I_{SERS} increases, until it maximizes when the PZC is achieved. Further increase of the AgNRs' negative potential would then lead to a decrease in I_{SERS} of BPE, as cations in the solution then out-compete BPE for adsorption to the negatively charged AgNRs. In fact, from Figure 5.4, this is exactly what is observed. The I_{SERS} slowly increases starting from $V_{step} = -1.2$ V and reaches a maximum I_{SERS} when $V_{step} \approx -1.6$ V,

increasing to -2.0 V leads to a drastic decrease in I_{SERS} ; as might be expected with a PZC-based process, this behavior is more-or-less ‘symmetrical’ about $V_{step} = -2.0V$, i.e. incrementally decreasing V_{step} lower than -1.6 V causes a concomitant drop in I_{SERS} (the right hand side of Figure 5.4). In addition, the controlled modulation seen in Figure 5.5A is suggestive of PZC effects, i.e. at $V_{step} = -1.8V$, I_{SERS} is large due to higher adsorption of neutral BPE (because charge is minimal on the metal surface). Yet, increasing V_{step} to -2.2 V mitigates I_{SERS} as the negatively charged surface leads to desorption of BPE, and favors physisorption of Na^+ cations. From Figure 5.5B, however, the presence of DI water appears to negate this PZC effect. The study mentioned previously [126] shows a large increase in I_{SERS} at the PZC (-0.6 V vs. Ag/AgCl), but also demonstrates a drastic drop in I_{SERS} when the applied potential was made more negative to -1.2 V (vs. Ag/AgCl). This appears to concur with our results, at least qualitatively.

5.3.3 Manipulation of Analyte Orientation, Bonding Polarization, and Charge-Transfer

The previous section discussed how the applied electrical potential and E-field may induce selective transport, adsorption, and desorption of BPE, which may be used to explain the changing I_{SERS} observed as the applied voltage is modulated. Yet, the SERS signal is more than a simple quantitative measure of analyte number adsorbed on the surface. The SERS signal is also sensitive to the orientation and configuration of the adsorbed analyte due to the surface selection rules we already discussed. The spectral response is also dependent upon polarization of the molecular bonds in the analyte, as well as the charge transfer interaction between the adsorbed analyte and the metal surface [137]. All of these factors can be modulated when a

potential is applied to the metal surface. We must therefore be mindful of these effects before we can ascertain an accurate explanation for the observed behavior.

5.3.3.1 Analyte Orientation and Surface Selection Rules

Applying a voltage to the AgNRs may induce reorientation of the adsorbed BPE [123, 140]. Such a molecular rearrangement will greatly affect the resulting SERS signal in a non-trivial manner. This is due to the ‘surface selection rules’, which states that vibrational modes containing a polarizability tensor component perpendicular to the metal surface will be preferentially enhanced, while the components parallel to the surface will not be enhanced [100, 101, 141]. Therefore a change in molecular orientation relative to the metal surface will result primarily in a change in the relative peak intensities.

Indeed, closer examination of Figure 5.2 reveals that the intensities of the four major BPE bands do not respond uniformly to an applied voltage. Distinctly different trends are observed for I_{1010} , I_{1200} , I_{1610} , and I_{1640} at low (-0.8 V), intermediate (-2.0V), and high (-2.8V) voltages. These trends are highlighted in Figure 5.6, as the peak ratios relative to I_{1200} (i.e. I_{1010}/I_{1200} , I_{1610}/I_{1200} , and I_{1640}/I_{1200}) are plotted. This figure uses the same data from Figure 5.2.

Comparing Figure 5.2 and Figure 5.6, the modulation behavior of the relative peak ratios echoes much of the modulation behavior observed for the peak intensities. For example, from Figure 5.6A, we see that when $V_{step} \leq -0.8$ V the peak ratios remain unchanged upon application of V_{step} , or upon stepping back to V_0 (Figure 5.5A); likewise, very little modulation is seen in the corresponding I_{SERS} plot of Figure 5.2C. On the other hand, as V_{step} becomes more negative (e.g. -2.0 V), the I_{1610}/I_{1200} and I_{1010}/I_{1200} ratios become larger, while I_{1640}/I_{1200} remains unchanged (Figure 5.6B). From this figure, which corresponds to the data in Figure 5.2F, we can see that

the peak ratios demonstrate a rapid change (over ~ 10 s, which is also observed with the I_{SERS} in Figure 5.2) upon application of V_{step} . Likewise, a very rapid change is also observed after returning to V_0 . Yet some key differences between the modulated behavior of the peak intensities and peak ratios are also evident. First, after V_{step} is initially applied, the peak ratios only show a change in a single direction, i.e. they do not reflect the slow decaying phase eventually observed in the corresponding I_{SERS} plot of Figure 5.2F. Moreover, the large surge (i.e. when $t > 800$ s) in I_{SERS} that occurs for all four peaks after returning to V_0 does not manifest as any obvious change in the peak intensity ratios in Figure 5.6B. *This suggests that the increase in I_{SERS} during this intensity surge is only due to increased analyte adsorption, and not due to analyte re-orientation.* That is, more BPE apparently adsorbs to the AgNRs *after* the V_{step} has been applied. At still larger negative voltages (e.g. -2.8 V; Figure 5.6C) the I_{1640}/I_{1200} continues to remain unchanged, while the other two peak ratios demonstrate an initial rapid change similar to that observed at -2.0 V. However, rather than continuing to increase slowly (as they did for the -2.0 V treatment), these two peak ratios begin to steadily decrease after a few moments, which is very reminiscent behavior observed for the corresponding I_{SERS} behavior in Figure 5.2H. Reducing the V_{step} back to 0 V and these peak ratios modulate rapidly, which also strongly echoes the trend observed for the corresponding I_{SERS} data from Figure 5.2H.

Tian et al. has previously investigated the analyte reorientation of the analyte thiourea (TU) in ClO_4^- adsorbed on a polarized Ag electrode [123]. TU demonstrated a high I_{SERS} at -0.9 V (vs Ag/AgCl); upon stepping the voltage down to -0.3 V the I_{SERS} of all TU peaks show a decrease in intensity over time. Some of these peaks demonstrated a rapid change in intensity (within ~ 1 s) before I_{SERS} stabilized. Meanwhile, other peaks decreased rapidly initially but then showed a much slower decay over the next several minutes. Furthermore, regardless of the decay

profile observed after decreasing the voltage to -0.3 V, upon stepping the potential back to -0.9 V, all of peaks demonstrated a very rapid increase back to the initial I_{SERS} observed prior to the voltage step.

The authors explain that electronic polarization and charge transfer effects (see next section) have extremely small time scales, and their corresponding SERS effects are expected to manifest ‘instantaneously’. However, molecular reorientation is anticipated to be a much slower process, which can potentially be monitored within the time resolution used in the study (0.5s). Thus, the authors infer that the observed time-dependent I_{SERS} behavior is a result of the

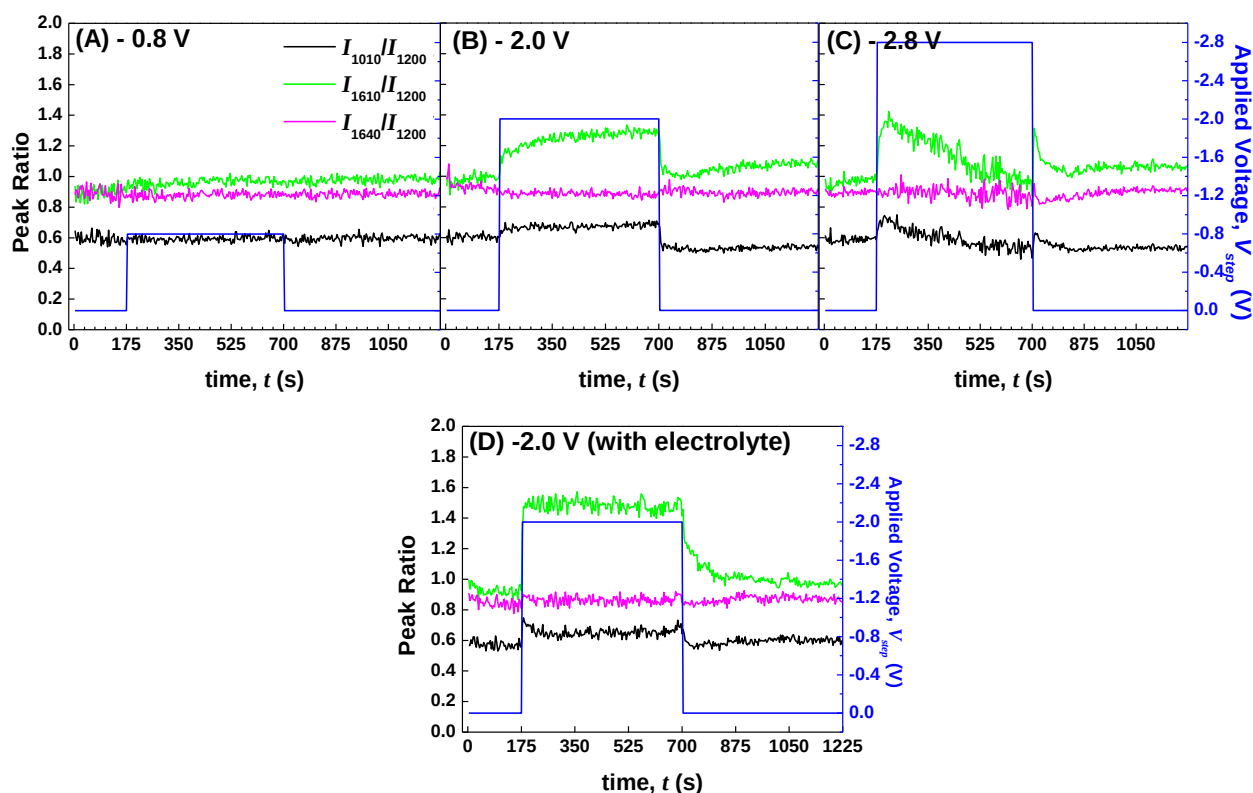


Figure 5.6. Ratios of I_{1010} , I_{1610} , and I_{1640} relative to I_{1200} for V_{step} equal to A) -0.8 V, B) -2.0 V, and C) -2.8V using the same data from Figure 5.2 C), F), and H), respectively, and D) with electrolyte using the same data from Figure 5.3B. The peak ratio is scaled by the left y-axis, and V_{step} is scaled by right y-axis.

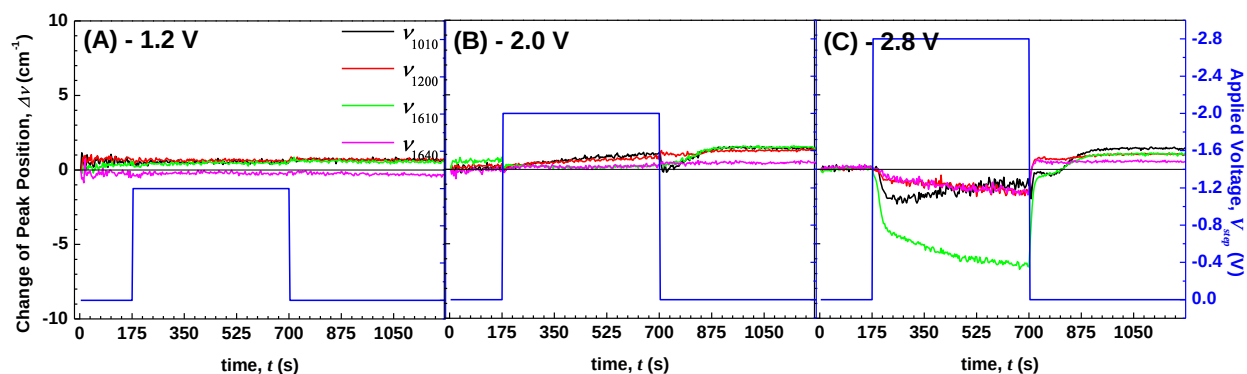


Figure 5.7. Peak position shifts ($\Delta\nu$) of ν_{1010} , ν_{1200} , ν_{1610} , and ν_{1640} for V_{step} equal to A) -1.2 V, B) -2.0 V, and C) -2.8V acquired from the same spectral data from Figure 5.2 C), F), and H), respectively. The change in peak position is scaled by the left y-axis, and the V_{step} is scaled by right y-axis.

adsorbed analyte undergoing a rearrangement on the polarized electrode surface. Moreover, the fact that different rates of change are observed for different peaks suggests that the adsorbates are not behaving as rigid bodies. The peaks that respond rapidly to the decreased potential correspond to the modes associated with the coordinated sulfur atom (i.e. bonds close to the metal surface). In contrast, the peaks that show a slower decay correspond to modes associated with the amino groups (i.e. further from the surface) that associate more with the surrounding solution. A change of orientation for the amino groups may therefore require the re-structuring of the surrounding solvent and electrolyte molecules.

Although the study by Tian et al. does not directly monitor peak ratios, we can compare the findings in that study to those of BPE in Figures 5.2 and 5.3. For the BPE in DI water, we can see behavior that is reminiscent of that for amine peaks of TU in ClO_4^- buffer, i.e. a rapid change in intensity followed by a slower change after applying V_{step} . Likewise, upon removing the potential step (returning to V_0), the I_{SERS} changes very rapidly, regardless of how fast the initial change was during V_{step} , which is what Tian et al. observed when changing the potential from -0.3 to -0.7 V. On the other hand, for BPE in phosphate buffer (Figure 5.3B) we see a

very rapid change in I_{SERS} during the voltage step for all four of the measured peaks. Based on the aforementioned logic proposed by Tian et al, the BPE reorientation in DI water may be slower during V_{step} , compared to that of the BPE in phosphate buffer, which may reorient much faster when a potential is applied, and hence the much faster spectral modulation. From Figure 5.3 we can also see that the I_{SERS} of all four peaks are lower when phosphate buffer is used. However, the I_{1010}/I_{1200} and I_{1610}/I_{1200} ratios achieve higher values and at a faster rate with phosphate buffer compared to the sample in DI water (Figure 5.6D). These observations indicate that the electrolyte aids in the reorientation process. In either case, (in DI water or phosphate buffer), we can see that the 1200 and 1640 cm^{-1} peaks are modulating/increasing together; hence, I_{1640}/I_{1200} remains constant regardless of the applied voltage. On the other hand, the 1010 and 1610 cm^{-1} increase in intensity more than the 1200 and 1640 cm^{-1} peaks during V_{step} . The 1610 cm^{-1} vibrational mode is strongly polarized along the long axis of the BPE molecule [78], and considering the fact that BPE adsorbs in a more vertical orientation relative to the surface, this may explain why this vibrational mode is more sensitive to the applied E-field. Unfortunately, however, a detailed physical interpretation of the BPE reorientation is very difficult, and computational methods such as density functional theory (DFT) will be required to ascertain how the voltage-induced molecular orientation of BPE will affect the SERS signal [95].

5.3.3.2 Bond Polarization

In addition to re-orientation, a charged metal surface will also affect the polarizability derivative of the bonds $\partial\alpha/\partial Q$, where α is the molecular polarizability, and Q represents the atomic position of the atoms of the bond. Changing the polarizability derivative will affect the intrinsic Raman response of each vibrational mode separately. For the TU study mentioned

previously, the derivatives of the bond polarizability have been calculated at different applied potentials, and shown to become larger with increasing negative potential [142]. This is due to an increase in the space within the bonds that the electrons may move. Likewise, the relative intensity of most of the TU peaks increases as the potential is made more negative from -0.4 to -0.9 V.

This polarization can also affect the vibrational frequency and therefore modulate the position of the measured Raman peaks. As an illustrative example, take the case of thiocyanate ($\text{N}\equiv\text{C}-\text{S}$) adsorb to a Ag surface via coordination of the S and Ag atoms. Upon application of a negative potential to the surface, the σ electron donation from the coordinated S atom diminishes and the bond weakens. Because weaker bonds produce vibrational modes with lower ν_{AgS} , we will observe a red shift of the S-Ag bond when a sufficiently negative potential is applied to the Ag surface. Likewise, upon the ‘injection’ of negative charge into the molecule the formation of $^-\text{N}=\text{C}=\text{S}$ will be promoted, and thereby reduce the bond order of the N-C bond, and thus decrease ν_{CN} [124].

To observe if bond polarization is affecting the BPE signal, Figure 5.7 plots the change in the peak positions $\Delta\nu$ from the same data shown in Figure 5.2. When $V_{\text{step}} \leq -1.2$ V, the peak positions of all four bands remain completely unchanged before, during, or after the voltage step (Figure 5.7A). When $V_{\text{step}} = -2.0$ V, only minor changes are observed (i.e. $\Delta\nu < 2 \text{ cm}^{-1}$), and the most notable change is a permanent red shift of all the peaks by $\sim 1 \text{ cm}^{-1}$ after removing the voltage (Figure 5.7B). When V_{step} increases to more negative values of -2.8 V, as shown in Figure 5.7C, all peaks show a rapid blue shift within the first minute or so immediately after applying V_{step} , with $\Delta\nu_{1610}$ being the most drastic. That peak then continues to blue shift over the next several minutes until $\Delta\nu_{1610} \approx 7 \text{ cm}^{-1}$. $\Delta\nu_{1200}$ and $\Delta\nu_{1640}$ respond together (just like I_{1200} and

I_{1640} ; see Figure 5.6) during V_{step} , and show a slow blue shift until $\Delta\nu = -2 \text{ cm}^{-1}$. However, the $\Delta\nu_{1010}$ peak initially blue shifts but then slowly begins red shifting. After removing the applied voltage, all the peaks return to a position very close to that prior to V_{step} ($\Delta\nu \approx 0$)

We should point out that other reports have demonstrated similar behavior for BPE. For example, the ν_{1610} and ν_{1010} peaks of BPE adsorbed to Ag film over nanospheres (AgFON) demonstrate a slight red shift of several cm^{-1} at -0.7 V compared to -0.3 V (vs. Ag/AgCl), yet the study does not specify the cause of these frequency shifts [143]. BPE and pyridine coordinate to the Ag surface in an identical manner (via lone-pair nitrogen of a pyridine ring), thus their polarization behaviors may be expected to be similar, yet a direct comparison of polarization properties between BPE and pyridine may not be completely appropriate.

Form Figure 5.7, we can clearly see that the peak shifts are only very minor when $V_{step} \leq -2.0 \text{ V}$, which suggests that the effect of bond polarization is not significant. Only at larger negative potentials ($V_{step} = -2.8 \text{ V}$) do we see notable shifts in the peak frequencies, which suggestin a larger degree of bond polarization. Although development of a bond polarization model specific for BPE is beyond the scope of this discussion, we have provided here a preliminary assessment of the influence that bond polarization may have on the SERS spectra during voltage treatments.

5.3.3.3 Charge Transfer

One other possible factor is the resonant charge-transfer (CT). The CT effect is believed to be responsible for the chemical enhancement (CHEM) that gives rise to an additional 10^1 - 10^2 SERS enhancement beyond the substrate's intrinsic EM enhancement [14]. However, isolating and quantifying the CHEM factor has been a rather elusive endeavor due to its coupling with the

EM effect. The process of resonant CT between adsorbate and metal during optical excitation is dependent on the Fermi level of the metal as well as the electronic structure/energy levels of the adsorbate [137]. Because an applied potential can change the Fermi level of the metal electrode, the CHEM effects can be augmented or attenuated accordingly. Studies have demonstrated that the CHEM enhancement pyridine can be increased via making the Ag electrode more negative [144]. Moreover, increasing/decreasing the CHEM effect does not necessarily lead to a uniform response in the SERS spectra, as some modes will be more/less enhanced than others. However, to date the literature does not provide any studies that explicitly determine the effects of CHEM enhancement of BPE adsorbed onto a Ag electrode.

5.3.4 Other Effects

Another possibility is that the BPE is being electrochemically reduced, yet the literature does not appear to explicitly discuss this possibility. Pyridine, an analogue of BPE, has been used in many SERS electrocell experiments yet the prospect of electrochemical reduction has not been stated, rather a reduced signal intensity is attributed to desorption of the pyridine from the surface [126].

Drawing on the discussions from Chapter 4, we may also anticipate that a very high surface coverage of BPE could lead to a reduced signal intensity due to the dielectric effect [97], which was observed with MPh. Thus, at a large negative voltages ($V > -2.0\text{V}$), the BPE adsorption rate and surface coverage may in fact be much greater, leading to a decrease in I_{SERS} . To provide a rough approximation of the maximum BPE surface coverage possible, the following assumptions are used 1) the total number of BPE molecules in the sample solution is $11\mu\text{L} \times (1 \times 10^{-5} \text{ M}) = 7 \times 10^{13}$ molecules, 2) assuming that 7×10^{15} BPE molecules can adsorb

per cm^2 of flat Ag surface [98], 3) the geometrical area A of the AgNR film in each cell = $\pi r^2 = 12.6 \text{ mm}^2$ where r is the radius of the well, and 4) conservatively estimating the actual AgNR surface area A_s is ~ 10 -fold greater than the geometrical surface area, $A_s = 10 \cdot A$. Given these assumptions, and assuming that every BPE molecule in the sample solution is adsorbed onto the AgNR surface, we find that only ~ 0.01 monolayers is achieved, which is below the surface coverage expected to yield a dielectrically-induced drop of I_{SERS} [97].

Most of the aforementioned voltage-induced effects result from a change in the behavior and/or properties of the analyte; however, an applied voltage may also cause changes in the optical response (and therefore SERS response) of the AgNRs themselves. Applying a potential will change the charge density within metal nanostructures [137]. A negative potential (with respect to the PZC) will result in higher charge density and therefore a blue shift of the surface plasmon resonance frequency (SPR); likewise, a potential positive of the PZC will result in a red-shift of the SPR. For the experiments presented here, blue-shifting the AgNR optical response may have allowed for better resonant coupling with the 785 nm excitation, which may explain the increased SERS intensity. Additionally, the increased electron density in the conduction band of the metal nanostructures (when applying a negative potential) will have a high polarizability, and therefore produce a large enhancement effect [137]. In either case, these changes in enhancement are expected to result in changes in the overall SERS intensity (such as that seen when $V_{\text{step}} = -0.8\text{V}$), and not relative changes in the peak intensity (such as that seen when $V_{\text{step}} \geq -1.6\text{V}$). Further investigation will be needed before the influence of these effects can be confidently attributed to the results observed in our studies.

5.4 AgNR Lab-on-a-Chip Microfluidic Electrocell Device

So far we have demonstrated that AgNRs could be successfully incorporated into a simple electrocell to improve the SERS detection of BPE by varying the applied potential. However, that device was utilized strictly for proof-of-concept studies. One particular design limitation of the aforementioned SERS-chip electrocell device is that only one cell could be treated at a time. This is because all of the cells are electrically connected, and the individual cells could not be individually addressed. This means that the electrical current through one cell will effect the voltage of all the other cells. In addition, each cell contained a fixed sample solution, whereby additional solutions or reagents could not be feasibly introduced without disassembling the device. Yet, *in situ* detection applications may require multiple processing steps, which makes the aforementioned device cumbersome. This section therefore outlines the design and fabrication of a microfluidic LOC device containing multiple, independently addressable AgNR electrocells.

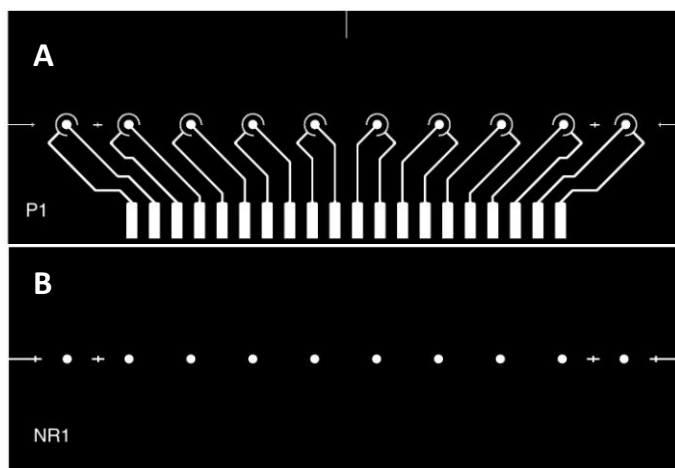


Figure 5.8. 1 in x 3 in electrode mask pattern for used for positive-tone photoresist lift-off. The white portions represent the metal pattern deposited onto the glass microscope slide; A) the mask for the working electrodes, counter electrodes wires, and contact pads; B) the mask for selective deposition of AgNRs onto central working electrode. Each mask is 1 × 3 inches in size.

We should first point out that although OAD-generated films have many technological applications, they do face a few technological challenges. First, the OAD requires a direct line-of-site pathway between the evaporating source and the substrate. This means that the AgNRs must be can only be deposited onto a relatively smooth, flat surface. Additionally, OAD films are rather delicate, and are very susceptible to mechanical damage. AgNRs (especially those used for SERS) are also sensitive to thermal and chemical damage. This means that OAD-generated AgNR films must be processed at relatively low temperature and not in the presence of corrosive chemicals.

To construct AgNR microelectrodes onto a substrate, such as a standard glass microscope slide, the AgNR films must be deposited onto a conductive/metal WE, and a CE must also be incorporated. The design proposed here consists of a 1 mm diameter circular WE that is circumvented by a “C” shaped counter electrode with a 2.5 mm diameter; 200 μm -wide wires run from these electrodes to contact pads positioned at the edge of the glass slide. Ten such electrode configurations are constructed as an array along the length of the microscope slide. Figure 5.8A shows a picture of a mask used to pattern the positive tone photoresist for fabrication of the aforementioned electrode design. Alignment marks are also included to align with the second mask pattern, which will allow selective deposition of AgNRs onto the 1 mm WE electrode.

The general process flow to fabricate this device entails several consecutive lithography/lift-off procedures, which are presented in Figure 5.9. Using this process, the following procedure was performed in a cleanroom. First, the glass microscope slide are cleaned in heated piranha ($\text{H}_2\text{SO}_4\text{:H}_2\text{O}_2$ 4:1), followed by thorough rinsing in ultra pure (UP) 18 $\text{M}\Omega\cdot\text{cm}$

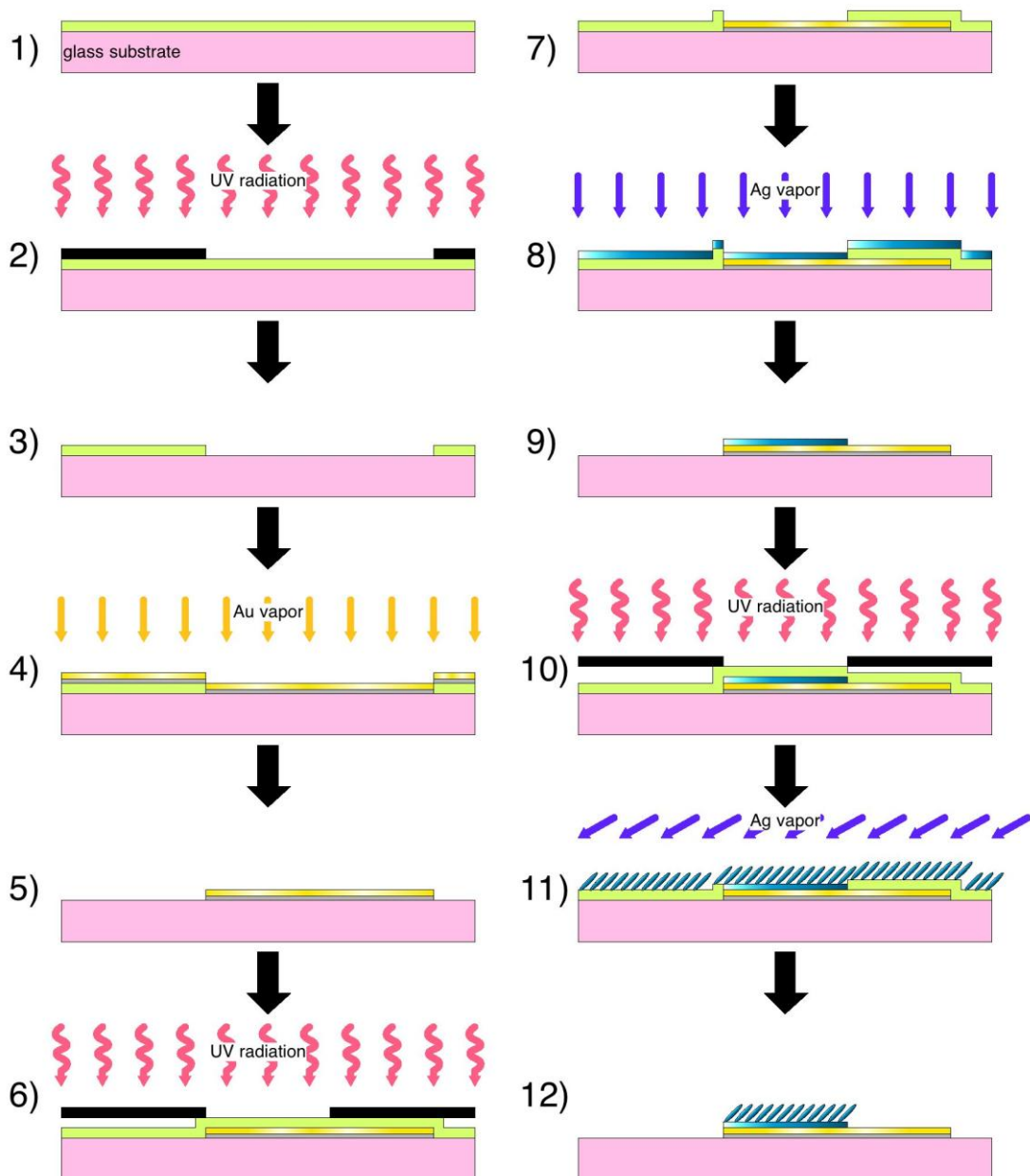


Figure 5.9. Process flow for OAD AgNR deposition onto metal electrodes. See main text for description.

water and blow drying with N_2 . To assure the surface is moisture-free before proceeding, the glass substrate is baked on a hot plate at 100-200° C for ≥ 30 minutes. After cooling the glass slide back to room temperature, Shipley S1313 positive-tone photoresist is spin coated onto the substrate at 5000 RPM for 30 s and then baked for 60 s at 115°C to yield a flat, smooth

photoresist film 1-2 μm thick (Figure 5.9, Step 1). This is followed by 30 s UV exposure using a MA6 (Zeiss) mask aligner and high resolution transparency masks (20,000 dpi, CAD/Art Services Inc.) containing the pattern shown in Figure 5.8A (Figure 5.9, Step 2). After exposure, the substrates are immersed into Microposit MF-321 developer (Shipley) for 60s seconds with sonication to remove the exposed resist. Afterwards, the substrates are rinsed with UP water and blow dried (Figure 5.9, Step 3).

After loading the substrates into the e-beam deposition chamber (with the patterned side directly facing the source material) the chamber is pumped down to 10^{-5} - 10^{-6} Torr before starting the deposition. First, 2-10 nm of Ti is deposited at 1-2 $\text{\AA}/\text{s}$ to act as adhesion layer. Then 50-200 nm of either Pt or Au is deposited at a rate of $\sim 1\text{\AA}/\text{s}$ (Figure 5.9, Step 4). After the deposition the photoresist and metal overlayer are removed with a lift-off process, by immersing the into acetone and placed into a sonication bath briefly for ~ 10 -30 seconds. Then the substrates are thoroughly rinsed with acetone, isopropanol, and water before blow-drying (Figure 5.9, Step 5).

To selectively deposit the AgNRs onto the 1 mm diameter central (working) electrodes, the same basic procedure used to fabricate the electrode arrays is used here with a few minor changes. First, the patterned substrate is not cleaned with piranha, as this will potentially etch the metal electrode. Secondly, after spin coating the S1818 photoresist onto the electrode pattern and baking, a different mask consisting only of 1 mm diameter circles and alignment marks (Figure 5.8B) is used. Using the mask aligner, the alignment marks of this mask are aligned to metal marker deposited onto the glass substrate during the previous steps. This allows the 1 mm diameter circles of the AgNR-patterning mask to be aligned precisely with the 1 mm diameter Au (or Pt) working electrodes. The substrate is then exposed (Figure 5.9, Step 6). After developing, the 1mm diameter Au working electrode is exposed while the rest of the substrate is

coated (Figure 5.9, Step 7). The substrate is placed back into the e-beam deposition chamber where Ag is deposited at a rate of 2.0-4.0 Å/s onto the Au working electrode to a final thickness of 100-200 nm (Figure 5.9, Step 8). The same lift off process from Step 5 is repeated again (Figure 5.9, Step 9). The substrate is again spin coated with photoresist and exposed using the mask in Figure 5.8B (Figure 5.9, Step 10). After developing the substrate and exposing the working electrode, the substrate is loaded into the e-beam chamber, with the surface oriented at 86° relative to the vapor direction. After the chamber pressure reaches 10^{-6} Torr, Ag is deposited at 86° at a rate of 2.0-4.0 Å/s to a final QCM thickness of 1000-2000 nm (Figure 5.9, Step 11).

For lift-off, the AgNR-coated substrates are allowed to incubate in acetone for a prolonged time to allow the photoresist film under the AgNR film to completely dissolve. Sonication cannot be used (as it normally would be for lift off) because this will completely destroy the delicate AgNR film. Therefore, after a prolonged incubation in acetone, the substrate is then treated to a stringent rinsing with acetone, and then alcohol (such as propanol, ethanol, or methanol), using a forceful stream from a squirt bottle. This method is sufficient to remove stubborn pieces of the photoresist or AgNR film not directly deposited onto the metal surface of the 1 mm working electrode, yet the method is gentle enough so as to not mechanically degrade the AgNR film. After a final rinse in methanol, the substrate is blow dried and ready to be used for subsequent processing (Figure 5.9, Step 12). Figure 5.10 shows SEM images of the AgNR microelectrodes and Pt counterelectrode.

After the planar array of AgNR electrodes have been fabricated, microfluidic channels/cells can be incorporated to complete the device. The microfluidic channels are fabricated by patterning PDMS with a photolithography-generated SU-8 mold [76]. This

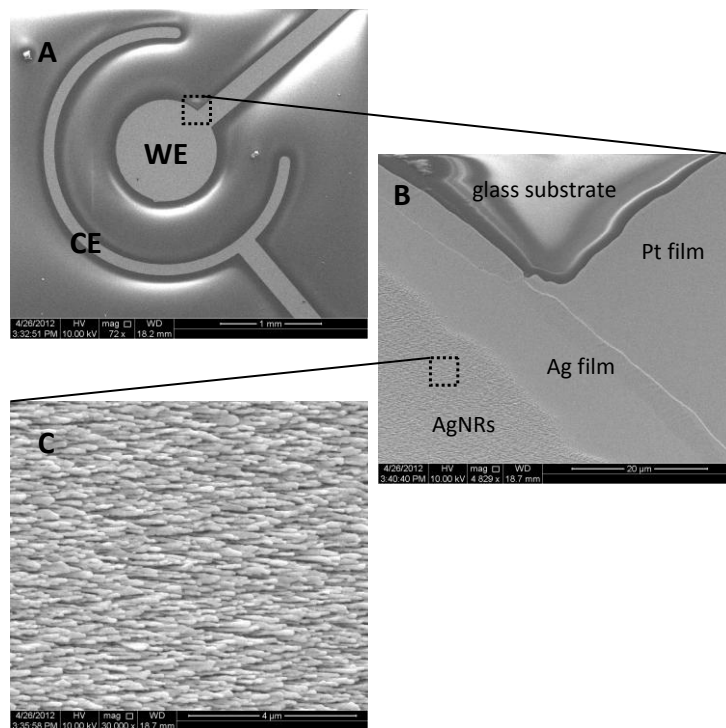


Figure 5.10. SEM images of one of the AgNR working electrode (WE) and a Pt counter electrode (CE) on the glass substrate. A) low magnification showing both WE and CE electrodes, B) higher magnification showing the different layers (Pt, Ag, and AgNRs) and 3) high resolution image showing intact AgNR film.

technique is straightforward and well established, and has been widely used in the literature to make a variety of microfluidic devices. The process starts by producing a photoresist mold that contains the desired microchannel pattern. SU-8 is a negative-tone photoresist traditionally used due to its ability to produce a thick film and therefore produce tall feature heights ($\geq 100 \mu\text{m}$). In addition, SU-8 is a robust photoresist with desirable mechanical properties that allow for it to be used in applications that required permanent structures (such as a mold) [145]. The mold pattern used here consists of an array of 3 mm diameter circular wells spaced apart so that they align with the aforementioned working/counter electrode array. The mold also consists of inlet and outlet channels to each cell connected to reservoirs. The mask used to expose the pattern is shown in Figure 5.11.

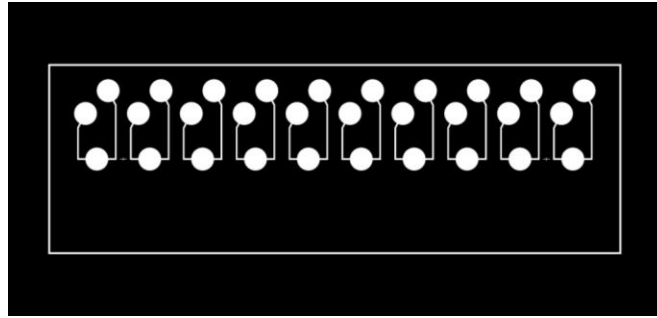


Figure 5.11. Mask design used to expose SU-8 for fabrication of the microchannel array mold.

The channel/cell mold is fabricated as follows. First, a 4 inch silicon wafer is cleaned in heated base-piranha (H_2O : NH_4OH : H_2O_2 , 5:1:1), then rinsed, blow dried, and baked on a hot plate at 200°C for ≥ 30 minutes to remove adsorbed moisture. The substrate is spin coated with SU-8 at 1000 RPM for 30 seconds yielding an film thickness of $\sim 100\ \mu\text{m}$, and then soft baked at 65° for ~ 5 min and 95°C for ~ 20 minutes on a hot plate. Next, the coated wafer is exposed using the MA6 mask aligner for approximately 30 seconds depending on the SU-8 thickness. The wafer is then post-baked at 65° for ~ 5 min and then 95°C for ~ 10 minutes. Afterwards, the substrate is placed into SU-8 developer for several minutes to allow dissolution of the unexposed resist, followed by rinsing with acetone and water before finally blow drying.

The completed SU-8 patterned wafer is then used as a mold for PDMS. The PDMS is prepared in the same way as described in Section 2.2. Several milliliters of liquid PDMS is poured onto SU-8 channel pattern at the center wafer and the PDMS is allowed to gravimetrically spread across the wafer. The resulting PDMS thickness is approximately 1 mm thick. After the PDMS has stopped spreading, the wafer is heated at $\sim 100^\circ\text{C}$ for several minutes to allow the PDMS to cure. Then a rectangular slab of PDMS containing the channel array is cut from the PDMS film. The PDMS slab is briefly (≥ 1 minute) exposed to O_2 plasma in an

inductively coupled plasma (ICP) chamber (Harrick Plasma, Inc.). This process activates silane groups on the surface of the PDMS, which will allow for a permanent, covalent bond between the PDMS and a glass surface [76]. After O₂ plasma treatment, the 3 mm cells (imprinted in the PDMS) are aligned to the AgNR electrode pattern on the glass slide. After several hours, the PDMS has formed a permanent, water-tight seal with the glass substrate.

One should note, however, that PDMS shrinks ~10% upon removal from the mold. This is troublesome when a microchannel/cell pattern that is to align precisely with the AgNR electrode pattern. One way to circumvent this issue is to first cut a thin (~ 1mm) slab of PDMS and then attach (via O₂ plasma treatment) this ‘pre-shrunk’ piece of PDMS onto the PDMS film still on the SU-8 micro mold pattern. The pre-shrunk PDMS piece will keep PDMS slab containing the microchannels from shrinking after removing from the SU-8 mold thereby preserving the desired channel array dimensions. Additional slabs of PDMS can be covalently bonded on top of the portion of the PDMS with the inlet/outlet reservoirs so as to allow more secure and reliable attachment of the inlet/outlet needles. A picture of the completed device is shown in Figures 5.12 and 5.13.

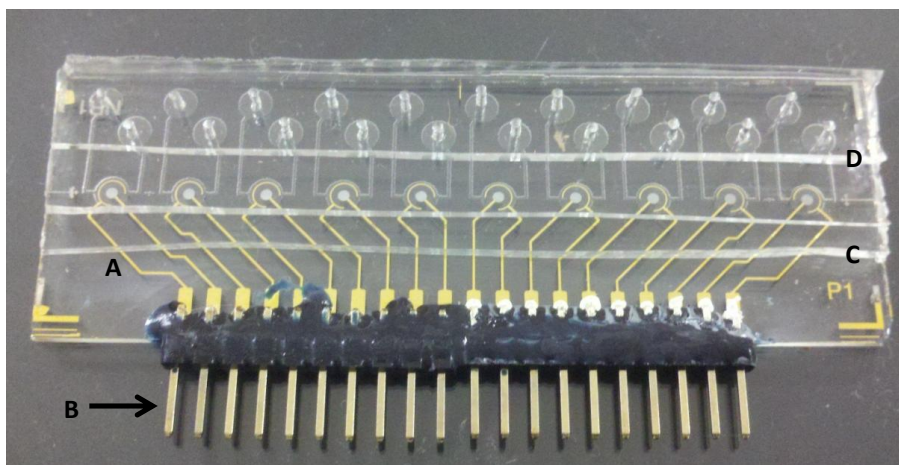


Figure 5.12. Picture of completed 1 × 3 inch AgNR LOC device. A - Au connection wire insides PDMS cell, B - 20 pin header, C - PDMS slab containing the microchannels, and D - PDMS slabs for reinforcement.

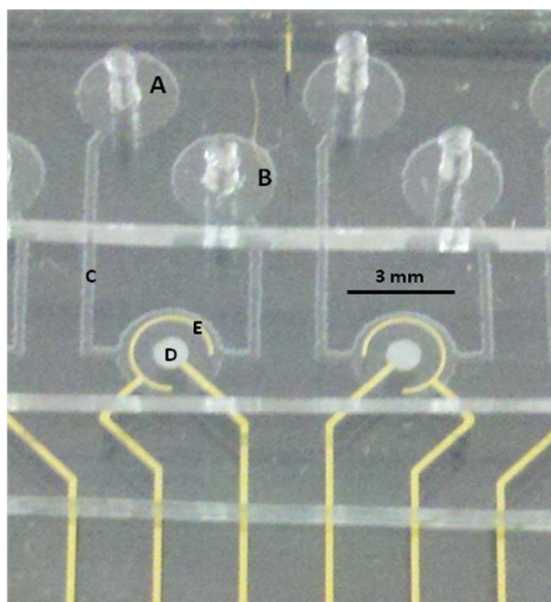


Figure 5.13. Higher magnification image of individual cells showing A - inlet reservoir, B - outlet reservoir, C - microchannel in PDMS, D - AgNR SERS sensor/working electrode, E - Au counter electrode.

5.5. Summary and Conclusions

In this chapter we have examined how external E-fields have been utilized for non-SERS detection to improve the kinetics and stringency of DNA hybridization with DNA probes immobilized onto microelectrodes. We have also discussed how AgNRs can be incorporated an electrocell for *in situ* SERS analysis, and how the SERS detection of a test analyte, e.g. BPE, can be improved by applying a negative potential to the AgNR film. To explain the physical causes of these observations, we reviewed the relevant literature and examined how applied voltages could modulate the SERS signal by inducing electrophoretic transport as well as increasing the adsorption capacity of BPE with the AgNR surface. We also looked at how a charged metal surface could potentially induce changes in BPE signal through modulation of molecular orientation, bond polarization, and charge transfer mechanisms. Lastly, we highlighted a novel

design for an AgNR-based SERS lab-on-a-chip device and discussed the fabrication of prototype. Thus, we have showed that AgNRs can be feasibly utilized in an electrocell setup for improved SERS detection. Additionally, the AgNR microelectrodes can be fabricated and incorporated into a microfluidic device. Therefore, this chapter, in conjunction with the previous chapters has laid the groundwork for the development of a lab-on-a-chip SERS device capable of *in situ* label-free miRNA analysis.

Chapter 6

CONCLUSIONS AND FUTURE WORK

This dissertation has investigated the use of surface-enhanced Raman scattering as an analytical platform for direct, label-free detection of nucleic acids, and AgNR substrates are shown to play a critical role in the label-free SERS detection of miRNA. The fact that the AgNRs provide the high enhancement and superior reproducibility, many of the substrate-related issues impeding SERS analysis can be overcome. A method to further improve the SERS signal quality of these substrates via a dynamic rastering measurement technique has also been proposed. In addition, the AgNR substrate fabrication process permits sufficient versatility to develop customized AgNR SERS devices. Thus, the fabrication, characterization, and optimization of a patterned multi-well array SERS chip have been presented. This simple device has thus been used as the SERS platform on which many parallel miRNA hybridization detection experiments are performed.

We have discussed how label-free nucleic acid detection is still very much in a nascent state, and highlighted much of the current controversy found in the scientific literature. The debate has centered primarily on the component signals, i.e. the band assignments and relative Raman cross-sections of the four different nucleic acids. Moreover, numerous physical interpretations have been proposed to explain the qualitative and quantitative variation of the spectral signal observed by different researchers. The issues of direct label-free Raman detection of miRNA hybridization have also been thoroughly discussed, namely the ambiguity of the signal resulting from the high degree of chemical similarity between a DNA probe before and

after hybridization with a miRNA target. The spectral signals of the pure nucleic acid components, i.e. the nucleotides, have been measured, and these source spectra have been shown to be primarily responsible for spectral characteristics of the measured DNA spectra. A very high degree of spectral similarity is seen for a DNA probe before and after hybridization with a clinically relevant miRNA target sequence, let-7f. However, independent confirmation using an extrinsic label indicates that specific miRNA capture is indeed occurring at the AgNR surface. To ascertain more definitive and quantitative analysis of these highly similar spectra, a straightforward linear least squares (LS) regression model is developed. Using the source spectra acquired from the pure nucleotide samples, and assuming that the measured DNA/miRNA signal is a linear combination of the source signals, the physical ratios of the nucleotide components within the immobilized sequence(s) could be ascertained. This method has produced rather accurate and reliable results allowing one to quantitatively differentiate between hybridized and single-stranded probe DNA sequences.

The possibility of using AgNRs for *in situ* miRNA detection, namely for incorporation into a lab-on-a-chip (LOC) SERS device, is also investigated extensively. We have discovered that the AgNRs are rather dynamic nanostructures when immersed in liquid and subsequently dried, as the AgNRs become bundled together by capillary forces induced by the drying solvent. When the AgNRs are functionalized with a hydrophilic thiol, de-bundling can be induced by immersion into a polar solvent.

The last section of this dissertation discusses the feasibility of incorporating the AgNR substrate into a microelectrocell, where the detection capability for the model analyte BPE was improved when a negative voltage was applied to the AgNRs. Lastly, a more sophisticated LOC-style electrocell, integrated with a microfluidic system, is also fabricated. This LOC device

provides the potential for up to 10 different samples to be independently processes and analyzed in parallel.

This dissertation presents the necessary technical foundation for the future development of a label-free miRNA SERS detection platform, with the eventual translation into a AgNR/LOC device. Equipped with the understanding and knowledge bestowed in this dissertation, such a proposition can be realized and possible future developments are below presented.

The scope of miRNA detection can be expanded beyond the proof of principle experiments presented in the current work. One of the ultimate goals is to convert the AgNR substrate into a label-free microarray platform. This would entail immobilizing many different types of DNA probes onto different locations of the AgNR substrate; each probe location can therefore be used to detect a specific complementary miRNA target sequence. The aforementioned SERS chip could be used to hold up to forty different probes; larger arrays containing still more sequences (i.e. hundreds or thousands) could be produced using robotic spotting procedures, similar to that used for traditional microarrays. The LS data analysis technique presented herein could also be further optimized. This would entail refinement of the source signals, i.e. using thiol-modified homogenous polynucleotide sequences as opposed to free nucleotides. This could allow measurement of more accurate source signals. Moreover, further refinement of the DNA/miRNA spectral preprocessing will help assure a more accurate LS fitting of the source signals. In addition, special probe sequences that are complementary to miRNA, but do not contain nucleotides, could also be immobilized onto the AgNRs instead of the traditional DNA probes used in this dissertation. One possible alternative is peptide nucleic acids (PNA). The lack of nucleotides in these molecules should allow for more obvious spectral differentiation before and after the PNA probes hybridize with target miRNA.

Because the AgNR hot spots (formed via solvent-induced bundles) have been discovered in this dissertation, such knowledge can be used as a new engineering paradigm for AgNR substrate development. Actively controlling the bundling process *in situ*, without the need for drying, would be a desirable quality. This effect could be accomplished in principle by utilizing various polymers that can expand/contract under external stimulus such as an electric potential or changes in pH/ionic content of the sample solution. A network of polymeric fibers underneath the AgNRs, or cross the top of the AgNR film could allow mechanical actuation of the AgNR film to reduce the spacing between the AgNRs, and therefore allow active control over hot spot formation.

For the AgNR electrocell studies, a highly controlled, well-designed experimental setup could be very beneficial for fundamental studies. A large scale electrocell could be used, allowing for the incorporation of a reference electrode and large counter electrode. Coupling real time *in situ* SERS measurement with cyclic voltammetry or differential capacitance measurements would provide an immense amount of quantitative data. In addition, the newly proposed LOC microfluidic SERS electrocell could be used to test the effects of an applied voltage on the detection of DNA/miRNA in solution. Strategies based on non-SERS voltage-assisted detection, such as improved DNA hybridization with μ s-pulses could be implemented with AgNRs. More quantitative control could be realized within the microcell by using a third Pt or Ag microelectrode as a pseudo-reference electrode.

REFERENCES

1. Nilsen, T. W., *Mechanisms of microRNA-mediated gene regulation in animal cells*. Trends in Genetics, 2007. **23**(5): p.243.
2. Stefani, G. and Slack, F. J., *Small non-coding RNAs in animal development*. Nature Reviews Molecular Cell Biology, 2008. **9**(3): p.219.
3. Jiang, Q. H., Wang, Y. D., Hao, Y. Y., Juan, L. R., Teng, M. X., Zhang, X. J., Li, M. M., Wang, G. H. and Liu, Y. L., *miR2Disease: a manually curated database for microRNA deregulation in human disease*. Nucleic Acids Research, 2009. **37**(p.D98).
4. Mitchell, P. S., Parkin, R. K., Kroh, E. M., Fritz, B. R., Wyman, S. K., Pogosova-Agadjanyan, E. L., Peterson, A., Noteboom, J., O'briant, K. C., Allen, A., Lin, D. W., Urban, N., Drescher, C. W., Knudsen, B. S., Stirewalt, D. L., Gentleman, R., Vessella, R. L., Nelson, P. S., Martin, D. B. and Tewari, M., *Circulating microRNAs as stable blood-based markers for cancer detection*. Proceedings of the National Academy of Sciences, 2008. **105**(30): p.10513.
5. Qavi, A. J., Kindt, J. T. and Bailey, R. C., *Sizing up the future of microRNA analysis*. Analytical and Bioanalytical Chemistry, 2010. **398**(6): p.2535.
6. Lagos-Quintana, M., Rauhut, R., Lendeckel, W. and Tuschl, T., *Identification of novel genes coding for small expressed RNAs*. Science, 2001. **294**(5543): p.853.
7. Raymond, C. K., Roberts, B. S., Garrett-Engele, P., Lim, L. P. and Johnson, J. M., *Simple, quantitative primer-extension PCR assay for direct monitoring of microRNAs and short-interfering RNAs*. Rna-a Publication of the Rna Society, 2005. **11**(11): p.1737.
8. Git, A., Dvinge, H., Salmon-Divon, M., Osborne, M., Kutter, C., Hadfield, J., Bertone, P. and Caldas, C., *Systematic comparison of microarray profiling, real-time PCR, and next-*

- generation sequencing technologies for measuring differential microRNA expression.* Rna-a Publication of the Rna Society, 2010. **16**(5): p.991.
9. Sipova, H., Zhang, S. L., Dudley, A. M., Galas, D., Wang, K. and Homola, J., *Surface Plasmon Resonance Biosensor for Rapid Label-Free Detection of Microribonucleic Acid at Subfemtomole Level.* Analytical Chemistry, 2010. **82**(24): p.10110.
 10. Sassolas, A., Leca-Bouvier, B. D. and Blum, L. J., *DNA biosensors and microarrays.* Chemical Reviews, 2008. **108**(1): p.109.
 11. Le Ru, E. C., Principles of surface-enhanced Raman spectroscopy : and related plasmonic effects. 1st ed. ed.; Amsterdam ;: Elsevier, 2009.
 12. Moskovits, M., *Surface-enhanced Raman spectroscopy: a brief retrospective.* Journal of Raman Spectroscopy, 2005. **36**(6-7): p.485.
 13. Vo-Dinh, T., *Surface-enhanced Raman spectroscopy using metallic nanostructures.* Trac-Trends in Analytical Chemistry, 1998. **17**(8-9): p.557.
 14. Kambhampati, P., Child, C. M., Foster, M. C. and Campion, A., *On the chemical mechanism of surface enhanced Raman scattering: Experiment and theory.* Journal of Chemical Physics, 1998. **108**(12): p.5013.
 15. Kneipp, K., Wang, Y., Kneipp, H., Perelman, L. T., Itzkan, I., Dasari, R. and Feld, M. S., *Single molecule detection using surface-enhanced Raman scattering (SERS).* Physical Review Letters, 1997. **78**(9): p.1667.
 16. Nie, S. M. and Emery, S. R., *Probing single molecules and single nanoparticles by surface-enhanced Raman scattering.* Science, 1997. **275**(5303): p.1102.
 17. Willets, K. A. and Van Duyne, R. P., Localized surface plasmon resonance spectroscopy and sensing. In Annual Review of Physical Chemistry, 2007; Vol. 58, p. 267.

18. Vo-Dinh, T., Dhawan, A., Norton, S. J., Khoury, C. G., Wang, H. N., Misra, V. and Gerhold, M. D., *Plasmonic Nanoparticles and Nanowires: Design, Fabrication and Application in Sensing*. Journal of Physical Chemistry C, 2010. **114**(16): p.7480.
19. Natan, M. J., *Concluding remarks - Surface enhanced Raman scattering*. Faraday Discussions, 2006. **132**: p.321.
20. Fleischmann, M., Hendra, P. J. and McQuilla, A. J., *Raman-Spectra Of Pyridine Adsorbed At A Silver Electrode*. Chemical Physics Letters, 1974. **26**(2): p.163.
21. Yang, K. H., Liu, Y. C. and Yu, C. C., *Simple Strategy To Improve Surface-Enhanced Raman Scattering Based on Electrochemically Prepared Roughened Silver Substrates*. Langmuir, 2010. **26**(13): p.11512.
22. Lee, P. C. and Meisel, D., *Adsorption And Surface-Enhanced Raman Of Dyes On Silver And Gold Sols*. Journal of Physical Chemistry, 1982. **86**(17): p.3391.
23. Munro, C. H., Smith, W. E., Garner, M., Clarkson, J. and White, P. C., *Characterization Of The Surface Of A Citrate-Reduced Colloid Optimized For Use As A Substrate For Surface-Enhanced Resonance Raman-Scattering*. Langmuir, 1995. **11**(10): p.3712.
24. Freeman, R. G., Grabar, K. C., Allison, K. J., Bright, R. M., Davis, J. A., Guthrie, A. P., Hommer, M. B., Jackson, M. A., Smith, P. C., Walter, D. G. and Natan, M. J., *Self-Assembled Metal Colloid Monolayers - An Approach To Sers Substrates*. Science, 1995. **267**(5204): p.1629.
25. Kahl, M., Voges, E., Kostrewa, S., Viets, C. and Hill, W., *Periodically structured metallic substrates for SERS*. Sensors and Actuators B-Chemical, 1998. **51**(1-3): p.285.

26. Oran, J. M., Hinde, R. J., Abu Hatab, N., Retterer, S. T. and Sepaniak, M. J., *Nanofabricated periodic arrays of silver elliptical discs as SERS substrates*. Journal of Raman Spectroscopy, 2008. **39**(12): p.1811.
27. Van Duyne, R. P., Hulteen, J. C. and Treichel, D. A., *Atomic-force microscopy and surface-enhanced raman-spectroscopy .1. ag island films and ag film over polymer nanosphere surfaces supported on glass*. Journal of Chemical Physics, 1993. **99**(3): p.2101.
28. Zhang, X. Y., Zhao, J., Whitney, A. V., Elam, J. W. and Van Duyne, R. P., *Ultrastable substrates for surface-enhanced Raman spectroscopy: Al₂O₃ overlayers fabricated by atomic layer deposition yield improved anthrax biomarker detection*. Journal of the American Chemical Society, 2006. **128**(31): p.10304.
29. Fan, M. K., Andrade, G. F. S. and Brolo, A. G., *A review on the fabrication of substrates for surface enhanced Raman spectroscopy and their applications in analytical chemistry*. Analytica Chimica Acta, 2011. **693**(1-2): p.7.
30. Gong, X., Bao, Y., Qiu, C. and Jiang, C. Y., *Individual nanostructured materials: fabrication and surface-enhanced Raman scattering*. Chemical Communications, 2012. **48**(56): p.7003.
31. Banholzer, M. J., Millstone, J. E., Qin, L. D. and Mirkin, C. A., *Rationally designed nanostructures for surface-enhanced Raman spectroscopy*. Chemical Society Reviews, 2008. **37**(5): p.885.
32. Alessio, P., Constantino, C. J. L., Aroca, R. F. and Oliveira, O. N., *Surface-Enhanced Raman Scattering: Metal Nanostructures Coated With Langmuir-Blodgett Films*. Journal of the Chilean Chemical Society, 2010. **55**(4): p.469.

33. Dou, X., Takama, T., Yamaguchi, Y., Hirai, K., Yamamoto, H., Doi, S. and Ozaki, Y., *Quantitative analysis of double-stranded DNA amplified by a polymerase chain reaction employing surface-enhanced Raman spectroscopy*. Applied Optics, 1998. **37**(4): p.759.
34. Graham, D. and Faulds, K., *Quantitative SERRS for DNA sequence analysis*. Chemical Society Reviews, 2008. **37**(5): p.1042.
35. Graham, D., Mallinder, B. J., Whitcombe, D. and Smith, W. E., *Surface enhanced resonance Raman scattering (SERRS) - A first example of its use in multiplex genotyping*. Chemphyschem, 2001. **2**(12): p.746.
36. Isola, N. R., Stokes, D. L. and Vo-Dinh, T., *Surface enhanced Raman gene probe for HIV detection*. Analytical Chemistry, 1998. **70**(7): p.1352.
37. Mahajan, S., Richardson, J., Brown, T. and Bartlett, P. N., *SERS-Melting: A New Method for Discriminating Mutations in DNA Sequences*. Journal of the American Chemical Society, 2008. **130**(46): p.15589.
38. Strelau, K. K., Kretschmer, R., Moller, R., Fritzsche, W. and Popp, J., *SERS as tool for the analysis of DNA-chips in a microfluidic platform*. Analytical and Bioanalytical Chemistry, 2010. **396**(4): p.1381.
39. Vodinh, T., Houck, K. and Stokes, D. L., *Surface-Enhanced Raman Gene Probes*. Analytical Chemistry, 1994. **66**(20): p.3379.
40. Brabec, V. and Niki, K., *Raman-Scattering From Nucleic-Acids Adsorbed At A Silver Electrode*. Biophysical Chemistry, 1985. **23**(1-2): p.63.
41. Kneipp, K. and Flemming, J., *Surface Enhanced Raman-Scattering (Sers) Of Nucleic-Acids Adsorbed On Colloidal Silver Particles*. Journal of Molecular Structure, 1986. **145**(1-2): p.173.

42. Gearheart, L. A., Ploehn, H. J. and Murphy, C. J., *Oligonucleotide adsorption to gold nanoparticles: A surface-enhanced raman spectroscopy study of intrinsically bent DNA*. Journal of Physical Chemistry B, 2001. **105**(50): p.12609.
43. Dong, L. Q., Zhou, J. Z., Wu, L. L., Dong, P. and Lin, Z. H., *SERS studies of self-assembled DNA monolayer - characterization of adsorption orientation of oligonucleotide probes and their hybridized helices on gold substrate*. Chemical Physics Letters, 2002. **354**(5-6): p.458.
44. Green, M., Liu, F. M., Cohen, L., Kollensperger, P. and Cass, T., *SERS platforms for high density DNA arrays*. Faraday Discussions, 2006. **132**: p.269.
45. Wu, C. Y., Lo, W. Y., Chiu, C. R. and Yang, T. S., *Surface enhanced Raman spectra of oligonucleotides induced by spermine*. Journal of Raman Spectroscopy, 2006. **37**(8): p.799.
46. Barhoumi, A., Zhang, D., Tam, F. and Halas, N. J., *Surface-enhanced Raman spectroscopy of DNA*. Journal of the American Chemical Society, 2008. **130**(16): p.5523.
47. Barhoumi, A., Zhang, D. M. and Halas, N. J., *Correlation of Molecular Orientation and Packing Density in a dsDNA Self-Assembled Monolayer Observable with Surface-Enhanced-Raman Spectroscopy*. Journal of the American Chemical Society, 2008. **130**(43): p.14040.
48. Barhoumi, A. and Halas, N. J., *Label-Free Detection of DNA Hybridization Using Surface Enhanced Raman Spectroscopy*. Journal of the American Chemical Society, 2010. **132**(37): p.12792.

49. Papadopoulou, E. and Bell, S. E. J., *DNA reorientation on Au nanoparticles: label-free detection of hybridization by surface enhanced Raman spectroscopy*. Chemical Communications, 2011. **47**(39): p.10966.
50. Papadopoulou, E. and Bell, S. E. J., *Label-Free Detection of Single-Base Mismatches in DNA by Surface-Enhanced Raman Spectroscopy*. Angewandte Chemie-International Edition, 2011. **50**(39): p.9058.
51. Papadopoulou, E. and Bell, S. E. J., *Label-Free Detection of Nanomolar Unmodified Single- and Double-Stranded DNA by Using Surface-Enhanced Raman Spectroscopy on Ag and Au Colloids*. Chemistry-a European Journal, 2012. **18**(17): p.5394.
52. Prado, E., Daugey, N., Plumet, S., Servant, L. and Lecomte, S., *Quantitative label-free RNA detection using surface-enhanced Raman spectroscopy*. Chemical Communications, 2011. **47**(26): p.7425.
53. Rusciano, G., De Luca, A. C., Pesce, G., Sasso, A., Oliviero, G., Amato, J., Borbone, N., D'errico, S., Piccialli, V., Piccialli, G. and Mayol, L., *Label-Free Probing of G-Quadruplex Formation by Surface-Enhanced Raman Scattering*. Analytical Chemistry, 2011. **83**(17): p.6849.
54. Xu, T. T., Huang, J. A., He, L. F., He, Y., Su, S. and Lee, S. T., *Ordered silicon nanocones arrays for label-free DNA quantitative analysis by surface-enhanced Raman spectroscopy*. Applied Physics Letters, 2011. **99**(15).
55. Bell, S. E. J. and Sirimuthu, N. M. S., *Surface-enhanced Raman spectroscopy (SERS) for sub-micromolar detection of DNA/RNA mononucleotides*. Journal of the American Chemical Society, 2006. **128**(49): p.15580.

56. Herne, T. M. and Tarlov, M. J., *Characterization of DNA probes immobilized on gold surfaces*. Journal of the American Chemical Society, 1997. **119**(38): p.8916.
57. Muniz-Miranda, M., Gellini, C., Pagliai, M., Innocenti, M., Salvi, P. R. and Schettino, V., *SERS and Computational Studies on MicroRNA Chains Adsorbed on Silver Surfaces*. Journal of Physical Chemistry C, 2010. **114**(32): p.13730.
58. Wachter, E. A., Moore, A. K. and Haas, J. W., *Fabrication Of Tailored Needle Substrates For Surface-Enhanced Raman-Scattering*. Vibrational Spectroscopy, 1992. **3**(1): p.73.
59. Chaney, S. B., Shanmukh, S., Dluhy, R. A. and Zhao, Y. P., *Aligned silver nanorod arrays produce high sensitivity surface-enhanced Raman spectroscopy substrates*. Applied Physics Letters, 2005. **87**(3): p.031908.
60. Driskell, J. D., Shanmukh, S., Liu, Y., Chaney, S. B., Tang, X. J., Zhao, Y. P. and Dluhy, R. A., *The use of aligned silver nanorod Arrays prepared by oblique angle deposition as surface enhanced Raman scattering substrates*. Journal of Physical Chemistry C, 2008. **112**(4): p.895.
61. Hawkeye, M. M. and Brett, M. J., *Glancing angle deposition: Fabrication, properties, and applications of micro- and nanostructured thin films*. Journal of Vacuum Science & Technology A, 2007. **25**(5): p.1317.
62. Zhu, Y., Dluhy, R. A. and Zhao, Y. P., *Development of silver nanorod array based fiber optic probes for SERS detection*. Sensors and Actuators B-Chemical, 2011. **157**(1): p.42.
63. Singh, J. P., Chu, H., Abell, J., Tripp, R. A. and Zhao, Y., *Flexible and mechanical strain resistant large area SERS active substrates*. Nanoscale, 2012.

64. Abell, J. L., Garren, J. M. and Zhao, Y. P., *Dynamic Rastering Surface-Enhanced Raman Scattering (SERS) Measurements on Silver Nanorod Substrates*. Applied Spectroscopy, 2011. **65**(7): p.734.
65. Driskell, J. D., Primera-Pedrozo, O. M., Dluhy, R. A., Zhao, Y. P. and Tripp, R. A., *Quantitative Surface-Enhanced Raman Spectroscopy Based Analysis of MicroRNA Mixtures*. Applied Spectroscopy, 2009. **63**(10): p.1107.
66. Driskell, J. D., Seto, A. G., Jones, L. P., Jokela, S., Dluhy, R. A., Zhao, Y. P. and Tripp, R. A., *Rapid microRNA (miRNA) detection and classification via surface-enhanced Raman spectroscopy (SERS)*. Biosensors & Bioelectronics, 2008. **24**(4): p.917.
67. Driskell, J. D. and Tripp, R. A., *Label-free SERS detection of microRNA based on affinity for an unmodified silver nanorod array substrate*. Chemical Communications, 2010. **46**(19): p.3298.
68. Kudelski, A. and Bukowska, J., *Influence Of Photochemical Effects On Irreversible Loss Of Active-Sites On Sers Active Silver Electrode*. Spectrochimica Acta Part a-Molecular and Biomolecular Spectroscopy, 1995. **51**(4): p.573.
69. Pang, Y. S., Hwang, H. J. and Kim, M. S., *Reversible temperature dependence in surface-enhanced Raman scattering of 1-propanethiol adsorbed on a silver island film*. Journal of Physical Chemistry B, 1998. **102**(37): p.7203.
70. Suh, J. S., Jang, N. H., Jeong, D. H. and Moskovits, M., *Adsorbate photochemistry on a colloid surface: Phthalazine on silver*. Journal of Physical Chemistry, 1996. **100**(2): p.805.
71. Viets, C. and Hill, W., *Laser power effects in SERS spectroscopy at thin metal films*. Journal of Physical Chemistry B, 2001. **105**(27): p.6330.

72. Yeo, B. S., Schmid, T., Zhang, W. H. and Zenobi, R., *A strategy to prevent signal losses, analyte decomposition, and fluctuating carbon contamination bands in surface-enhanced Raman spectroscopy*. Applied Spectroscopy, 2008. **62**(6): p.708.
73. De Jesus, M. A., Giesfeldt, K. S. and Sepaniak, M. J., *Use of a sample translation technique to minimize adverse effects of laser irradiation in surface-enhanced Raman spectrometry*. Applied Spectroscopy, 2003. **57**(4): p.428.
74. De Jesus, M. A., Giesfeldt, K. S. and Sepaniak, M. J., *Improving the analytical figures of merit of SERS for the analysis of model environmental pollutants*. Journal of Raman Spectroscopy, 2004. **35**(10): p.895.
75. Abell, J. L., Driskell, J. D., Dluhy, R. A., Tripp, R. A. and Zhao, Y. P., *Fabrication and characterization of a multiwell array SERS chip with biological applications*. Biosensors & Bioelectronics, 2009. **24**(12): p.3663.
76. Sia, S. K. and Whitesides, G. M., *Microfluidic devices fabricated in poly(dimethylsiloxane) for biological studies*. Electrophoresis, 2003. **24**(21): p.3563.
77. Beavers, K. R., Marotta, N. E. and Bottomley, L. A., *Thermal Stability of Silver Nanorod Arrays*. Chemistry of Materials, 2010. **22**(7): p.2184.
78. Yang, W. H., Hulteen, J., Schatz, G. C. and Vanduyne, R. P., *A surface-enhanced hyper-Raman and surface-enhanced Raman scattering study of trans-1,2-bis(4-pyridyl)ethylene adsorbed onto silver film over nanosphere electrodes. Vibrational assignments: Experiment and theory*. Journal of Chemical Physics, 1996. **104**(11): p.4313.
79. Chu, H. Y., Liu, Y. J., Huang, Y. W. and Zhao, Y. P., *A high sensitive fiber SERS probe based on silver nanorod arrays*. Optics Express, 2007. **15**(19): p.12230.

80. Abell, J. L., Driskell, J. D., Dluhy, R. A., Tripp, R. A. and Zhao, Y. P. In *Optimization of a multi-well array SERS chip*, 2009; F. F. Nicholas, Jr and S. S. Venkataraman, Eds. SPIE: 2009; p 732103.
81. Negri, P., Marotta, N. E., Bottomley, L. A. and Dluhy, R. A., *Removal of Surface Contamination and Self-Assembled Monolayers (SAMs) from Silver (Ag) Nanorod Substrates by Plasma Cleaning with Argon*. *Applied Spectroscopy*, 2011. **65**(1): p.66.
82. Abell, J. L., Garren, J. M., Driskell, J. D., Tripp, R. A. and Zhao, Y. P., *Label-Free Detection of Micro-RNA Hybridization Using Surface-Enhanced Raman Spectroscopy and Least-Squares Analysis*. *Journal of the American Chemical Society*, 2012. **134**(31): p.12889.
83. Gong, P., Lee, C. Y., Gamble, L. J., Castner, D. G. and Grainger, D. W., *Hybridization behavior of mixed DNA/alkylthiol monolayers on gold: Characterization by surface plasmon resonance and P-32 radiometric assay*. *Analytical Chemistry*, 2006. **78**(10): p.3326.
84. Sandström, P., Boncheva, M. and Åkerman, B., *Nonspecific and Thiol-Specific Binding of DNA to Gold Nanoparticles*. *Langmuir*, 2003. **19**(18): p.7537.
85. Maskos, U. and Southern, E. M., *Oligonucleotide Hybridizations On Glass Supports - A Novel Linker For Oligonucleotide Synthesis And Hybridization Properties Of Oligonucleotides Synthesized Insitu*. *Nucleic Acids Research*, 1992. **20**(7): p.1679.
86. Solomun, T., Mix, R. and Sturm, H., *Immobilization of Silanized DNA on Glass: Influence of the Silane Tether on the DNA Hybridization*. *Acs Applied Materials & Interfaces*, 2010. **2**(8): p.2171.

87. Moody, B. and Mccarty, G., *Statistically Significant Raman Detection of Midsequence Single Nucleotide Polymorphisms*. Analytical Chemistry, 2009. **81**(5): p.2013.
88. Peterson, A. W., Heaton, R. J. and Georgiadis, R. M., *The effect of surface probe density on DNA hybridization*. Nucleic Acids Research, 2001. **29**(24): p.5163.
89. Bryant, M. A. and Pemberton, J. E., *Surface Raman-Scattering Of Self-Assembled Monolayers Formed From 1-Alkanethiols At Ag*. Journal of the American Chemical Society, 1991. **113**(10): p.3629.
90. Kudelski, A., *Structures of monolayers formed from different HS-(CH₂)₂-X thiols on gold, silver and copper: comparative studies by surface-enhanced Raman scattering*. Journal of Raman Spectroscopy, 2003. **34**(11): p.853.
91. Roush, S. and Slack, F. J., *The let-7 family of microRNAs*. Trends in Cell Biology, 2008. **18**(10): p.505.
92. Weistein, E. W., *Least Squares Fitting*. MathWorld -- A Wolfram Web Resource <http://mathworld.wolfram.com/LeastSquaresFitting.html>.
93. Abell, J. L., Driskell, J. D., Tripp, R. A. and Zhao, Y., Current Progress on Surface-Enhanced Raman Scattering Chemical/Biological Sensing. In Nanoparticles for Nanomedicine and Bioelectronic Devices, American Chemical Society: 2012.
94. Lee, H. M., Kim, M. S. and Kim, K., *Surface-Enhanced Raman-Scattering Of Ortho-Mercaptophenol And Para-Mercaptophenol In Silver Sol*. Vibrational Spectroscopy, 1994. **6**(2): p.205.
95. Sriram, S., Bhaskaran, M., Chen, S. J., Jayawardhana, S., Stoddart, P. R., Liu, J. Z., Medhekar, N. V., Kalantar-Zadeh, K. and Mitchell, A., *Influence of Electric Field on*

- SERS: Frequency Effects, Intensity Changes, and Susceptible Bonds*. Journal of the American Chemical Society, 2012. **134**(10): p.4646.
96. Kudelski, A., *Chemisorption of 2-mercaptoethanol on silver, copper, and gold: Direct Raman evidence of acid-induced changes in adsorption/desorption equilibria*. Langmuir, 2003. **19**(9): p.3805.
 97. Zeman, E. J., Carron, K. T., Schatz, G. C. and Vanduyne, R. P., *A Surface Enhanced Resonance Raman-Study Of Cobalt Phthalocyanine On Rough Ag Films - Theory And Experiment*. Journal of Chemical Physics, 1987. **87**(7): p.4189.
 98. Norrod, K. L., Sudnik, L. M., Rousell, D. and Rowlen, K. L., *Quantitative comparison of five SERS substrates: Sensitivity and limit of detection*. Applied Spectroscopy, 1997. **51**(7): p.994.
 99. Moskovits, M. and Suh, J. S., *Surface Geometry Change In 2-Naphthoic Acid Adsorbed On Silver*. Journal of Physical Chemistry, 1988. **92**(22): p.6327.
 100. Moskovits, M., *Surface Selection-Rules*. Journal of Chemical Physics, 1982. **77**(9): p.4408.
 101. Moskovits, M., Dilella, D. P. and Maynard, K. J., *Surface Raman-Spectroscopy Of A Number Of Cyclic Aromatic-Molecules Adsorbed On Silver - Selection-Rules And Molecular-Reorientation*. Langmuir, 1988. **4**(1): p.67.
 102. Schmidt, M. S., Hubner, J. and Boisen, A., *Large Area Fabrication of Leaning Silicon Nanopillars for Surface Enhanced Raman Spectroscopy*. Advanced Materials, 2012. **24**(10): p.OP11.

103. Kim, A., Ou, F. S., Ohlberg, D. a. A., Hu, M., Williams, R. S. and Li, Z. Y., *Study of Molecular Trapping Inside Gold Nanofinger Arrays on Surface-Enhanced Raman Substrates*. Journal of the American Chemical Society, 2011. **133**(21): p.8234.
104. Fan, J. G. and Zhao, Y. P., *Characterization of watermarks formed in nano-carpet effect*. Langmuir, 2006. **22**(8): p.3662.
105. Zhao, Y. P. and Fan, J. G., *Clusters of bundled nanorods in nanocarpet effect*. Applied Physics Letters, 2006. **88**(10).
106. Fan, J. G. and Zhao, Y. P., *Nanocarpet Effect Induced Superhydrophobicity*. Langmuir, 2010. **26**(11): p.8245.
107. Fan, J. G., Dyer, D., Zhang, G. and Zhao, Y. P., *Nanocarpet effect: Pattern formation during the wetting of vertically aligned nanorod arrays*. Nano Letters, 2004. **4**(11): p.2133.
108. Fan, J. G. and Zhao, Y. P., *Spreading of a water droplet on a vertically aligned Si nanorod array surface*. Applied Physics Letters, 2007. **90**(1).
109. Fan, J. G., Fu, J. X., Collins, A. and Zhao, Y. P., *The effect of the shape of nanorod arrays on the nanocarpet effect*. Nanotechnology, 2008. **19**(4): p.045713.
110. Song, C.
111. Kovarik, M. L., Gach, P. C., Ornoff, D. M., Wang, Y. L., Balowski, J., Farrag, L. and Allbritton, N. L., *Micro Total Analysis Systems for Cell Biology and Biochemical Assays*. Analytical Chemistry, 2012. **84**(2): p.516.
112. Rios, A., Zougagh, M. and Avila, M., *Miniaturization through lab-on-a-chip: Utopia or reality for routine laboratories? A review*. Analytica Chimica Acta, 2012. **740** p.1.

113. Edman, C. F., Raymond, D. E., Wu, D. J., Tu, E. G., Sosnowski, R. G., Butler, W. F., Nerenberg, M. and Heller, M. J., *Electric field directed nucleic acid hybridization on microchips*. Nucleic Acids Research, 1997. **25**(24): p.4907.
114. Heller, M. J., Forster, A. H. and Tu, E., *Active microelectronic chip devices which utilize controlled electrophoretic fields for multiplex DNA hybridization and other genomic applications*. Electrophoresis, 2000. **21**(1): p.157.
115. Cabeca, R., Rodrigues, M., Prazeres, D. M. F., Chu, V. and Conde, J. P., *The effect of the shape of single, sub-ms voltage pulses on the rates of surface immobilization and hybridization of DNA*. Nanotechnology, 2009. **20**(1): p. 015503.
116. Fixe, F., Branz, H. M., Louro, N., Chu, V., Prazeres, D. M. F. and Conde, J. P., *Immobilization and hybridization by single sub-millisecond electric field pulses, for pixel-addressed DNA microarrays*. Biosensors & Bioelectronics, 2004. **19**(12): p.1591.
117. Fixe, F., Cabeca, R., Chu, V., Prazeres, D. M. F., Ferreira, G. N. M. and Conde, J. P., *Electric-field-pulse-assisted covalent immobilization of DNA in the nanosecond time scale*. Applied Physics Letters, 2003. **83**(7): p.1465.
118. Fixe, F., Chu, V., Prazeres, D. M. F. and Conde, J. P., *Single base mismatch detection by microsecond voltage pulses*. Biosensors & Bioelectronics, 2005. **21**(6): p.888.
119. Zhou, Q., Liu, Y. J., He, Y. P., Zhang, Z. J. and Zhao, Y. P., *The effect of underlayer thin films on the surface-enhanced Raman scattering response of Ag nanorod substrates*. Applied Physics Letters, 2010. **97**(12).
120. Delahay, P., Pourbaix, M. and Vanrysselberghe, P., *Potential-Ph Diagram Of Silver - Construction Of The Diagram - Its Applications To The Study Of The Properties Of The*

- Metal, Its Compounds, And Its Corrosion*. Journal of the Electrochemical Society, 1951. **98**(2): p.65.
121. Ibrahim, M. a. M., Hassan, H. H., Abd El Rehim, S. S. and Amin, M. A., *The electrochemical behaviour of polycrystalline silver electrodes in Na₂CO₃ solution and the effect of ClO₄⁻ ions*. Journal of Solid State Electrochemistry, 1999. **3**(7-8): p.380.
 122. Jovic, B. M. and Jovic, V. D., *Electrochemical formation and characterization of Ag₂O*. Journal of the Serbian Chemical Society, 2004. **69**(2): p.153.
 123. Tian, Z. Q., Li, W. H., Mao, B. W. and Gao, J. S., *Surface-Enhanced Raman-Spectroscopic Studies On Structural Dynamics Of Coadsorption Of Thiourea And ClO₄⁻ At Ag Electrodes*. Journal of Electroanalytical Chemistry, 1994. **379**(1-2): p.271.
 124. Weaver, M. J., Barz, F., Gordon, J. G. and Philpott, M. R., *Surface-Enhanced Raman-Spectroscopy Of Electrochemically Characterized Interfaces - Potential Dependence Of Raman-Spectra For Thiocyanate At Silver Electrodes*. Surface Science, 1983. **125**(2): p.409.
 125. Pothier, N. J. and Force, R. K., *Surface-Enhanced Raman-Spectroscopy At A Silver Electrode As A Real-Time Detector In Flowing Streams*. Applied Spectroscopy, 1992. **46**(1): p.147.
 126. Pothier, N. J. and Force, R. K., *Detection Of Biologically Important Compounds In Flowing Aqueous Streams By Surface-Enhanced Raman-Spectroscopy At A Silver Electrode*. Applied Spectroscopy, 1994. **48**(4): p.421.
 127. Bard, A. J. and Faulkner, L. R., *Electrochemical Methods; Fundamentals and Applications*. 2nd ed. ed.; John Wiley & Sons, Inc.: Hoboken, 2001.

128. Bockris, J. O. M. and Khan, S. U. M., *Surface Electrochemistry: A Molecular Level Approach*. 1st ed. ed.; Plenum Press: New York, 1993.
129. Polk, B. J., Stelzenmuller, A., Mijares, G., Maccreehan, W. and Gaitan, M., *Ag/AgCl microelectrodes with improved stability for microfluidics*. *Sensors and Actuators B-Chemical*, 2006. **114**(1): p.239.
130. Zhou, J. H., Ren, K. N., Zheng, Y. Z., Su, J., Zhao, Y. H., Ryan, D. and Wu, H. K., *Fabrication of a microfluidic Ag/AgCl reference electrode and its application for portable and disposable electrochemical microchips*. *Electrophoresis*, 2010. **31**(18): p.3083.
131. Sanchez-Cortes, S. and Garcia-Ramos, J. V., *Influence of coverage in the surface-enhanced Raman scattering of cytosine and its methyl derivatives on metal colloids: chloride and pH effects*. *Surface Science*, 2001. **473**(1-2): p.133.
132. Papadopolou, E. and Bell, S. E. J., *Surface-Enhanced Raman Evidence of Protonation, Reorientation, and Ag(+) Complexation of Deoxyadenosine and Deoxyadenosine-5' -Monophosphate (dAMP) on Ag and Au Surfaces*. *Journal of Physical Chemistry C*, 2011. **115**(29): p.14228.
133. Zhu, X., Wang, N., Zhang, R., Song, W., Sun, Y. P., Duan, G. P., Ding, W., Zhang, Z. R. and Yang, H. F., *pH-dependent surface-enhanced Raman scattering observation of sulfanilamide on the silver surface*. *Journal of Raman Spectroscopy*, 2009. **40**(12): p.1838.
134. Leopold, N., Cinta-Pinzaru, S., Szabo, L., Ilesan, D., Chis, V., Cozar, O. and Kiefer, W., *Raman and SERS study of metoclopramide at different pH values*. *Journal of Raman Spectroscopy*, 2010. **41**(3): p.248.

135. Liu, F. F., Gu, H. M., Lin, Y., Qi, Y. J., Dong, X., Gao, J. X. and Cai, T. T., *Surface-enhanced Raman scattering study of riboflavin on borohydride-reduced silver colloids: Dependence of concentration, halide anions and pH values*. Spectrochimica Acta Part a-Molecular and Biomolecular Spectroscopy, 2012. **85**(1): p.111.
136. Morrow, R. and Mckenzie, D. R., *The time-dependent development of electric double-layers in pure water at metal electrodes: the effect of an applied voltage on the local pH*. Proceedings of the Royal Society a-Mathematical Physical and Engineering Sciences, 2012. **468**(2137): p.18.
137. Wu, D. Y., Li, J. F., Ren, B. and Tian, Z. Q., *Electrochemical surface-enhanced Raman spectroscopy of nanostructures*. Chemical Society Reviews, 2008. **37**(5): p.1025.
138. Li, D., Li, D. W., Fossey, J. S. and Long, Y. T., *Portable Surface-Enhanced Raman Scattering Sensor for Rapid Detection of Aniline and Phenol Derivatives by On-Site Electrostatic Preconcentration*. Analytical Chemistry, 2010. **82**(22): p.9299.
139. Cho, H. S., Lee, B., Liu, G. L., Agarwal, A. and Lee, L. P., *Label-free and highly sensitive biomolecular detection using SERS and electrokinetic preconcentration*. Lab on a Chip, 2009. **9**(23): p.3360.
140. Luo, Z. X., Loo, B. H., Cao, X. Q., Peng, A. D. and Yao, J. N., *Probing the Conformational Transition of 2,2 '-Bipyridyl under External Field by Surface-Enhanced Raman Spectroscopy*. Journal of Physical Chemistry C, 2012. **116**(4): p.2884.
141. Moskovits, M. and Suh, J. S., *Surface Selection-Rules For Surface-Enhanced Raman-Spectroscopy - Calculations And Application To The Surface-Enhanced Raman-Spectrum Of Phthalazine On Silver*. Journal of Physical Chemistry, 1984. **88**(23): p.5526.

142. Liu, Z. J. and Wu, G. Z., *Surface-enhanced Raman scattering of thiourea adsorbed on the silver electrode: bond polarizability derivatives as elucidated from the Raman intensities*. Chemical Physics Letters, 2004. **389**(4-6): p.298.
143. Hulteen, J. C., Young, M. A. and Van Duyne, R. P., *Surface-enhanced hyper-Raman scattering (SEHRS) on Ag film over nanosphere (FON) electrodes: Surface symmetry of centrosymmetric adsorbates*. Langmuir, 2006. **22**(25): p.10354.
144. Avila, F., Ruano, C., Lopez-Tocon, I., Arenas, J. F., Soto, J. and Otero, J. C., *How the electrode potential controls the selection rules of the charge transfer mechanism of SERS*. Chemical Communications, 2011. **47**(14): p.4213.
145. Lorenz, H., Despont, M., Fahrni, N., Brugger, J., Vettiger, P. and Renaud, P., *High-aspect-ratio, ultrathick, negative-tone near-UV photoresist and its applications for MEMS*. Sensors and Actuators a-Physical, 1998. **64**(1): p.33.

APPENDICES

Appendix A

Linear Least Squares Regression Model

The LS regression fits the parameters d and b for a function $f(x) = d + bx$ to the binomial data values (x_i, y_i) , where i is the number of data points measured. The sum of the vertical difference between each measured value y_i and the model is referred to as the residual R . Squaring this value gives the sum of squares

$$R^2(d, b) = \sum_{i=1}^n [y_i - (d + bx_i)]^2 \quad (3.3)$$

The LS solution is ascertained by determining the parameter values d and b that minimizes R^2 , which is accomplished when the derivative of R^2 with respect to each parameter is equal to zero, i.e.,

$$\frac{\partial R^2}{\partial d} = 0 \quad (A.1)$$

$$\frac{\partial R^2}{\partial b} = 0 \quad (A.2)$$

where

$$\frac{\partial R^2}{\partial d} = -2 \sum_{i=1}^n [y_i - (d + bx_i)] \quad (A.3)$$

$$\frac{\partial R^2}{\partial b} = -2 \sum_{i=1}^n [y_i - (d + bx_i)] x_i \quad (A.4)$$

Which leads to

$$nd + b \sum_{i=1}^n x_i = \sum_{i=1}^n y_i \quad (A.5)$$

$$n \sum_{i=1}^n x_i d + b \sum_{i=1}^n x_i^2 = \sum_{i=1}^n y_i x_i \quad (A.6)$$

Which in matrix form yields

$$\begin{bmatrix} n & \sum x_i \\ \sum x_i & \sum x_i^2 \end{bmatrix} \begin{bmatrix} d \\ b \end{bmatrix} = \begin{bmatrix} \sum y_i \\ \sum x_i y_i \end{bmatrix} \quad (\text{A.7})$$

Matrix algebra can then be used to solve for d and b .

Mathematica Code:

```
Clear["Global`*"]

data=Import["C:\Documents and Settings\Administrator\Desktop\Justin\Least Squares DNA SERS
fitting\Spectral Data for LS\3-30-12 hyb spectra for least squares.txt","Table"]; (*import
data*)

SourceSpec=Import["C:\Documents and Settings\Administrator\Desktop\Justin\Least Squares DNA SERS
fitting\A,C,G,U Source Spectra (from 11-9-11).txt","Table"]; (*import source spectra*)

(* n1m1 n1m2 n1m3 . . n1m40
   n2m1 . . . .
   n3m1 . . . .
   . . . .
   . . . .
   n1247m1 . . . . n1247m40 *)

n=1247; (* # of spectral points*)
m=41; (*last column(i.e. spectrum) # of sample*)
xaxis=data[[2;;n+1,1]]; (* xaxis column*)

amp=SourceSpec[[2;;n+1,2]]; (*define source signals*)
cmp=SourceSpec[[2;;n+1,3]];
gmp=SourceSpec[[2;;n+1,4]];
ump=SourceSpec[[2;;n+1,5]];

output=Table[
  specdata=data[[2;;n+1,j]]; (*define spectral data for LS*)
  LinearSolve[
    Transpose[
      List[
        (*define 5x5 source signal matrix*)
        intVector=List[n,  $\sum_{i=1}^n \text{amp}[i]$ ,  $\sum_{i=1}^n \text{cmp}[i]$ ,  $\sum_{i=1}^n \text{gmp}[i]$ ,  $\sum_{i=1}^n \text{ump}[i]$  ],
        ampVector=List[ $\sum_{i=1}^n \text{amp}[i]$ ,  $\sum_{i=1}^n \text{amp}[i]^2$ ,  $\sum_{i=1}^n \text{amp}[i] + \text{cmp}[i]$ ,  $\sum_{i=1}^n \text{amp}[i] + \text{gmp}[i]$ ,
 $\sum_{i=1}^n \text{amp}[i] + \text{ump}[i]$  ],
        cmpVector=List[ $\sum_{i=1}^n \text{cmp}[i]$ ,  $\sum_{i=1}^n \text{cmp}[i] + \text{amp}[i]$ ,  $\sum_{i=1}^n \text{cmp}[i]^2$ ,  $\sum_{i=1}^n \text{cmp}[i] + \text{gmp}[i]$ ,
 $\sum_{i=1}^n \text{cmp}[i] + \text{ump}[i]$  ],
        gmpVector=List[ $\sum_{i=1}^n \text{gmp}[i]$ ,  $\sum_{i=1}^n \text{gmp}[i] + \text{amp}[i]$ ,  $\sum_{i=1}^n \text{gmp}[i] + \text{cmp}[i]$ ,  $\sum_{i=1}^n \text{gmp}[i]^2$ ,
 $\sum_{i=1}^n \text{gmp}[i] + \text{ump}[i]$  ],
        umpVector=List[ $\sum_{i=1}^n \text{ump}[i]$ ,  $\sum_{i=1}^n \text{ump}[i] + \text{amp}[i]$ ,  $\sum_{i=1}^n \text{ump}[i] + \text{cmp}[i]$ ,  $\sum_{i=1}^n \text{ump}[i] + \text{gmp}[i]$ ,
 $\sum_{i=1}^n \text{ump}[i]^2$  ],
        1],
        solutionVector=List[ $\sum_{i=1}^n \text{specdata}[i]$ ,  $\sum_{i=1}^n \text{specdata}[i] + \text{amp}[i]$ ,  $\sum_{i=1}^n \text{specdata}[i] + \text{cmp}[i]$ ,
 $\sum_{i=1}^n \text{specdata}[i] + \text{gmp}[i]$ ,  $\sum_{i=1}^n \text{specdata}[i] + \text{ump}[i]$  ] (*define measured spectrum vector*)
      ],{j,2,m}];
```

```

samplernames=data[[1,2;;m]] (*define sample names for output table*)
output1=MapThread[Prepend,{output,samplernames}]; (*construct/export table for least squares
coefficients*)
output2=Grid[Prepend[output1,{"sample","intercept","A","C","G","U"}]]
Export["C:\Documents and Settings\Administrator\Desktop\Justin\Least Squares DNA SERS
fitting\Least Squares Coefficients\3-1-12 DNA least squares fitting
coefficients.txt",output2,"Table"]

```

Appendix B

Spectra and Molecular Structure of MPh and MH

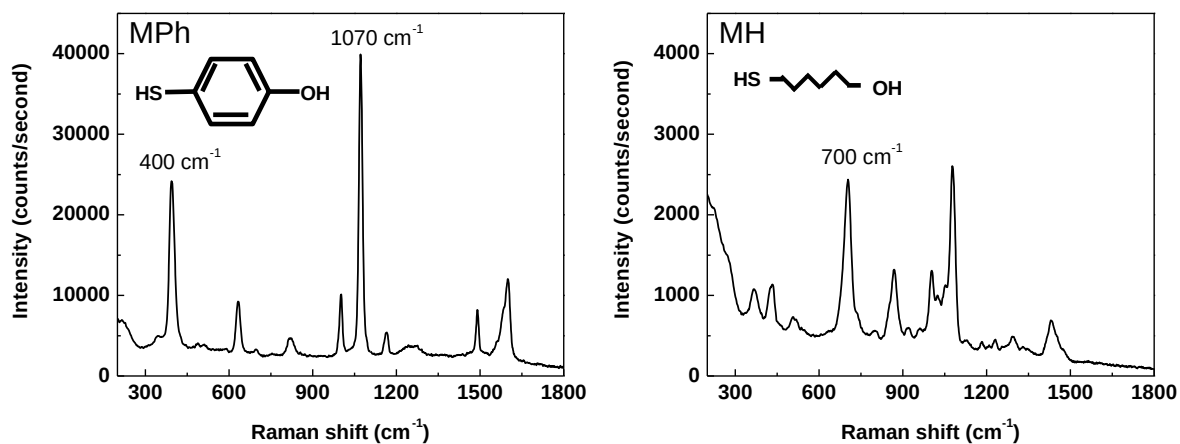


Figure B.1. Spectra (when AgNRs are dry) and molecular structure of MPh and MH. The marked peaks are those used for quantitative analysis in Chapter 4.