TANDEM OPTICAL DETECTION STRATEGIES BASED ON SURFACE ENHANCED RAMAN SCATTERING (SERS) SPECTROSCOPY

A Dissertation

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by

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Surface enhanced Raman scattering (SERS) spectroscopy is a powerful and widely used analytical tool.\textsuperscript{1-2} Not only does SERS show promise for detecting small amounts of analytes, but combining SERS with other advanced analytical methods may add insight into nanoscale static and dynamic phenomena.

In this thesis, the history and advantages of SERS are first addressed in Chapter 1, as the technique is the principal approach which enables all of this work. In the following chapters, two different approaches to develop new measurement methodologies based on SERS are introduced. First, surface plasmon resonance (SPR) spectroscopy is correlated with SERS in the Kretschmann configuration, as demonstrated by successfully recording SPR sensorgram and SERS spectra separately and simultaneously, which is introduced in Chapter 2.\textsuperscript{3} As a critical test of the measurement, the observed association constants (K_A) for streptavidin/biotin and streptavidin functionalized gold nanoparticles (STV-AuNPs)/biotin were found to agree with literature values. In addition to the typical SPR function, in the Kretschmann configuration of SPR-SERS spectroscopy, Raman
scattering from STV-AuNPs/biotinylated gold thin film was enhanced by a combination of localized surface plasmon (LSPR) from AuNPs and surface plasmon polaritons (SPS) from thin Au film. In these experiments, the SERS spectra were simultaneously detected with SPR sensorgram, providing chemical insight into binding events, which were not detectable by SPR alone. By analyzing the spectra with multivariate curve resolution (MCR) analysis, SPR-SERS spectroscopy demonstrated its potential to distinguish specific binding signals from the net sensorgram, thereby expanding the capabilities of SPR spectroscopy.

Chapter 3 introduces SERS in tandem with amperometry exploited to monitor the behavior of single Ag nanoparticles (AgNPs) functionalized with Raman reporter, 1,4-bis(2-methylstyryl)benzene (bis-MSB), when they are transported to, and captured in, single nanopores.\(^4\) To that end, highly ordered solid state nanopore electrodes arrays (NEAs) were fabricated to contain periodic arrays of nanopores, each housing a single recessed Au ring electrode. The NEAs were used to electrostatically capture and trap single bis-MSB AgNPs, characterized by simultaneous amperometry and SERS spectroscopy of AgNP oxidation and enhanced Raman scattering by bis-MSB at silver-gold hot spots, respectively. The frequency and magnitude of oxidation current spikes increased with stepwise increases in DC voltage, and characteristic bis-MSB SERS spectra were observed. Under AC excitation, on the other hand, two distinctly different types of SERS signals were observed, independent of frequency and amplitude: (1) strong, transient (< 10 s) spectra and (2) slow (> 100 s) monotonically diminishing
spectra. We hypothesize that the former behavior results from AgNP aggregates, while the latter occurs due to multiple incomplete AgNP oxidation events in succession. These results show that attoliter-volume NEAs are competent to acquire concurrent SERS spectra and amperometry of single nanoparticles and that together these measurements can illuminate the collision dynamics of the nanoparticles in confined environments.

Overall, this work illustrates how combining SERS with other analytical tools yields measurements with improved sensitivity and characterization potential. The possibility of combining with surface plasmon polariton measurements and nanopore amperometry produces a versatile technique which should be applicable to many fields.
## CONTENTS

Figures .................................................................................................................. iv

Tables ..................................................................................................................... ix

Acknowledgments .................................................................................................... x

Chapter 1: Introduction ............................................................................................... 1
  1.1 Raman Scattering ............................................................................................. 1
  1.2 Surface Enhanced Raman Scattering (SERS) Background ............................. 3
    1.2.1 Surface Plasmons ....................................................................................... 3
    1.2.2 Enhancement Mechanism ......................................................................... 5
  1.3 Applications ...................................................................................................... 6

Chapter 2: Combined SERS to Surface Plasmon Resonance (SPR) Spectroscopy .... 7
  2.1 Backgrounds ....................................................................................................... 7
    2.1.1 Surface Plasmon Resonance (SPR) ............................................................ 7
    2.1.2 SPR Sensorgram ....................................................................................... 11
  2.2 SPR-SERS Spectroscopy ..................................................................................... 12
    2.2.1 Previous Works .......................................................................................... 12
    2.2.2 Instrumentation .......................................................................................... 14
    2.2.3 Substrate Preparation ............................................................................... 19
      2.2.3.1 Materials .............................................................................................. 19
      2.2.3.2 Self-Assembled Monolayer (SAM) ....................................................... 20
      2.2.3.3 Flow Channel Fabrication .................................................................. 21
  2.3 Results ................................................................................................................ 21
    2.3.1 SPR Detection ............................................................................................. 21
    2.3.2 SERS Detection with Angle Scanning ....................................................... 26
    2.3.3 Simultaneous SPR-SERS Detection ........................................................... 28
    2.3.4 Multivariate Curve Resolution (MCR) Analysis ......................................... 35
  2.4 Conclusion and Future Works ......................................................................... 40

Chapter 3: Capture of Single Silver Nanoparticles in Nanopore Arrays Detected by
  Simultaneous Amperometry and SERS ................................................................. 41
  3.1 Backgrounds ...................................................................................................... 41
  3.2 Nanopore Electrodes Arrays (NEAs) ............................................................... 44
  3.3 Functionalization of Nanoparticles with Raman Reporter ............................... 52
    3.3.1 1,4-bis(2-methylstyryl)benzene (bis-MSB) ................................................. 52
3.3.2 Viologen Paraquat ................................................................. 62
3.3.3 Methyl-benzene thiol (MBT) ................................................... 65
3.4 Electrochemical and Raman Measurements ................................. 67
  3.4.1 Spectroelectrochemical Behavior of Nanoparticles – DC Excitation
      ................................................................................................. 68
  3.4.2 Spectroelectrochemical Behavior of Nanoparticles – AC Excitation
      ................................................................................................. 78
3.5 Conclusion .................................................................................. 84

Chapter 4: Conclusions and Future Directions ....................................... 85
  4.1 Summary of Completed Works ..................................................... 85
  4.2 Future Directions of Combined SPR-SERS Spectroscopy .............. 86
  4.3 Future Directions of Capturing Nanoparticles in NEAs by Simultaneous
      Amperometry and SERS ................................................................ 88

References ......................................................................................... 90
FIGURES

Figure 1.1 Jablonski diagram of scattered light. Left is energy diagram of Rayleigh scattering resulted from elastic collision, while middle and right diagrams illustrate the energy of photons was lost (middle, Stokes Raman) or gained (right, anti-Stokes Raman) by inelastic collision.................................................................2

Figure 1.2 Schematic illustrations of (a) surface plasmon polaritons (SPPs) and (b) localized surface plasmon resonance (LSPR). Reprinted from ref 9. Copyright 2014 American Chemical Society............................................................4

Figure 2.1 P-polarized light path in the ray-optics approximation in the Kretschmann configuration.................................................................................................................................................................................................8

Figure 2.2 Schematic diagram of the SPR effect in the Kretschmann configuration with a gold thin film.................................................................................................................................................................................................10

Figure 2.3 Schematic explanation of the way sensorgram is recorded. When SPR angle shifts by molecular binding on the surface (A and B), the reflectivity at fixed angle increases in time scale (C).................................................................................................................................................................................................................................................................11

Figure 2.4 A diagram of the SPR-SERS instrument. (BS = a beam splitter, PD = a photodiode, CCD = charge coupled device)..............................................................................................................................................................15

Figure 2.5 Theoretical SPR angle at air/gold (50 nm thickness)/N-BK7 prism interface is 72.3 degree (green solid line), while air/gold/sapphire prism has 55.1 degree of SPR angle (red solid line). The goniometer in our instrument enables scanning from 30º to 60º, so we chose a sapphire prism whose SPR angle falls within the accessible range. .................................................................................................................................................................................................16

Figure 2.6 Schematic description of the calculation to adjust an incident angle and a mechanical angle in a gold/glass/prism media.................................................................................................................................................................................................17

Figure 2.7 Experimental SPR curve (solid lines) obtained from the SPR-SERS spectroscopy is compared to theoretical value (dotted line). SPR curves in air phase are illustrated blue line while those in PBS butter are delineated in red line. .................................................................................................................................................................................................18
Figure 2.8 Schematic illustration of (A) biotin thiol/MUOH mixed SAM and (B) pure MUOH SAM on gold substrate.

Figure 2.9 (A) The diagrams illustrate the sequential steps of STV-AuNP binding to thiolated biotin monolayer on gold. (B) SPR angles at each step correlated to A. (C) SPR sensogram for the sequential steps.

Figure 2.10 (A) SPR sensogram are detected with increasing concentrations of STV diluted in PBS buffer. The average increase in reflectivity of each concentration is taken on the plateau of the sensogram and plotted in (B). In the same way, reflectivity changes of different concentrations of STV-AuNPs are recorded (C), and the average increasing values are plotted as (D).

Figure 2.11 The SERS spectra in air from STV-AuNPs (0.1 nM) drop coated on biotin/MUOH gold film measured while scanning the incident laser angle. (A) SERS spectra at specified angles. (B) SPR reflectivity (orange) compared to integrated SERS intensity (blue) along the angle.

Figure 2.12 Simultaneous detection of (A) SPR and (B) SERS from STV-AuNPs on a biotin/MUOH mixed monolayer on a gold film. The inset next to (A) is a scheme of the mixed monolayer. (C) Selected SERS spectra at specific time points.

Figure 2.13 Simultaneous detection of (A) SPR and (B) SERS from STV-AuNPs (0.3 nM) on an MUOH monolayer (without biotin) on a gold film. The inset next to (A) is a scheme of the monolayer. (C) Selected SERS spectra at specific time points.

Figure 2.14 SERS spectra from the biotin/MUOH mixed SAM (blue line, t = 665s and black line, t=700 s, respectively) and from the MUOH control surface (green line, t=845s).

Figure 2.15 MCR components (A) and their scores (B) of SERS spectra from STV-AuNPs on the biotin thiol/MUOH SAM. C and D are MCR components and scores of SERS, respectively, from STV-NPs on MUOH control surface. SPR sensorgram is below the scores to clarify the correspondence to the SPR experiment.

Figure 2.16 A scree plot of the MCR component number versus fitting value for SERS spectra from STV-AuNPs on the biotin thiol/MUOH SAM. From component 5, the percentage of error shows low value, which justifies that five components of MCR can fit the data properly.

Figure 2.17 Illustrations to explain the cases of specific (left) and non-specific (right) binding, where STV is indicated as purple, and biotin as pentagon diagram tethered on the gold surface.
Figure 3.1 Schematic diagrams illustrating an overview of NEA fabrication. Gold deposition (200 nm wide 100 nm of thick) and patterning on clean room cleaned glass cover slip (A), followed by milling nanopores with focused ion beam (FIB) on SiNx-coated gold substrate (B).

Figure 3.2 Schematic diagram of the NEA device integrated with Raman microscope and potentiostat. The NEAs, working as a working electrode, are exposed to bis-MSB-tagged-AgNPs colloids and contacted with Pt Quasi-reference electrode (QRE). The other end of gold substrate is connected to the potentiostat with metal wire.

Figure 3.3 (A) Schematic cross-sectional diagram of a single nanopore in an NEA with AgNPs captured by applied voltage. The potentiostat is used to control the potential at the Au ring working electrode vs. a Pt quasi-reference electrode (QRE). 532 nm incident field illuminated from the top. (B and C) show SEM images of nanopores. (D) cross-sectional image of the NEAs.

Figure 3.4 Simulated electric field and particle trajectories of AgNPs in the nanopores under steady-state conditions. The finite element simulations were conducted by applying constant potential, \( E = +0.5 \text{ V} \) (A) or \( +1.0 \text{ V} \) (B).

Figure 3.5 Possible diffraction-limited laser beam spots on NEAs.

Figure 3.6 UV-absorption of 80 nm citrate coated AgNPs (blue), bis-MSB solution (yellow), and AgNPs after functionalization with bis-MSB (orange).

Figure 3.7 UV-absorption of the PEG-coated AgNPs both before (40nm, blue) and after (orange) mixing with bis-MSB solution.

Figure 3.8 Examples of hydrogen bonding induced NP aggregation (A) Schematic illustration showing the configuration of hydrogen bonding. Reprinted from ref 93. Copyright 2004 American Chemical Society. (B) TEM image of aggregated gold nanorods induced by hydrogen bonding. Reprinted from ref 94. Copyright 2005 Institute of physics.

Figure 3.9 Procedures to functionalize AgNPs with Raman reporters.

Figure 3.10 (A) Schematic diagram illustrating the functionalization of AgNPs, first with 11-mercapto-1-undecanol, and then with 1,4-Bis(2-methylstyryl)benzene. (B) SERS spectrum from bis-MSB functionalized AgNPs. (C) SEM image of bis-MSB-tagged AgNPs (40 nm).

Figure 3.11 Various form of viologen dication. Viologen paraquat (right) is used in this work. Chloride ion (green dots) makes the molecules readily attached on the silver surface. Reprinted from ref 98. Copyright 2009 American Chemical Society.
Figure 3.12 (A) Reference Raman spectra of AgNPs on silicon wafer with (a) 1000 ppm, (b) 100 ppm, and (c) 50 ppm of viologen. Reprinted from ref 98. Copyright 2015 Institute of physics. (B) SERS spectrum from viologen paraquat functionalized AgNPs. Spectra were randomly taken from three different spots in the same substrate. ..........................................................64

Figure 3.13 (A) Reference Raman spectra of MBT. Reprinted from ref 100. Copyright 2009 American Chemical Society. (B) SERS spectrum from MBT functionalized AgNPs.................................................................66

Figure 3.14 Plots illustrating applied voltage schemes. (A) DC voltage program. Steady state potentials were applied in 0.1 V increments from +0.8 V to +1.4 V for 60 s each. (B) AC voltage program showing initial voltage (Init E) and amplitude (Amp). Sample interval (2 s) includes data sampling interval and impedance acquisition time (0.1739 s)..............................................................................69

Figure 3.15 Simultaneously detected current amperometric traces (A and D) and SERS heat maps (B and E) and waterfall plots (C and F) obtained under DC excitation at voltages from +0.8 V to +1.0 V (A-C) and +1.1 V to +1.3 V (D-F). SERS signals (acquisition time = 0.5 s) at all voltages are plotted on the same scale, and bis-MSB reference spectra are plotted on the right. All experiments were performed with bis-MSB AgNPs (40 nm) in 10 mM KNO₃.................................................................70

Figure 3.16 Simultaneously detected current trace (A) and SERS signals (B and C) at +1.4 V. (B) and (C) represent the SERS data in heat map and waterfall plot formats, respectively. SERS experiments (acquisition time = 0.5 s) were performed with bis-MSB AgNPs (40 nm) in 10 mM KNO₃ supporting electrolyte. .............................................................................................................71

Figure 3.17 Histograms of oxidative current transient amplitudes derived from amperometric traces in Figure 3.15 at DC voltage levels from +0.9 V to +1.3 V vs. Pt QRE. Gaussian fits to the data are given by the yellow lines. Dotted lines indicate a current level 3σ from the mean. The position of the maximum, the integrated area of the histogram, and the number of peaks exceeding 3σ are summarized in Table 3.1..............................................................................73

Figure 3.18 Amperometric traces acquired at potentials from +0.8 V to +1.4 V, with average (AVG), AVG + standard deviation (σ), and AVG + 3σ indicated for each data set. Spikes which exceed AVG + 3σ are indicated with asterisks. .................74

Figure 3.19 Nanopore status before (A) and after (B and C) +0.8 V DC voltage was applied for 60 s. A shows SEM image of NEAs before any voltage was applied. B and C were acquired after +0.8 V was applied for 60 s. Except for a couple of nanopores containing nanoparticles (indicated by red arrows in C), the majority of the pores are empty. .................................................................................................................77
Figure 3.20 SERS heat map (A) and waterfall plot (B) showing detection of bis-MSB-tagged AgNPs on NEAs under AC excitation ($V_0 = +0.8$ V, $\Delta V = +0.05$ V, $f = 28.84$ kHz). Selected SERS spectra at specific time points are plotted in C (red line = type A, black line = type B). Acquisition time for each spectrum is 0.5 s. 79

Figure 3.21 Heat map (A) and waterfall plot (B) SERS spectra of bis-MSB-tagged AgNPs on NEAs under AC excitation ($V_0 = +0.8$ V, $\Delta V = +0.005$ V, $f = 28.84$ kHz). (C) Selected SERS spectra at specific time points (red line = type A, black line = type B). Acquisition time for each spectrum is 0.5 s. 80

Figure 3.22 Waterfall plot SERS spectra of bis-MSB-tagged AgNPs on NEAs under AC excitation. ($V_0 \pm \Delta V, f$) = (0.8 ± 0.1 V, 28.84 kHz) (A); (0.8 ± 0.5 V, 28.84 kHz) (B); (0.8 ± 0.1 V, 20.0 kHz) (C); (0.8 ± 0.1 V, 10.00 kHz) (D). Acquisition time for each spectrum is 0.5 s. 81

Figure 3.23 Schematic diagram illustrating proposed mechanisms giving rise to type A and type B spectra. 83

Figure 4.1 Plasmon-waveguide resonance biosensors with lipid (A) and increased sensitivity of Au-SPR compared to plasmon waveguide resonance biosensors (B). Reprinted from ref 105. Copyright 2011 Elsevier. 86

Figure 4.2 Capturing AgNPs with a dual-ring nanopore system (left) and the threshold voltage detection (right). Reprinted from ref 76. Copyright 2018 American Chemical Society. 88
TABLES

Table 2.1 Refractive indices (RI) of matters.................................................................8
Table 2.2 Streptavidin binding to biotin thiolated surface.....................................26
Table 3.1 Amperometry characteristics ..................................................................75
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CHAPTER 1: INTRODUCTION

1.1 Raman Scattering

Spontaneous inelastic light scattering was discovered by C. V. Raman in 1928 and subsequently named for him. When the light flux encounters other particles (i.e. atoms, molecules, etc.), the majority of photons collide elastically, conserving the net kinetic energy before and after the encounters (Rayleigh scattering). On the other hand, the scattered light from inelastic collision, Raman scattering, normally loses a part of its energy in the encounter (Figure 1.1). Since this partial energy level is matched with rotation-vibrational energy, containing structural information of molecules, Raman scattering is a useful spectroscopic method to identify the chemical characteristics of molecules.

Despite its obvious benefits, the low intensity of Raman scattering limits its versatility. To overcome this drawback, various ways to enhance the intensity of scattered light have been developed. One of the most widely used methods of enhancement is surface enhanced Raman scattering (SERS), which is obtained by exploiting metal substrates, as introduced in section 1.2.
Figure 1.1 Jablonski diagram of scattered light. Left is energy diagram of Rayleigh scattering resulted from elastic collision, while middle and right diagrams illustrate the energy of photons was lost (middle, Stokes Raman) or gained (right, anti-Stokes Raman) by inelastic collision.
1.2 Surface Enhanced Raman Scattering (SERS) Background

1.2.1 Surface Plasmons

Plasmons are quantized oscillations of conduction electrons in metals, disturbed from their equilibrium status. A surface plasmon resonance (SPR) is a collective excitation of the plasmon located near the surface of metals, which is typically excited by interaction with electromagnetic radiation. There are largely two forms or surface plasmons depending on the shape of plasmonic materials (Figure 1.2) – Surface plasmon polaritons (SPPs) and localized surface plasmon resonance (LSPR). SPPs propagate along the thin (~50 nm) metal-dielectric interface, and exhibit an evanescent field which decays exponentially in the z-direction away from the surface. SPP resonances are very sensitive to the refractive index of the surrounding media and exhibit a characteristic dispersion (energy-momentum) relationship which facilitates angle tuning. Thus SPPs on thin films (Kretschmann configuration) have been extensively exploited to study kinetics of biological binding interactions using SPR spectroscopy, which is further discussed in Chapter 2.

On the other hand, when light interacts with nano-scale metallic structures smaller than the wavelength of incident field, collective resonant plasmons are induced which oscillate within the metallic particles. These collective oscillations are known as LSPRs. Recent advances in nanotechnology have enabled detailed studies of variations in LSPR caused by geometry, size, material and surroundings of metal substrate. Monitoring LSPR wavelength shift is the most commonly used measurement of changes in the local dielectric environment.
Figure 1.2 Schematic illustrations of (a) surface plasmon polaritons (SPPs) and (b) localized surface plasmon resonance (LSPR). Reprinted from ref 9. Copyright 2014 American Chemical Society.
1.2.2 Enhancement Mechanism

Since Fleischmann et al. discovered the extraordinarily high Raman scattering of pyridine in the vicinity of a silver surface in 1974, researchers have conducted intensive studies to elucidate the mechanism of SERS.\(^\text{16}\) One or both of two competing effects are typically invoked to explain SERS. Chemical (or charge transfer) enhancement results when the Raman cross section of the molecule is strengthened through electronic coupling with a metal.\(^\text{17}\) The other major mechanism is electromagnetic (EM) field enhancement.\(^\text{18-20}\) In EM enhancement, when molecules are in the vicinity of a metal substrate, surface plasmons resonate with incident and scattered radiation field, thus leading to amplification of the Raman signal.

The enhancement factor (EF) by EM enhancement can be described phenomenologically as:

\[
EF_{\text{SERS}}(\omega_\nu) = \frac{|E_{\text{out}}(\omega)|^2|E_{\text{out}}(\omega-\omega_\nu)|}{E_0^4} = \frac{[I_{\text{SERS}}(\omega_\nu)/N_{\text{surf}}]}{[I_{\text{NRS}}(\omega_\nu)/N_{\text{ex}}]} \tag{1}
\]

where \(E_{\text{out}}(\omega)\) is the incident field amplitude, \(E_{\text{out}}(\omega - \omega_\nu)\) is the EM field amplitude of Stokes’ Raman shifted radiation, \(I_{\text{SERS}}\) is the enhanced Raman intensity normalized by number of the molecules coupling to the metal, \(N_{\text{surf}}\), the normal Raman intensity, \(I_{\text{NRS}}\), and the number of excited molecules, \(N_{\text{ex}}\).\(^\text{11,21}\) The total SERS enhancement factor by LSPR can be as high as 14 orders of magnitude \((10^{14})\),\(^\text{20}\) yet it is hard to reproducibly measure EF, because controlling the morphology of hot spots at the nanoscale still remains difficult to achieve. In this context, technical advances in nanofabrication have led to various new ways to manipulate controllable metal structures including nanopore arrays,\(^\text{22-23}\) as will further be discussed in Chapter 3.
1.3 Applications

Since rotational-vibrational modes of the molecules provide chemical insights into their structure and characteristics, surface enhanced Raman scattering (SERS) spectroscopy is in high demand as a non-destructive, label-free and surface sensitive spectroscopy.\textsuperscript{1-3, 24} For example, the large enhancement enables SERS to identify samples consisting only of a single or a few molecules and to distinguish their properties from ensemble average behavior.\textsuperscript{25} Also, the use of sensitive detection by SERS can be exploited to characterize biomolecules, ranging from DNA to proteins to lipids.\textsuperscript{26-28} Furthermore, other applications - from evaluating polymer properties to identifying pigment mixtures in paintings – show the versatility of the SERS methodology.\textsuperscript{29-30} Above all, one of the most compelling attributes of SERS is the compatibility of its platform with a wide variety of samples. Generally novel metal nanostructures are used as SERS platforms to generate surface plasmons and support the Raman enhancement, allowing this technique to be coupled with other analytical methods. Two coupling examples that will be highlighted in this thesis are combining SERS with (1) surface plasmon resonance (SPR) spectroscopy and (2) amperometric trace detection on nanopores electrodes arrays (NEAs). The former benefits classic SPR angle detection for evaluating protein-ligand affinity as well as SPPs from gold thin film as Raman excitation, while the latter takes advantage of confinement in attoliter volumes in order to examine the behavior of a single or a few particles.
CHAPTER 2:
COMBINED SERS TO SURFACE PLASMON RESONANCE (SPR)
SPECTROSCOPY

Material presented in this chapter is taken, in part, from

J. Kim et al., Analytical Chemistry, 2017, 89.

2.1 Backgrounds

2.1.1 Surface Plasmon Resonance (SPR)

Classic SPR measurements are based on the excitation of SPPs in the Kretchmann configuration at the interface of two different media. When p-polarized light travels through a dense medium (i.e. glass, sapphire) into a less dense dielectric matter, the fraction of refraction and reflection follows Snell’s law (Equation 2) in the case that the incident angle ($\Theta_i$) is smaller than the critical angle ($\Theta_c$). On the other hand, if the light travels with an incident angle larger than a critical angle, the reflected light undergoes total internal reflection (TIR) (Figure 2.1). This results in an evanescent field decaying in the direction perpendicular to the prism-dielectric interface. This happens because the refractive index of the prism is higher than that of the surrounding dielectric, so that the light velocity in prism is slower than those in air/water.
Snell’s law \[ \frac{\sin \theta_t}{\sin \theta_i} = \frac{n_p}{n_d} \] (2)

where (\(\theta_i\)) is the refraction angle of transmitted light, \(n_p\) is the refractive index of prism, and \(n_d\) is that of the dielectric. Refractive indices (RI) are listed in Table 2.1.\(^{33}\)

**TABLE 2.1**

REFRACTIVE INDICES (RI) OF MATTERS

<table>
<thead>
<tr>
<th></th>
<th>Air</th>
<th>Water</th>
<th>PBS†</th>
<th>Gold</th>
<th>Sapphire</th>
<th>Glass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Refractive Indices</td>
<td>1.000</td>
<td>1.332</td>
<td>1.334</td>
<td>0.173 + 3.442i</td>
<td>1.766</td>
<td>1.52</td>
</tr>
</tbody>
</table>

†PBS: Phosphate-buffered Saline, pH 7.4

*NOTE: refractive indices at 632.8 nm wavelength.

![Diagram of light paths](image)

Figure 2.1 P-polarized light path in the ray-optics approximation in the Kretschmann configuration.
In order to give rise to SPR in the Kretschmann configuration, a thin metal film is required at the prism-dielectric interface to produce SPPs (Figure 2.2). At a specific angle where the wave vector of the evanescent field is equivalent to those of SPPs from metallic thin film, such as gold, SPR can occur as evidenced by a reduction of the reflected light.\textsuperscript{34-35} The specific angle is known as the SPR angle and it is critically sensitive to the RI values of the surrounding media as described by:

$$\theta_{SPR} = \sin^{-1} \left( \frac{1}{n_p} \sqrt{\frac{n_a^2 n_g^2}{n_a^2 + n_g^2}} \right)$$

where \( n_g \) is refractive index of gold.
Figure 2.2 Schematic diagram of the SPR effect in the Kretschmann configuration with a gold thin film.
2.1.2 SPR Sensorgram

As introduced in Chapter 1, SPR sensorgrams provide information about the molecule loading at the gold surface within the evanescent field range. Using the angle-dependent reflectance curve to identify SPR angle shift, SPR sensorgrams are recorded by fixing the excitation angle at the maximum slope of reflectivity (Figure 2.3 B), and recording the resulting reflection with time. This provides the greatest change in signal associated with changes in surface coverage of Kretschmann configuration i.e. the maximum sensitivity. Sensorgrams were constructed by monitoring the change in reflected light intensity as a function of time. Increases in reflectivity are attributed to surface association (position ① to ② in Figure 2.3), while decreases in reflectivity indicate molecules lost from the surface.

Figure 2.3 Schematic explanation of the way sensorgram is recorded. When SPR angle shifts by molecular binding on the surface (A and B), the reflectivity at fixed angle increases in time scale (C).
2.2 SPR-SERS Spectroscopy

2.2.1 Previous Works

SPR spectroscopy is a powerful analysis technique with high sensitivity and one of the most widely used methods for determining molecular affinity.\textsuperscript{37-38} Despite its advantages, non-specific interactions remain a challenging problem in SPR detection, and a number of attempts have been made to address this challenge.\textsuperscript{39-40} As one approach to address this challenge, we suggested combining SPR with Raman spectroscopy and demonstrated its implementation.\textsuperscript{3}

The idea of enhancing Raman signals with SPPs on flat metallic surfaces in a Kretschmann configuration originated in late 1960,\textsuperscript{41-43} and more recently, Etchegoin \textit{et al.} and Smith \textit{et al.} independently reported enhanced Raman signals from organic molecules such as Nile blue and pyridine.\textsuperscript{8,44} In their work, using flat metallic surfaces provides reproducible signals, but the enhancement factor of SPPs is relatively low, $10^3$-$10^4$, compared to those provided by LSPRs up to $10^{14}$.\textsuperscript{7,11,43,45} To address this problem, Xu \textit{et al.} and Chen \textit{et al.} combined SPR and SERS using silver nanoparticles (AgNPs) on a silver film in order to use LSPRs from the particles as well as SPPs from the film.\textsuperscript{46-48} In their work, Xu \textit{et al.} showed the detection of 4-mercaptopyridine, while Chen \textit{et al.} determined the secondary structures of oligonucleotides at the surface. Later, the Xu group detected protein/ligand complexes by assembling AgNPs over a dye-labeled biotin and avidin sandwich array. The results showed a 6-fold SERS enhancement and 2.5-fold increase in SPR sensitivity.\textsuperscript{49} In addition to those groups, others have also reported improved SPR sensitivity with nanoparticles.\textsuperscript{50-51}
Here, we further develop this combination platform in the Kretschmann configuration, which enables simultaneous SPR-SERS detection. To that end, we exploit streptavidin-functionalized gold nanoparticle (STV-AuNP) binding to a thiolated biotin monolayer placed in a flow channel attached on a gold film. The streptavidin (STV) and biotin complex provides a well-understood model system to assess specific and nonspecific binding due to its high affinity and well-known SERS spectra.\textsuperscript{27, 52-55} By using metallic nanoparticles, we anticipate exploiting LSPRs as well as SPPs from the thin film, leading to enhanced Raman signals from STV-biotin interaction located in particle-film hot spots. In addition, the material choice of gold ensures chemical stability and less toxicity than silver in case of biological applications.\textsuperscript{44, 56} Multivariate curve resolution (MCR) analysis of the observed SERS spectra with SPR sensorgram exhibits a potential to differentiate the specific binding of STV-AuNPs and biotin from nonspecific adsorption and background signals.
2.2.2 Instrumentation

Figure 2.4 depicts the configuration used for SPR-SERS spectroscopy. A hemicylindrical sapphire prism (RI 1.7, Team Photon Inc.) was positioned at the center of a dual-arm goniometer. Unlike classic SPR instruments, which have a rotating sample coupled with a movable detector to collect the reflected light, the goniometer in this work keeps the sample position fixed and enables efficient collection of Raman scattering from the sample. The goniometer uses two motorized rotational stages from Thorlabs Inc. that are stacked and aligned vertically. One arm of the goniometer consists of a fiber coupled 632.8 nm HeNe laser (Melles Griot). Cross polarizers in the beam path control the intensity of the laser beam. A polarizing beam splitter (N-SF1, Thorlabs Inc.) provides p-polarized radiation at the sample interface. Lastly, a convex lens (focal length 35 mm) provides a gentle focus onto the gold film. A fluidic channel is positioned on the gold surface overlapping the incident light focal spot, allowing particle colloids to flow over the biotin thiolated gold film. Fabrication steps for fluidic channel and self-assembled monolayer are discussed in 2.2.3.
Figure 2.4 A diagram of the SPR-SERS instrument. (BS = a beam splitter, PD = a photodiode, CCD = charge coupled device)
Each of the goniometer stages can move from 30° to 60°, enabling scanning over a wide range of angles with an angular resolution of 0.01 degree. Our choice of sapphire prism is based on the theoretical calculation of SPR angle (Figure 2.5). Common glass prism (N-BK7) requires > 70° of movement range to detect the SPR angle in air, while the SPR angle from a sapphire prism is 55.1°, which falls within 30° to 60° range, the accessible range of our instrumentation. Calculations are performed using SPR 4-Phase Fresnel Reflectivity Calculation program provided by Corn and coworkers. RI values are listed in Table 2.1.

Figure 2.5 Theoretical SPR angle at air/gold (50 nm thickness)/N-BK7 prism interface is 72.3 degree (green solid line), while air/gold/sapphire prism has 55.1 degree of SPR angle (red solid line). The goniometer in our instrument enables scanning from 30° to 60°, so we chose a sapphire prism whose SPR angle falls within the accessible range.
The incident radiation reflected from the gold interface is collected with a planoconvex lens (focal length 25.4 mm) and focused onto a silicon photodiode (Thorlabs Inc.) in the other arm of the goniometer. To minimize noise, a low pass electronic filter is applied to the output of the photodiode after analog-digital conversion. The goniometer angle and SPR data acquisition is controlled by LabView (National Instrument Corporation), including adjustment of difference between the incident light and mechanical angle. As Figure 2.6 describes, the light path refracts when it travels through the interface of the gold film with the glass cover. The program converts mechanical angle to incident angle based on Snell’s law of refraction (Equation 1).

\[
\alpha + \theta_3 = 90^\circ
\]

Snell’s law
\[
\frac{n_2 \cdot \sin \theta_2}{n_3} = n_3 \cdot \sin \theta_3 = n_3 \cdot \sin(90^\circ - \alpha)
\]

\[
= n_3 \cdot \cos(\alpha)
\]

\[
n_2 = 1.5, n_3 = 1.7
\]

\[
\therefore \theta_2 = \sin^{-1}\left(\frac{1.7}{1.5} \cos \alpha\right)
\]

Figure 2.6 Schematic description of the calculation to adjust an incident angle and a mechanical angle in a gold/glass/prism media.
Figure 2.7 shows the reflectivity observed from 50 nm-thick gold film on the sapphire prism to demonstrate SPR detection ability of the setup. The measured SPR angles are 36.4° in air and 55.4° in PBS buffer, in excellent agreement with calculated results, 36.4° in air and 55.1° in PBS, showing only slight mechanical error and from a difference in the substrate. Small variations likely result from different gold substrates reflecting slight uncontrollable roughness variations. The results successfully prove that the homemade SPR-SERS spectroscopy can detect the SPR angle during angle scanning.

![Graph showing SPR reflectivity](image)

Figure 2.7 Experimental SPR curve (solid lines) obtained from the SPR-SERS spectroscopy is compared to theoretical value (dotted line). SPR curves in air phase are illustrated blue line while those in PBS butter are delineated in red line.
With respect to SERS detection, enhanced Raman scattering from the gold film is collected by an objective lens (40 X, 0.75 NA, Olympus) mounted normal to the sample surface. A 633 nm dichroic mirror and 635 nm edge filter cut off Rayleigh scattering, leading Raman scattering to another objective lens (10 X, 0.25 NA) connected to a fiber coupled spectrograph (Kaiser Holoscope f/2) with charge coupled device (CCD, Andor Technology Ltd.).

Since the dichroic mirror is tilted at 45º, some portion of the light is reflected and detected on the CMOS camera (Thorlabs Inc.). An LED lamp and beam splitter are used before the camera to visualize the sample interface within the laser focal spot on gold surface.

2.2.3 Substrate Preparation

2.2.3.1 Materials

The gold film used in this work was 50 nm thick with a titanium adhesion layer on a cover glass (0.13- 0.16 mm thick), purchased from Platypus Technologies. STV-AuNPs (60 nm) and biotin functionalized polyethylene glycol thiol (25 mg, herein referred to as biotin thiol) were purchased from Nanocs Inc. Streptavidin (25 mg, Life Technologies) was diluted in phosphate buffered saline (PBS, pH 7.4, 0.1X). Sylgard-184 polydimethylsiloxane (PDMS) substrate was purchased from Dow corning corporation. Other chemicals were purchased and used as received from Sigma-Aldrich.
2.2.3.2 Self-Assembled Monolayer (SAM)

A gold surface was derivatized with mixed self-assembled monolayers (SAM) of biotin thiol and 11-mercaptop-1-undecanol (MUOH). In order to make a uniform SAM on the surface, the gold film was immersed into a mixed solution of biotin thiol (0.05 mM) and MUOH (0.45 mM) in a 1:1 volume ratio for 24 h, then rinsed with ethanol and dried in argon gas. Afterwards, the film was re-immersed in ethanol, sonicated for 3 min and dried with argon gas. The resulting mixed monolayer is illustrated schematically in Figure 2.8 A.

MUOH monolayers were also prepared for control experiments to correct for non-specific binding of streptavidin. The gold substrate is functionalized as above, except using a 0.45 mM MUOH solution instead of the mixed solution.

![Schematic illustration of (A) biotin thiol/MUOH mixed SAM and (B) pure MUOH SAM on gold substrate.](image-url)
2.2.3.3 Flow Channel Fabrication

The flow channel (12 mm × 2 mm × 0.3 mm) was made using single coated adhesive scotch tape (3M) on a flat glass slide. PDMS elastomer and curing agent were mixed in volume ratio of 10 to 1, poured on the mold and cured at 65 °C for one hour. PDMS substrate was then cut into pieces of ~20 mm × 10 mm and oxidized in plasma cleaner for two minutes. The oxidized channel was placed on the biotin/MUOH functionalized gold film. Analyte solutions (PBS, streptavidin and STV-AuNPs) were injected using a syringe pump through a capillary (O.D. 150 μm and I.D. 75.9 μm, obtained from Polymicro technologies).

2.3 Results

2.3.1 SPR Detection

As introduced in 2.2.1, the choice of streptavidin (STV) and biotin for the protein-ligand model is based on the well-known fact that the STV-biotin complex has an extraordinarily high association constant.53-54 The change in the SPR response was verified by monitoring the biotin thiol monolayer on the gold surface and subsequent absorption of STV-AuNPs (Figure 2.9).
Figure 2.9 (A) The diagrams illustrate the sequential steps of STV-AuNP binding to thiolated biotin monolayer on gold. (B) SPR angles at each step correlated to A. (C) SPR sensorgram for the sequential steps.
One specific representative experiment gave the following observation. When PBS buffer only flows in the PDMS channel, the SPR angle is 56.34° indicated as (a) in Figure 2.9. The angle shifts to 56.50° as biotin thiol molecules are linked to the gold surface (b), leading increased reflectivity in the sensorgram. Subsequently, PBS buffer resuspends loosely bound biotin molecules, resulting in a reflectivity decrease (c) as the SPR angle shifts back to 56.42°. Lastly, STV-AuNPs attach to the surface-bound biotin increasing the SPR angle to 56.66° and increasing the reflectivity. The sensorgram increase indicates the amount of molecular binding within the evanescent field.

In order to measure the association constant of STV-biotin and STV-AuNPs-biotin quantitatively, reflectivity changes from different concentrations of free STVs and STV-AuNPs were monitored over time at a fixed angle to produce a series of sensorgrams (Figure 2.10 (a), (b), and (c) (d), respectively). To that end, the solutions of STV and STV-AuNPs in PBS buffer were injected into the flow channel (flow rate 3 μL/min) onto the biotin thiol/MUOH SAM gold film. Note that STVs indicate untethered free streptavidin protein while STV-AuNPs mean streptavidin functionalized gold nanoparticles, and that the concentrations of the free protein assume that all streptavidin exists as tetramers.

The average increased reflectivity (R) was taken from the steady-state part of sensorgram for each solution, and the values are summarized in Table 2.2. These values were fitted to a Langmuir adsorption isotherm (Equation 4) to determine the association constant, $K_A$.\(^{59}\)

\[
R = R_{max} \left( \frac{C K_A}{C K_A + 1} \right)
\]  
(4)
where C is concentration of the analytes and \( R_{\text{max}} \) is maximum value of reflectivity increase.

By fitting the SPR response (\( \Delta R \)) with STV concentration to a Langmuir isotherm (Equation 4), the association constant is calculated as \( K_A = 2 \pm 1 \times 10^7 \text{ M}^{-1} \), which is in good agreement with the results reported by Tang et al.\textsuperscript{59} Similarly, the association constant of STV-AuNPs to biotinylated gold surface calculated from SPR sensorgram (Figure 2.10 C and D) is \( 2.4 \pm 0.3 \times 10^{10} \text{ M}^{-1} \). As a result, the association constant of biotinylated surface is larger for STV-AuNPs than for free STV due to avidity effects arising from the localized and increased protein coverage on the surface of functionalized nanoparticles.\textsuperscript{60}
Figure 2.10 (A) SPR sensorgrams are detected with increasing concentrations of STV diluted in PBS buffer. The average increase in reflectivity of each concentration is taken on the plateau of the sensorgram and plotted in (B). In the same way, reflectivity changes of different concentrations of STV-AuNPs are recorded (C), and the average increasing values are plotted as (D).
TABLE 2.2
STREPTAVIDIN BINDING TO BIOTIN THIOLATED SURFACE

<table>
<thead>
<tr>
<th></th>
<th>C</th>
<th>0.047</th>
<th>0.094</th>
<th>0.122</th>
<th>0.141</th>
<th>0.188</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔR</td>
<td>0.030 ± 0.001</td>
<td>0.39 ± 0.01</td>
<td>0.42 ± 0.01</td>
<td>0.41 ± 0.01</td>
<td>0.43 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>STV-AuNPs</th>
<th>C</th>
<th>0.037</th>
<th>0.075</th>
<th>0.15</th>
<th>0.30</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔR</td>
<td>0.0080 ± 0.001</td>
<td>0.038 ± 0.002</td>
<td>0.046 ± 0.002</td>
<td>0.050 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

*C: Concentration of the solution in μM, R: increase reflectivity from the bottom line in sensorgram

** STV: streptavidin diluted in PBS buffer, STV-AuNPs: streptavidin functionalized AuNPs in PBS buffer

2.3.2 SERS Detection with Angle Scanning
The adsorption of STV-AuNPs to biotinylated gold film enables detection of Raman bands characteristic of STV/biotin binding. To demonstrate SERS detection with SPR-SERS spectroscopy, 10 μL of 0.1 nM STV-AuNPs was dropped on the biotin thiolated gold film and dried at room temperature. Figure 2.11 illustrates the increased SERS response of the STV-AuNPs when excited by the SPPs at a laser focal spot. The excitation angle was scanned from 33º to 40º in 0.5º increments, using a 3 s acquisition at each angle.
Figure 2.11 The SERS spectra in air from STV-AuNPs (0.1 nM) drop coated on biotin/MUOH gold film measured while scanning the incident laser angle. (A) SERS spectra at specified angles. (B) SPR reflectivity (orange) compared to integrated SERS intensity (blue) along the angle.
In Figure 2.11, the spectra have been treated with baseline subtraction and normalization before integration. The intensity of the SERS signal is observed to increase as the angle approaches the SPR angle, and it shows the maximum response at the SPR angle (35.6°). The blue line in Figure 2.11B indicates the integration of the SERS signal and the orange line is the simultaneously detected SPR reflectivity. The SPR angle (35.6°) with lowest reflectivity matches the highest integrated intensity observed in the SERS measurement. This agrees with previous angle-dependent SERS measurements conducted independently by Smith et al. and Etchegoin et al.\textsuperscript{8,44} In addition to increased Raman scattering, the observed background arising from sapphire and silica surface in our work also matches the previous study by Smith et al.\textsuperscript{51} According to the reference, the main contribution of background is from the sapphire prism and the SiO\textsubscript{2} cover slip that supports gold film. As the incident angle is close to the SPR angle, the intensity of SPPs increase, and it leads to increase in background as well.

A small difference in the SERS intensity at the SPR angle (35.6°) and the angle of maximum slope (~ 35°) indicates that a significant evanescent field is present at a small range of angles near the SPR angle. In our SPR-SERS system, the angle remains fixed at the maximum slope during sensorgram recording as explained in Section 2.1.2. The results in Figure 2.11 indicate that the SPPs at the maximum slope are enough to excite SERS spectra nearly as efficiently as the excitation field at the SPR angle.

2.3.3 Simultaneous SPR-SERS Detection

In Figure 2.10, the SPR sensorgram obtained from functionalized nanoparticles shows larger fluctuations prior to the equilibrium portion of the curve relative to the
sensorgrams observed from free protein. This increased noise could arise from laser fluctuations; alternatively, this may reflect the more drastic changes in the RI arising from nanoparticles within the evanescent field, which may not be specifically bound to the film. Previous reports have shown that the SPR signal can be affected by a single nanoparticle in the detection volume.\(^{62-64}\) To address the problem, we conducted simultaneous SPR-SERS detection that might provide a means to differentiate between specific and nonspecific binding as shown in Figure 2.12.
Figure 2.12 Simultaneous detection of (A) SPR and (B) SERS from STV-AuNPs on a biotin/MUOH mixed monolayer on a gold film. The inset next to (A) is a scheme of the mixed monolayer. (C) Selected SERS spectra at specific time points.
STV and STV-AuNPs solution (0.3 nM) were injected into the flow channel on biotin thiol/MUOH SAM gold film (flow rate 3μL/min). While SPR reflectivity was recorded by photodiode, SERS spectra were simultaneously recorded by CCD detector. The SERS acquisition time was 5 s.

The correlated SPR/SERS signal observed from interaction between STV-AuNPs and the biotin/MUOH monolayer is shown in Figure 2.12. As STV-AuNPs interact with the surface, the SPR sensorgram (Figure 2.12 A) shows a Δ0.15 RU increase, synchronous with an increase in the SERS signal (Figure 2.12 B). At 500 s, PBS buffer is introduced to remove loosely bound particles including nonspecifically aggregated STV-AuNPs. After PBS washing at 500s, the observed equilibrium value is Δ0.052 RU. The detected SERS signal decreases in a correlated fashion, which indicates that SERS can provide additional information about chemical interactions associated with STV-AuNPs binding to biotin. The SERS spectra at specific time points are shown in Figure 2.12 C.

At 25 s, no evident binding has occurred, either in the SPR trace or the SERS spectra. From 70 s, the observed SERS bands are consistent with previous reports of streptavidin; peaks at 1290-1300 cm\(^{-1}\) and 1440-1460 cm\(^{-1}\) are assigned to methylene (CH\(_2\)) and methyl (CH\(_3\)) deformations, while the peaks at 1350 cm\(^{-1}\) and 1560 cm\(^{-1}\) are associated with C-N vibrations of tryptophan (Trp) in streptavidin.\(^{55}\) Peaks at 1045-1050 cm\(^{-1}\) and 1160-1170 cm\(^{-1}\), observed after PBS washing, are attributed to either biotin or streptavidin.\(^{27}\) The observed SERS signal in the equilibrium region is well conserved, as noted by the similarity of the spectra at 665 and 700 s in Figure 2.12 C. The SERS spectrum of the biotin/STV/AuNPs is reported to be markedly different from that of the
aggregated STV-AuNPs.\textsuperscript{55} In particular, bands observed at 1047-1053 and at 1132 and 1173 cm\textsuperscript{-1} associated with biotin provide clear evidence of the biotin/STV interaction.

To further assess the chemical specificity of the SERS spectrum, SPR-SERS was performed using a MUOH monolayer on the gold film without biotin (Figure 2.13). Even in the absence of biotin on the substrate, the SPR sensorgram shows increases, strongly suggesting that they result from nonspecific binding of the functionalized nanoparticles. Most of these particles are removed after washing with PBS; however, the sensorogram shows a small increase, Δ 0.02 RU, and SERS signals are still observed, indicating nanoparticles are still adsorbed to the surface. After 370 s, there are common peaks at 1530 -1540 cm\textsuperscript{-1} and 1440- 1450 cm\textsuperscript{-1} (CH\textsubscript{2} and CH\textsubscript{3}) attributable STV-AuNPs.\textsuperscript{55} Other time point spectra show sporadic peaks at 1220-1230cm\textsuperscript{-1} (700 and 845 s) and 1600 cm\textsuperscript{-1} (370 and 845 s). Peaks at 1140 and 1358 cm\textsuperscript{-1} temporarily emerge at 370 s and 535 s, then disappear after washing with PBS. Interestingly, several distinct peaks observed in the specific binding experiment are not present in the spectrum with a MUOH monolayer, as further specified in Figure 2.14.
Figure 2.13 Simultaneous detection of (A) SPR and (B) SERS from STV-AuNPs (0.3 nM) on an MUOH monolayer (without biotin) on a gold film. The inset next to (A) is a scheme of the monolayer. (C) Selected SERS spectra at specific time points.
Figure 2.14 illustrates differences in the SERS spectrum from the equilibrium region of the SPR sensorgram obtained from the biotin thiolated/MUOH SAM and the control surfaces. The signals are reprinted from Figure 2.12 and 2.13 C. Strong peaks at 1047-1053 cm\(^{-1}\) (biotin), 1132 cm\(^{-1}\) (C-N and valine), 1173 cm\(^{-1}\) (phenylalanine, tyrosine, valine) and 1242 cm\(^{-1}\) (ureido ring) are only shown in biotinylated surface but not on the control surface (indicated by the dotted lines). Among those, the peaks at 1132 cm\(^{-1}\) and 1173 cm\(^{-1}\) match previous studies of streptavidin and biotin complexes.\(^55\)

![Figure 2.14 SERS spectra from the biotin/MUOH mixed SAM (blue line, t = 665s and black line, t=700 s, respectively) and from the MUOH control surface (green line, t=845s).](image-url)
2.3.4 Multivariate Curve Resolution (MCR) Analysis

To further investigate the ability to discriminate between specific and non-specific binding, multivariate curve resolution (MCR) analysis was performed on the time dependent SERS data. MCR analysis computes the effect of pure components and the amount of their contribution to total data represented in scores. In this work, MCR analysis is used on the SERS spectra observed in 2.3.3 to analyze them into five pure components with their score (Figure 2.15). Features at 1335-1350, 1560, and 1590 cm\(^{-1}\) are assigned to tryptophan.\(^{55}\) The peaks around 1130-1149 cm\(^{-1}\) mainly come from valine and C-N functional group. Asterisks (*) indicate components from CH\(_2\) and CH\(_3\).\(^{55}\) SPR sensorgrams are given below the scores to clarify the correspondence to the SPR experiment.

We chose to use five components as indicated in a scree plot associated with MCR analysis (Figure 2.16). The scree plot shows the possible error value correlating the component numbers used in the analysis. This result justifies that using a 5-component of MCR provides low error values while maintaining a relatively simple system.

The MCR scores show the contribution of each component to the observed spectrum at each time point (Figure 2.15 B). The MCR analysis of the MUOH control surface is shown in Figure 2.15 C and D. The peaks at 1335-1350, and 1590 cm\(^{-1}\) are assigned to tryptophan and asterisks (*) indicate components from CH\(_2\) and CH\(_3\) modes.\(^{55}\)
Figure 2.15 MCR components (A) and their scores (B) of SERS spectra from STV-AuNPs on the biotin thiol/MUOH SAM. C and D are MCR components and scores of SERS, respectively, from STV-NPs on MUOH control surface. SPR sensorgram is below the scores to clarify the correspondence to the SPR experiment.
Figure 2.16 A scree plot of the MCR component number versus fitting value for SERS spectra from STV-AuNPs on the biotin thiol/MUOH SAM. From component 5, the percentage of error shows low value, which justifies that five components of MCR can fit the data properly.

Figure 2.17 Illustrations to explain the cases of specific (left) and non-specific (right) binding, where STV is indicated as purple, and biotin as pentagon diagram tethered on the gold surface.
To understand the different components, we consider possible sources of SERS signals. One possible source for non-specific SERS signal is nanoparticle aggregation. Aggregated AuNPs can evince a significant Raman signal, if they are within the evanescent field. Additionally, if these aggregated particles adsorb in the detection region, an SPR response would be observed. Using the combination of the SERS signal, SPR signal, and solution conditions, it is possible to use the MCR results to interpret the origin of the different signals. In Figure 2.15 A and B, component 3 is attributed to the SERS from non-specifically absorbed nanoparticles aggregates. As the aggregated particles are not tightly fixed on the surface, the SERS intensity would be expected to fluctuate as the nanoparticle containing solution flows. For example, the SERS spectrum at 70 s shows an out-of-trend higher intensity than other timepoints (Figure 2.12 C). Based on our hypothesis of aggregation, the corresponding MCR analysis at 70 s attributes this signal to component 3. Additionally, a spike is observed in the SPR sensorgram at 70 s, which is consistent with an aggregate at the surface. A similar trend is observed between 200-300 s for component 4. The MCR scores for both components 3 and 4 are almost zero after PBS washing, further associating these signals with nonspecific aggregates.

On the other hand, components 1 and 2 are considered to result from AuNPs binding to the surface, because their components not only remain after washing, but they also comprise a larger fraction of the total signal after 500 s. As the scores represent the fraction of the total signal, such that specific binding likely occurs before 500 s, but its relative contribution is diminished in the presence of background and non-specifically adsorbed particles and aggregates. After washing, the relative values of specifically-
bound components increase in percentage due to a diminished non-specific binding portion. The peaks observed in component 1 and 2 match reference spectra from biotin-STV-NPs complex\textsuperscript{55} confirming that they represent specific-binding. It is not clear why there are two components attributed to specific binding, but they may reflect specific binding of aggregates or possibly orientation differences with respect to the gap mode providing enhancement. It is, of course, also possible of 2 components in the MCR does not have a physical origin, but rather is a feature of the mathematical decomposition.

The assignment of non-specific binding and aggregates is further supported by the same MCR analysis performed with SERS from the control MUOH surface (Figure 2.15 C and D). Component 4 is highly similar to component 3 of biotin/MUOH surface in Figure 2.14 A and B, which was assigned to weakly adsorbed aggregates. The time dependent score of component 5 is also consistent with a weakly adsorbed aggregate. As expected, the scores of both components disappear after PBS washing. Meanwhile, component 2 and 3 are attributed to more strongly bound non-specific interactions of the STV-NPs onto the MUOH surface, whose score remains after washing. Here the SERS spectra show only peaks from streptavidin and can be discerned not to arise from specific interactions by the lack of peaks attributable to the biotin-STV complex.

Interestingly, the luminescence background previously observed at the SPR angle (Figure 2.11) is shown in MCR analysis of both biotin/MUOH and MUOH surface in common (component 5 and 1, respectively). The score of the luminescence associated component decreases as AuNPs interact to the surface. As the score is a relative measure of the total signal, this apparent decrease results from the other (i.e. Raman) components’ increasing contributions to the overall signal.
From the relative scores in the MCR analysis, we calculated the fraction of specific binding to nonspecific binding, as well as aggregation observed in the equilibrium region of the SPR sensorgram from 500 to 900 s. At each time point in Figure 2.15 B, the score for each component is divided by the sum of all 5 scores and Q residuals to calculate an average percentage. The fractions of component 1 and 2, which we attribute to specific binding are 31.11 and 57.09 %, respectively. The nonspecific binding and aggregation comprise 4.11 % (component 3) and 5.20 % (component 4). Background luminescence contributes 0.67 %, and 1.82 % remains unassigned in the residuals. Thus, 88.20 % of total signal is attributed to specific binding.

2.4 Conclusion and Future Works

In conclusion, we successfully demonstrated the combination of SPR-SERS to distinguish specific and non-specific binding from functionalized nanoparticles. Matching calculated SPR reflectivity curves and reproducing the $K_A$ of streptavidin for biotin in solution validated the measurement. The avidity effect of functionalizing a nanoparticle with streptavidin was demonstrated by measuring a $10^3$ higher affinity for the functionalized nanoparticles with maximum SERS intensity at the SPR angle. Using the SERS signal arising from the gap mode between the nanoparticle and SPR film, simultaneous SPR-SERS sensorograms were measured in real time without fluorescence labels. MCR analysis demonstrated that SPR-SERS instrument can differentiate between specific and non-specific binding. We believe that the combination of SPR-SERS and MCR had the potential to improve studies of nanoparticle affinity relevant to nanomedicine and other applications.
CHAPTER 3:
CAPTURE OF SINGLE SILVER NANOPARTICLES IN NANOPORE ARRAYS
DETECTED BY SIMULTANEOUS AMPEROMETRY AND SERS

Material presented in this chapter is taken, in part, from

J. Kim et al., Analytical Chemistry, 2019, Submitted.

3.1 Backgrounds

Recently, metal nanomaterials have garnered considerable attention due to their applications in fields as diverse as electrocatalysis, biomolecular sensing and environmental chemistry. Supporting these extensive applications requires understanding of the fundamental behavior of individual nanoparticles, in contrast to conventional ensemble studies that reflect averaging effects that convolve the characteristics of distinct subpopulations. To meet this challenge a number of approaches have been developed to investigate the behavior of single (or a few) nanoparticles. Examining the collisions between single nanoparticles and an electrode is one informative approach which can reveal the kinetics of direct electron transfer processes at the single particle level. For example, Compton and coworkers developed a method for addressing the oxidation of single nanoparticles impacting solid ultramicroelectrodes, and Zhang, White, and their coworkers studied individual nanoparticles impacting nanoelectrodes and suggested an oxidation mechanism involving
multiple collisional events of the same nanoparticle to explain their observations.\textsuperscript{67,70} Other groups have conducted statistical studies that illustrate how the electrochemical dynamics of individual nanoparticles contrast with ensemble behavior.\textsuperscript{71-73}

Recent technical developments in nanofabrication open up a wide range of possibilities for nanostructures which can facilitate experiments that add insight into collisions between nanoparticles and an electrode. Zero-dimensional (\textit{i.e.} all three dimensions at the nanoscale) nanopore electrodes are one representative example - due to their attoliter volumes and polarizable metallic substrate, these nanopore structures enable the observation of the particle-electrode interaction under well-controlled, confined conditions so that nanoparticle behavior can be isolated from bulk colloidal motions and quantified.\textsuperscript{74-75} Our laboratory has developed a series of recessed ring-disk and dual-ring nanopore electrode arrays (NEAs) and used these to explore nanoscale electrochemical phenomena of both molecular and particulate analytes.\textsuperscript{76-77} By investigating single nanoparticles \textit{in situ} and evaluating the oxidative current transients observed in amperometry, we showed that nanoparticle uptake into the nanopore can be gated by the potential applied to the top (nearest bulk solution) electrode. The observed threshold voltages were found to control nanoparticle access to the bottom (working) electrode and to determine the extent of direct electron transfer processes at the single particle level.\textsuperscript{76}

In addition to their utility in electrochemical experiments, NEAs are also compatible with optical measurements when one or both interfaces are transparent, \textit{i.e.} single- or dual-ring nanopore electrode configurations. These conditions, then, enable single entity optical experiments that can be combined with electrochemical
investigations by constraining the focal volume near or below the diffraction limit.\textsuperscript{22-23, 78}

Surface enhanced Raman scattering (SERS) spectroscopy, in particular, stands to benefit from these structures, since information about molecular structure and inter-molecular interactions can be acquired by exploiting the capacity of metallic nanopores to act as enhancing substrates, thereby amplifying the inelastic light scattering to detectable levels.\textsuperscript{79-80} Because Raman enhancement from localized surface plasmon resonance (LSPR) has been measured to be as large as $10^{14}$, the combination of SERS\textsuperscript{11} and confined nanostructures is of interest for possible applications to phenomena ranging from single nanoparticle studies to DNA sequencing.\textsuperscript{25, 45, 81}

Taking advantage of these characteristics, we have introduced simple single-ring nanostructures in which electrochemical measurements (amperometry) are coupled with SERS spectroscopy (EC-SERS) to detect the capture of single SERS-tagged nanoparticles and subsequent redox-active collisions with the nanopore electrodes.\textsuperscript{4} Solid state NEAs were fabricated with controlled pore size, interpore distance, and electrode spacing.\textsuperscript{22} and AgNPs were labeled with the electrochemically stable Raman reporter molecule, 1,4-bis(2-methylstyryl)benzene (bis-MSB). The behaviors of the nanoparticles were carefully characterized under both DC and AC voltage excitation by analyzing the current trajectory from the entire NEA and the bis-MSB SERS spectra from a single nanopore. The evidence obtained from these tandem signals, enables us to propose a mechanism to explain the collisional motion of nanoparticles under different applied potential conditions.
3.2 Nanopore Electrodes Arrays (NEAs)

3.2.1 Fabrication of NEAs

To start, NEAs with a single gold electrode in each nanopore were fabricated. The process scheme used to prepare NEAs is illustrated in Figure 3.1. Cleanroom cleaned glass coverslips (75 x 25 mm, 0.17 mm thick) were obtained from Schott Nexterion and patterned by photolithography with AZ5214E photoresist (PR). PR AZ5214E (AZ Electronic Materials) was purchased and used according to the manufacturer’s specifications. Commercial polydimethylsiloxane film (PDMS) was obtained from Specialty Manufacturing, Inc.

Au (100 nm) and Ti (10 nm) were deposited by electron-beam evaporation (UNIVEX 450B, Oerlikon) on the patterned glass coverslip, followed by an acetone lift-off to remove the photoresist. After ~120 nm of SiNₓ was deposited by plasma-enhanced chemical vapor deposition (PECVD 790, Plasma-Therm), a dual-source FIB instrument (Helios Nanolab 600, FEI Corp.) was used to mill an array of nanopore-confined ring electrodes. Nanopores were patterned in a 30 μm × 30 μm square array with a spacing of 500 nm. FIB milling was performed at 30 kV acceleration, 0.28 nA ion aperture, and 0.1 ms dwell time to produce the nanopore array. In order to connect the nanopores to the electrical and optical readout systems, device NEAs with large connection pads were fabricated.
Figure 3.1 Schematic diagrams illustrating an overview of NEA fabrication. Gold deposition (200 nm wide 100 nm of thick) and patterning on clean room cleaned glass cover slip (A), followed by milling nanopores with focused ion beam (FIB) on SiNx-coated gold substrate (B).

Figure 3.2 schematically depicts the integration of the NEA devices with the potentiostat and the confocal Raman microscope. A glass slide (75 mm by 25 mm, 1 mm thick) was first drilled to allow fluid contact to the NEAs, then cleaned with DI water and dried under N$_2$(g). The slide was attached to the nanopore arrays using double-sided adhesive tape (no. 5603, Nitto). A 0.030” thick PDMS template was cut and attached around the NEA opening to serve as a reservoir of AgNPs.
Figure 3.2 Schematic diagram of the NEA device integrated with Raman microscope and potentiostat. The NEAs, working as a working electrode, are exposed to bis-MSB-tagged-AgNPs colloids and contacted with Pt Quasi-reference electrode (QRE). The other end of gold substrate is connected to the potentiostat with metal wire.
3.2.2 Characterization of NEAs

NEAs were fabricated by consecutive steps of photolithography, film deposition and focused ion beam (FIB) milling, to produce an array of SiN$_x$-covered recessed nanopore ring electrodes, as shown schematically in Figure 3.3 A.$^{22,76}$ The whole array consists of 3721 nanopores in a 30 μm × 30 μm area with 500 nm inter-pore spacing. Figure 3.3 B and 3.3 C show plan view scanning electron micrograph (SEM) images of the entire array and a magnified view of the nanopores, respectively. Each nanopore contains a 100 nm thick single gold ring electrode to support AgNP electrochemistry, and because the pores were milled into a single planar Au film (viz. Figure 3.1), all of the ring electrodes display the same potential. In order to restrict the electrical signal solely to the AgNPs transported into the pore, the top surface of the Au electrode NEA is insulated with SiN$_x$. Figure 3.3 D illustrates a cross-section of the pores with the Au electrodes covered with a SiN$_x$ passivation layer. In a typical structure top and bottom diameters of the nanopores were measurable to be ~280 and ~173 nm, respectively, and the height to be ~192 nm. Assuming the pore is a truncated cone, we can use the equation 5 to calculate the volume.

$$Volume\ of\ Truncated\ cone = \frac{1}{3} \times \pi \times h \times (r_1^2 + r_2^2 + r_1 \times r_2)$$ (5)

where $r_1$ is the radius of the pore opening, $r_2$ is the radius of the pore bottom, and $h$ is the height. Therefore, the volume of the conical frustum shape of one nanopore is $\sim 7.9 \times 10^{-18}$ L = 7.9 aL.
Figure 3.3 (A) Schematic cross-sectional diagram of a single nanopore in an NEA with AgNPs captured by applied voltage. The potentiostat is used to control the potential at the Au ring working electrode vs. a Pt quasi-reference electrode (QRE). 532 nm incident field illuminated from the top. (B and C) show SEM images of nanopores. (D) cross-sectional image of the NEAs.
The AgNP concentration of $1.62 \times 10^{14}$ particles L$^{-1}$ indicates an average pore occupancy, $\langle n \rangle_{\text{pore}} = 0.0013$ and an average array occupancy, $\langle n \rangle_{\text{array}} = 4.7$, so at any given time under random filling conditions, the array should house an average of ~5 particles, and the likelihood of observing a nanoparticle in a single nanopore by optical means would be negligible. However, once the PDMS reservoir was filled with 400 μL of functionalized AgNPs colloids, a negative voltage applied to the Au working electrode was observed to greatly enhance transport of nanoparticles into the nanopores.\textsuperscript{76}

In order to predict the behavior of nanoparticles, theoretical modeling has been conducted. Numerical calculations were performed with a finite element method using COMSOL Multiphysics version 5.3. We used the ‘Electrostatics’, and ‘Charged Particle Tracking’ physics of COMSOL in a time-dependent mode to obtain AgNP trajectories. An NEA of 10 nanopores was simulated in a 2D geometry, consisting of a 100 nm thick recessed ring electrode and a 100 nm thick top insulating layer (SiNx). The geometry was adapted to match scanning electron micrographs (SEMs) of FIB cross-sections. The domain above the pores was drawn sufficiently large ($w = 1000 \mu m$, $h = 1000 \mu m$) to avoid interference from boundaries, and the mesh was refined both within the nanopores and in the region just above the pores to provide sufficient resolution. In this work, the SiNx pore walls were assigned a surface charge density, $\rho_{\text{surface}} = -1 \text{ mC/m}^2$.

Figure 3.4 shows the simulated electric field and particle trajectories of AgNPs in the nanopores under steady-state conditions. Finite element simulations were conducted under a constant potential at the working electrode, WE, $E_{\text{WE}} = +0.5 \text{ V}$, Figure 3.4 A, and $E_{\text{WE}} = +1.0 \text{ V}$, Figure 3.4 B. As Figure 3.4 A illustrates, at small positive potentials, $E_{\text{WE}}$, transport of AgNPs into the nanopores is limited. Using the nomenclature introduced
previously by this laboratory, these are gate-closed conditions. This behavior is expected, because the AgNPs are anionic and are repelled by the negatively charged nanopore surface. However, as EWE becomes more positive, EWE ≥ +1.0 V, it overcomes the negative surface charge, and the gate opens, allowing negatively charged AgNPs to enter the nanopores, Figure 3.4 B. The simulation results demonstrate that the positive potential not only gates, i.e. controls, the entry of AgNPs into the nanopore but also produces an electric field that can assist nanoparticle transport once the nanoparticle enters the pore.

Figure 3.4 Simulated electric field and particle trajectories of AgNPs in the nanopores under steady-state conditions. The finite element simulations were conducted by applying constant potential, $E = +0.5$ V (A) or +1.0 V (B).
Furthermore, once the AgNPs enter the nanopores they can be probed by both amperometry and by SERS. An important difference between these two signals is that the amperometric signal is integrated across the entire array of nanopores, because the Au ring electrodes are all derived from the same planar Au film, while the SERS spectra are acquired from a single nanopore. When the AgNPs collide with the gold surface, oxidation may occur, giving rise to transient spikes in the amperometric traces. At the same time, Raman scattering from bis-MSB reporter molecules is excited at 532 nm, and SERS enhancement is obtained, most likely arising across the gap between the AgNPs and the proximal Au electrode in the nanopore. Considering that the excitation wavelength ($\lambda$) is 532 nm and the numerical aperture, NA, of the objective is 1.0, the diffraction-limited laser spot diameter ($d$) is 649 nm, according to the equation (6).

$$d = 1.22 \times \frac{\lambda}{NA} \quad (6)$$

Figure 3.5 shows several possible diffraction-limited laser beam spots relative to the nanopores in the NEAs. Since the top diameter of the nanopore is ~280 nm, and the interpore spacing is 500 nm, these calculations support the assertion that the optical sampling area that covered by the illumination is ~ 1 nanopore.

![Figure 3.5 Possible diffraction-limited laser beam spots on NEAs.](image)
3.3 Functionalization of Nanoparticles with Raman Reporter

3.3.1 1,4-bis(2-methylstyryl)benzene (bis-MSB)

In order to obtain SERS signals from nanoparticles, AgNPs were functionalized with a Raman reporter. The first Raman reporter we used is bis-MSB, which has been widely used as optical label due to its strong excited state polarizability, which is ca. 10,000 times higher than that of the ground state.\textsuperscript{82-83} In addition, bis-MSB is electrochemically inactive, making it suitable to provide consistent Raman spectra independent of applied potential.

Three different strategies were employed to immobilize bis-MSB to the AgNP surface. First, we tried to physisorb bis-MSB on the AgNP surface. We anticipated that bis-MSB molecules would physically adsorb on the silver surface as previously demonstrated by Maynard \textit{et al.} in 1988,\textsuperscript{84} and supported by computational simulations.\textsuperscript{85-86} According to the literature, π-π bonding of benzene ring can adsorb to metal surface.

Silver nanoparticles (80 nm, citrate-stabilized, 90 pM or 5.4 \times 10^{10} particles/mL) were purchased from nanoComposix. Bis-MSB was obtained from Sigma-Aldrich and prepared in ethanol and acetone (pure, 200 proof, HPLC/spectrophotometric grade). AgNPs were mixed with 0.4 mM bis-MSB solution (details of the mixing process are discussed with Figure 3.9), and UV (ultra violet)- spectra of the resulting AgNPs were taken as shown in Figure 3.6.
Figure 3.6 UV- absorption of 80 nm citrate coated AgNPs (blue), bis-MSB solution (yellow), and AgNPs after functionalization with bis-MSB (orange).
In Figure 3.6, the blue line indicates the absorption spectrum from citrate-coated AgNPs, and the yellow line is obtained from bis-MSB solution for comparison. The orange line is obtained from AgNPs after mixing with bis-MSB solution. The peak of the resulting spectrum at 466 nm shows a 4 nm red-shift from AgNPs before treatment. This indicates that the diameter of AgNPs has slightly increased. Still, strong peak at 346 nm indicates that substantial amounts of bis-MSB remained unreacted in the colloidal solution.

For better functionalization, a second attempt was made to bring the reactants together at the surface by replacing negatively charged citrate with neutrally charged PEG (polyethylene glycol) coated AgNPs (40 nm, 90 pM or $5.4 \times 10^{10}$ particles/mL, nanoComposix). By doing so, it was expected that bis-MSB would adsorb on the PEG coated Ag surface. To test this hypothesis, the PEG particles were mixed with 0.4 mM of bis-MSB solution, and the UV-vis spectra shown in Figure 3.7 were acquired.
Figure 3.7 UV- absorption of the PEG-coated AgNPs both before (40nm, blue) and after (orange) mixing with bis-MSB solution.
Figure 3.7 shows UV absorption of PEG-coated AgNPs (blue line) and the same NPs after mixing with 0.4 mM bis-MSB (orange). The peaks of the spectra show a negligible red-shift with significantly decreased intensity. Because of the 1 nm shift is not significant, and the large decrease in intensity implies the possibility of nanoparticles aggregation. We tried a third strategy.

Lastly, we used the so-called organic pocket method. Rotello et al. introduced NPs functionalized with an alkanethiol terminated without zwitterionic headgroup, thus featuring hydrophobic interior and hydrophilic exterior. The idea is to use the hydrophobic layer to entrap organic ‘guest’ molecules inside. The NPs can be dissolved in polar solvent due to their ionic tail. When the NPs are re-dissolved in organic solvent the guest molecule can be released. Because of this partitioning feature, the hydrophobic pocket NPs are widely used in drug delivery.

In the functionalization process, the right choice of organic molecules and concentration control is crucial to avoid nanoparticle aggregation. If the concentration of the organic molecules with hydroxyl group is too high, hydrogen bonding can occur between the particles, leading to agglomeration as shown in Figure 3.8.
Figure 3.8 Examples of hydrogen bonding induced NP aggregation (A) Schematic illustration showing the configuration of hydrogen bonding. Reprinted from ref 93. Copyright 2004 American Chemical Society. (B) TEM image of aggregated gold nanorods induced by hydrogen bonding. Reprinted from ref 94. Copyright 2005 Institute of physics.
After various attempts in wide range of the concentration, we chose to use 0.03 mM 11-mercapto-1-undecanol (97%, MUD), obtained from Sigma-Aldrich and prepared in ethanol. The protocol of functionalization process is detailed in Figure 3.9. First, 1000 μL of 40 nm AgNPs was centrifuged at 10,000 rpm for 20 min (microcentrifuge RS-200, REVSCI); then the solvent was decanted, and the AgNPs were resuspended in ethanol and sonicated for 5 min. The colloid was spun down again, and the solvent was replaced with a 0.03 mM solution of MUD in ethanol. The displacement reaction was allowed to proceed for > 15 min during which time the MUD displaced citrate on the Ag surface. The resulting derivatized AgNPs were purified as just described. The solvent was decanted, and the AgNPs were resuspended in 0.4 mM bis-MSB solution and allowed to stand overnight. Then the colloid was centrifuged at 8000 rpm for 15 min. The majority of the solvent was decanted, leaving ~30 μL, to which 300 μL of 10 mM KNO₃ supporting electrolyte was added, and the particles were finally resuspended one more time, yielding 330 μL of ~270 pM functionalized AgNP solution.
By following the functionalization steps described above, the alkanethiol chains of MUD form a hydrophobic layer around the particles into which the bis-MSB can partition, yet the hydroxyl group of MUD helps the particles remain dispersed in ethanol. Once the nanoparticles were mixed with bis-MSB, the Raman reporters were encapsulated in the hydrophobic organic layer, as shown schematically in Figure 3.10 A. The 11-carbon alkyl chain of MUD provides sufficient volume for bis-MSB to be fully incorporated. Finally, functionalized AgNPs solution were concentrated and dispersed in potassium nitrate (KNO₃) supporting electrolyte to a final colloidal concentration of 270 pM (1.62 x 10¹⁴ particles/L). Potassium nitrate (KNO₃, Sigma-Aldrich) was prepared in deionized (DI, ρ = 18.2 MΩ cm) water obtained from a Milli-Q Gradient water purification system (Millipore).

In order to ascertain if functionalization was successful, we took Raman spectra from the AgNPs. To that end, 5 μL of the bis-MSB AgNP solution was dropped onto a
glass cover slip and dried, after which a SERS spectrum was observed from the nanoparticles, Figure 3.10 B. The Raman spectrum of the bis-MSB-tagged AgNPs is in excellent agreement with a bis-MSB reference spectrum,\textsuperscript{96} confirming that AgNPs are successfully functionalized with bis-MSB and that the bis-MSB SERS dominates background scattering from MUD. Hereafter, we refer to these functionalized nanoparticles as bis-MSB-tagged AgNPs.\textsuperscript{97}
Figure 3.10 (A) Schematic diagram illustrating the functionalization of AgNPs, first with 11-mercapto-1-undecanol, and then with 1,4-Bis(2-methylstyryl)benzene. (B) SERS spectrum from bis-MSB functionalized AgNPs. (C) SEM image of bis-MSB-tagged AgNPs (40 nm).
3.3.2 Viologen Paraquat

The second Raman reporter we tried is viologen paraquat (methyl viologen dichloride hydrate). Viologens are organic compounds with the formula of \((\text{C}_5\text{H}_5\text{NR})_2\text{H}^+\), widely used in synthesizing herbicides. Guerrini et al. detected SERS from viologen functionalized silver nanoparticles. Also, viologen can form charge-transfer complexes with silver surface in the presence of chloride ions (Figure 3.11 A). Thus, the Raman reporter can be readily attached on the surface of nanoparticles without extra treatment.

Figure 3.11 Various form of viologen dication. Viologen paraquat (right) is used in this work. Chloride ion (green dots) makes the molecules readily attached on the silver surface. Reprinted from ref 98. Copyright 2009 American Chemical Society.
The functionalization protocol follows Figure 3.9. 95 1000 μL of AgNPs (50 nm, citrate-stabilized, 90 pM or 5.4 x 10^10 particles/mL, nanoComposix) was centrifuged at 10,000 rpm for 20 min. After the solvent was decanted, the AgNPs were resuspended in ethanol and sonicated for 5 min. 95 The colloid was spun down again, and the solvent was replaced with 0.05 mM viologen paraquat (Sigma-Aldrich) diluted in ethanol. After >15 mins waiting for displacement reaction, the resulting derivatized AgNPs were centrifuged at 8000 rpm for 15 min. The majority of the solvent was decanted, leaving ~30 μL, to which 970 μL of supporting electrolyte or ethanol was added, and the particles were finally resuspended one more time, yielding functionalized AgNP solution.

Similar to the functionalization process with bis-MSB, Raman spectra from the AgNPs were obtained to ascertain if functionalization was successful. 5 μL of the resulting AgNPs colloidal solution was dropped onto a glass cover slip and dried, and SERS spectrum was observed from the nanoparticles (Figure 3.12). 99 The resulting Raman spectrum from paraquat functionalized AgNPs was a good match with the reference spectrum, indicating that AgNPs are successfully functionalized with viologen molecules.
Figure 3.12 (A) Reference Raman spectra of AgNPs on silicon wafer with (a) 1000 ppm, (b) 100 ppm, and (c) 50 ppm of viologen. Reprinted from ref 98. Copyright 2015 Institute of physics. (B) SERS spectrum from viologen paraquat functionalized AgNPs. Spectra were randomly taken from three different spots in the same substrate.
3.3.3 Methyl-benzene thiol (MBT)

The last Raman reporter we tried is 4-methyl-benzene-thiol (MBT, Sigma-Aldrich). MBT is widely used as model Raman reporter, since its thiol group can form strong bonds on the metal surface. Also, its simple structure provides predictable spectra.

Similarly, 40 nm of citrate coated AgNPs were functionalized following the steps described Figure 3.9. The Raman spectra from resulting AgNPs and their well-matched reference Raman are illustrated in Figure 3.13.

Out of three candidates of Raman reporters as described above, we chose to use bis-MSB for spectroelectrochemical experiments. It is because (1) the molecule has its unique peaks (1177, 1316, 1334, 1555, 1593, and 1627 cm\(^{-1}\)) due to its structure that are not too as simple as MBT. This makes bis-MSB a good indicator for particle-electrode collision. Also, (2) unlike viologen, the bis-MSB molecule is electrochemically stable so that consistent Raman peaks are expected under the changing the applied voltage, only its intensity would vary. This feature allows us to avoid complexity of considering changes in molecular shape, but to focus on the effect of the voltage on particle-electrode collision.
Figure 3.13 (A) Reference Raman spectra of MBT. Reprinted from ref 100. Copyright 2009 American Chemical Society. (B) SERS spectrum from MBT functionalized AgNPs.
3.4 Electrochemical and Raman Measurements

An electrochemical analyzer/potentiostat (CH Instruments, Model 750E and 842C) was used for electrochemical measurements. A Pt wire was used as an auxiliary/quasi-reference electrode (QRE); thus all potentials are reported vs. Pt QRE. The QRE was immersed in 400 μL of an AgNP solution contained in a PDMS reservoir and in direct fluid contact with the NEA. In order to apply voltages to the recessed ring electrode of the NEA, CH Instruments electrochemical workstation software version 16.07 was used; DC voltages were applied using the Multi-Potential Steps mode, Figure 3.13 A, and the AC Impedance-Time technique was used to apply AC voltage programs, Figure 3.13 B. An Alpha 300R scanning confocal Raman microscope (WITec Instruments Corp.) was used to obtain all SERS spectra. NEA devices were mounted on a motorized piezoelectric stage (ASR 100B120B, Zaber Technologies Inc.). Excitation radiation at 532 nm from solid-state diode pumped laser was used to irradiate the NEAs from the SiNx side of the structure through a water immersion objective lens (Nikon Fluor 60X, N.A. = 1), and Raman scattering was collected by the same objective (epi-illumination geometry). WITec Project software was used for microscope control and data acquisition. All electrical and optical data were processed by Matlab R2016a (Mathworks).
3.4.1 Spectroelectrochemical Behavior of Nanoparticles – DC Excitation

We first applied steady-state (DC) potentials to NEAs, as illustrated in Figure 3.14, and observed electrical and optical behavior of the AgNPs using both amperometry and SERS. Figure 3.15 illustrates the electrochemical and spectroscopic data obtained under DC excitation from +0.8 V to +1.0 V (Figure 3.15 A-C) and +1.1 V to +1.3 V (Figure 3.15 D-F) in 0.1 V increments. Also, Figure 3.16 illustrates the data acquired at +1.4 V. The signals were recorded from 5 to 55 s in order to provide for a quiescent settling time before changing the potential. Note that the amperometric data trace is detected from the whole NEA, while SERS signal is collected from a single nanopore. The data shown in Figure 3.15 reflect several phenomena which occur simultaneously. When bis-MSB-tagged AgNPs are taken up into the nanopore array, they may collide with the nanopore ring electrode giving rise to two effects. (1) The AgNPs may be oxidized, either partially or completely, during the collision, giving rise to the amperometric signals. (2) AgNPs may approach the gold electrode sufficiently closely to enhance the bis-MSB Raman signal by the SERS effect.
Figure 3.14 Plots illustrating applied voltage schemes. (A) DC voltage program. Steady state potentials were applied in 0.1 V increments from +0.8 V to +1.4 V for 60 s each. (B) AC voltage program showing initial voltage (Init E) and amplitude (Amp). Sample interval (2 s) includes data sampling interval and impedance acquisition time (0.1739 s).
Figure 3.15 Simultaneously detected current amperometric traces (A and D) and SERS heat maps (B and E) and waterfall plots (C and F) obtained under DC excitation at voltages from +0.8 V to +1.0 V (A-C) and +1.1 V to +1.3 V (D-F). SERS signals (acquisition time = 0.5 s) at all voltages are plotted on the same scale, and bis-MSB reference spectra are plotted on the right. All experiments were performed with bis-MSB AgNPs (40 nm) in 10 mM KNO₃.
Figure 3.16 Simultaneously detected current trace (A) and SERS signals (B and C) at +1.4 V. (B) and (C) represent the SERS data in heat map and waterfall plot formats, respectively. SERS experiments (acquisition time = 0.5 s) were performed with bis-MSB AgNPs (40 nm) in 10 mM KNO₃ supporting electrolyte.
The amperometric data were analyzed by quantifying the oxidative spikes and analyzing them. The histograms corresponding to the amperometric current spikes are shown in Figure 3.17, and Table 3.1 summarizes (a) the position of the maximum of the Gaussian fit to the histogram, (b) the integrated area of the histogram, and (c) the number of peaks with intensity deviating more than 3σ from the average. As shown in Figure 3.18, the data beyond +1.3V contains the influence of water electrolysis, so it is not analyzed. Nevertheless, several trends are clear. Both the frequency and the magnitude of the current spikes increase with potential up to +1.0 V, after which they plateau. This behavior is apparent in the shift of the histogram to larger currents, reflected in the position of the maximum of the Gaussian fit, Table 3.1, as well as the integrated area of the histogram. Furthermore, the number of very large peaks (exceeding 3σ) also increases in the same manner. It is interesting to note that both the number and the intensity of amperometric current spikes increases in the range +0.9 V ≤ E_{appl} < +1.2 V, although all three electrochemical activity indicators in Table 3.1 plateau above +1.1 V. This trend demonstrates that with increasing positive voltage more nanoparticles are transported to, and captured by, the NEA nanopores. Once captured they can subsequently undergo oxidation upon colliding with the gold electrode surface. Furthermore, because the amperometry is integrated over the entire array, the observation of well-defined current transients suggests that each transient corresponds to oxidation of a single nanoparticle, and the increase in integrated histogram area with increasing potential reflects more complete oxidation at higher potentials, as would be expected.
Figure 3.17 Histograms of oxidative current transient amplitudes derived from amperometric traces in Figure 3.15 at DC voltage levels from +0.9 V to +1.3 V vs. Pt QRE. Gaussian fits to the data are given by the yellow lines. Dotted lines indicate a current level 3σ from the mean. The position of the maximum, the integrated area of the histogram, and the number of peaks exceeding 3σ are summarized in Table 3.1.
Figure 3.18 Amperometric traces acquired at potentials from +0.8 V to +1.4 V, with average (AVG), AVG + standard deviation (σ), and AVG + 3σ indicated for each data set. Spikes which exceed AVG + 3σ are indicated with asterisks.
### TABLE 3.1

**AMPEROMETRY CHARACTERISTICS**

<table>
<thead>
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<th>Potential (V)</th>
<th>0.9</th>
<th>1.0</th>
<th>1.1</th>
<th>1.2</th>
<th>1.3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaussian Max(^a) (x 10(^{-10}) A)</td>
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<td>5.32</td>
<td>7.18</td>
<td>7.01</td>
<td>6.86</td>
</tr>
<tr>
<td>Integrated Area(^b) (a.u.)</td>
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<td>6.11</td>
<td>8.41</td>
<td>8.86</td>
<td>8.99</td>
</tr>
<tr>
<td>Counts &gt; 3(\sigma)</td>
<td>8</td>
<td>9</td>
<td>12</td>
<td>20</td>
<td>15</td>
</tr>
</tbody>
</table>

\(^a\)Position of the maximum of the Gaussian fit to the histogram of current spikes.

\(^b\)Integrated area of the histogram counts.
In addition to the electrochemical data, SERS spectra were also observed. At +0.8 V, SERS peaks appear almost immediately near 1600 and 1200 cm\(^{-1}\), which match bis-MSB reference peaks, but their intensities are weak and discontinuous in time. In contrast, relatively strong spectra are observed transiently, for example at 22.5 and 26.5 s. This suggests that at +0.8 V nanoparticles may be drawn into the nanopores, but also may continue to freely diffuse after colliding with the electrode. To test this hypothesis, SEM images were taken before and after application of +0.8 V of DC potential for 60 s to the NEAs in the presence of bis-MSB-tagged AgNPs. The images show that the majority of nanopores are empty even after +0.8 V was applied (Figure 3.19). At +0.9 V and +1.0 V, conspicuous SERS spectra appeared after 20 s exhibiting scattering near the bis-MSB characteristic band at 1600 cm\(^{-1}\). These spectra were observed continuously until the end of the experiment (60 s). This behavior is distinct from that observed at +0.8 V and indicates that at \(E_{appl} = +0.9\) or +1.0 V the nanoparticles are transported to, and subsequently captured by, the nanopores. Furthermore, the near constant intensity of the SERS spectra suggest that the bis-MSB-tagged Ag nanoparticle or nanoparticles remained in close proximity to the Au electrode surface without diffusing away during the entire time the voltage was applied. Finally, increasing the applied potential to +1.1 or +1.2 V, consistent SERS signals with strong bis-MSB peaks were observed from the beginning, suggesting that increasing the voltage increases the probability of transport and capture of bis-MSB-tagged AgNPs in the monitored nanopore. Upon increasing \(E_{appl}\) to +1.3 V, the SERS spectra show intense bands in the region where carbon artifacts are typically observed,\(^{101}\) and water electrolysis, evidenced by strong peaks at 1454, 1491 and 1550 cm\(^{-1}\) (Figure 3.16), is observed along with carbon at +1.4 V.\(^{101-102}\) Taken
together these SERS data suggest an interpretation in which successively larger positive potentials increase both the transport of bis-MSB-tagged AgNPs to the nanopores and the efficiency of their subsequent capture.

Figure 3.19 Nanopore status before (A) and after (B and C) +0.8 V DC voltage was applied for 60 s. A shows SEM image of NEAs before any voltage was applied. B and C were acquired after +0.8 V was applied for 60 s. Except for a couple of nanopores containing nanoparticles (indicated by red arrows in C), the majority of the pores are empty.
3.4.2 Spectroelectrochemical Behavior of Nanoparticles – AC Excitation

To investigate the effect of different temporal voltage programs, AC voltage excitation was applied, as shown in Figure 3.14 B. The initial voltage was set to +0.8 V, because we estimated that free diffusion and electromigration contribute nearly equally to mass transport at this potential. Frequencies in the range 10 kHz - 30 kHz were chosen, and amplitudes from $\Delta V = +0.005$ to +0.5 V were used. Furthermore, frequency-dependent impedance data on these NEAs produced a strong resonance near 28.8 kHz, so this frequency was chosen for special scrutiny in subsequent amperometric and SERS measurements under AC excitation.

Interestingly, two distinct SERS spectral behaviors were observed, as shown in Figure 3.20, hereafter designated as type A and type B spectra. Type A spectra are short-lived but intense, while type B spectra are less intense but persist over longer time windows, as long as ~150 s. Figure 3.20 shows an example of SERS spectra from bis-MSB-tagged AgNPs on NEAs under AC excitation of $V_0 \pm \Delta V = 0.8 \pm 0.05$ V at 28.84 kHz. A type B signal starts at about 18 s, with gradually decreasing intensity over time until ~141 s. On the other hand, a type A spectrum is observed starting at 204 s, that decays in less than 2 s. Its intensity is higher than those of type B spectra and displays characteristic bis-MSB Raman bands near 1600 cm$^{-1}$. Only these two types of spectra were repeatedly observed under AC excitation even at different amplitude and frequency conditions, as shown in Figure 3.21 and Figure 3.22.
Figure 3.20 SERS heat map (A) and waterfall plot (B) showing detection of bis-MSB-tagged AgNPs on NEAs under AC excitation ($V_0 = +0.8$ V, $\Delta V = +0.05$ V, $f = 28.84$ kHz). Selected SERS spectra at specific time points are plotted in C (red line = type A, black line = type B). Acquisition time for each spectrum is 0.5 s.
Figure 3.21 Heat map (A) and waterfall plot (B) SERS spectra of bis-MSB-tagged AgNPs on NEAs under AC excitation ($V_0 = +0.8$ V, $\Delta V = +0.005$ V, $f = 28.84$ kHz). (C) Selected SERS spectra at specific time points (red line = type A, black line = type B). Acquisition time for each spectrum is 0.5 s.
Figure 3.22 Waterfall plot SERS spectra of bis-MSB-tagged AgNPs on NEAs under AC excitation. \((V_0 \pm \Delta V, f) = (0.8 \pm 0.1 \text{ V}, 28.84 \text{ kHz})\) (A); \((0.8 \pm 0.5 \text{ V}, 28.84 \text{ kHz})\) (B); \((0.8 \pm 0.1 \text{ V}, 20.0 \text{ kHz})\) (C); \((0.8 \pm 0.1 \text{ V}, 10.00 \text{ kHz})\) (D). Acquisition time for each spectrum is 0.5 s.
In order to explain these observations, we put forth the following hypothesis, referencing Figure 3.23. First, we posit two types of entities that can enter the nanopores: aggregates, Figure 3.23 A, and isolated nanoparticles, Figure 3.23 B. The transient type A spectra can plausibly be explained by aggregated nanoparticles being transported to a position proximal to the nanopore, where they are held briefly and from which they can produce SERS spectra. However, they cannot be transported into the nanopore due to their size, so instead they diffuse away (Figure 3.23 A). In support of this explanation for type A behavior, the localized surface plasmon enhancement (LSPR) factor is known as $10^{10}$-$10^{11}$, higher than the enhancement factor from AgNPs-gold electrode junction$^7,45$. During the strong enhancement process, SERS from other molecules in the AgNP aggregate, e.g. MUD, can also be amplified, explaining the presence of multiple peaks in addition to the bis-MSB peaks in type A spectra.

On the other hand, type B SERS spectra exhibit lower intensities over longer time windows, consistent with a hypothesis that type B spectra are caused by enhancement at the AgNP-gold electrode nanogap.$^{103,104}$ As shown in Figure 3.23 B, transport of nanoparticles into the interior of the nanopore would allow them to collide with the Au electrode multiple times before they are completely oxidized. The spectral acquisition window (0.5 s) is long enough to allow the observation of many independent electrode collisions while the spectra are being acquired. Over time these multiple collisions of the bis-MSB-tagged AgNPs that cause SERS signals, also result in incomplete oxidation, which leads to the size of the nanoparticle being diminished and, ultimately to the complete oxidation of the AgNP. Since the size distribution of AgNPs is relatively monodisperse, $d_{avg} = 40$ nm, all type B spectra would be expected to exhibit similar life
times, and in fact the experimental observation is that type B spectra exhibit an average lifetime $\sim 130 \pm 43$.

Figure 3.23 Schematic diagram illustrating proposed mechanisms giving rise to type A and type B spectra.
3.5 Conclusion

In this work, we developed a dual function array of zero-dimensional nanopores capable of supporting both amperometric and SERS spectral studies of bis-MSB-tagged AgNPs. While electrochemical detection integrated behavior over the entire array of nanopores, Raman (SERS) spectra were acquired from an individual nanopore-confined electrode. To accomplish the parallel SERS and ampermometric detection modalities, AgNPs were functionalized with an electrochemically stable Raman reporter, bis-MSB, using an organic pocket method. Both amperometric and SERS signals revealed that increasingly positive DC potentials induce greater numbers of bis-MSB-tagged AgNPs to be taken up and captured by the NEAs. COMSOL simulations also showed the importance of applied electric field-induced electromigration on the overall transport of AgNPs into the nanopores. Under AC excitation, SERS spectra displayed two different behaviors: (A) strong, transient signals, which have tentatively been assigned to nanoparticle aggregates, and (2) less intense, but gradually diminishing (~150 s) spectra which are tentatively assigned to bis-MSB-AgNPs undergoing multiple successive incomplete nanoparticle oxidation events. These experiments show that concurrent SERS and amperometry measurements are advantageous, because they enable the characterization of nanoparticle-nanopore collision dynamics by nearly orthogonal chemical signals, thus demonstrating that optical-electrochemical dual mode detection may ultimately broaden the scope of single nanoparticle studies.
CHAPTER 4:
CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Summary of Completed Works

In this thesis, we highlighted two novel ways of coupling SERS to other versatile technologies. By combining SERS to SPR, simultaneous detection of molecular affinity and chemical characteristic has been demonstrated. By analyzing the data with MCR, the possibility to distinguish specific and non-specific binding is suggested. This approach will improve the precise detection for protein-ligand interaction, thus further contribute to nanomedicine and other applications.

On the other hand, a dual function array of nanopore arrays showed its capability to support both amperometric and SERS spectral studies. The behaviors of nanoparticles were examined under DC and AC forms of voltage, followed by building the hypothesis to explain their displays. The research proves that concurrent SERS and amperometry measurements have advantages characterizing nanoparticle-pore collision dynamics, which may open the way to deepen our knowledge in single nanoparticles study.

Overall, our works represent high potential of SERS as compatible analytical methods. Although SERS itself is real-time, label-free and sensitive analytical tools combining it to other spectroscopies allows us to take advantages of multiple detection methods, eventually providing us profound insights in vast applications.
4.2 Future Directions of Combined SPR-SERS Spectroscopy

As described in Chapter 2, SPR-SERS spectroscopy has wide potential as promising biosensor. In light of this, we suggest using the spectroscopy on other biological sample, such as lipid and blood.

Abbas et al. demonstrated that the ability of SPR spectroscopy is a sensitive detecting tool providing unique qualitative information of lipid bilayer membranes. According to literature, SPR is capable of detecting the different lipid layers at the interface film/fluid (Figure 4.1). If we could exploit SERS spectral study in addition to SPR detection, we believe that the chemical components of thin lipid layers can be analyzed with a surface sensitivity, distinguished from bulk sensitivity.

Figure 4.1 Plasmon-waveguide resonance biosensors with lipid (A) and increased sensitivity of Au-SPR compared to plasmon waveguide resonance biosensors (B). Reprinted from ref 105. Copyright 2011 Elsevier.
Another application we may consider is blood sample. In real-life detection, blood is one of the most approachable fluidic serums in clinical uses, but blood contains variety of other matrixes. These complex matrixes make it difficult to effectively distinguish the target substrate from background signals.

Many scientists conducted researches to detect target molecules in blood sample by using either SPR or SERS.\textsuperscript{106-108} One of the target material to detect is IgG and its antibodies, because it is reported that after exposure to infectious agent, a heterogeneous group of patients undergo severe bacterial infections due to failure to produce IgG antibodies.\textsuperscript{109} Fischer \textit{et al.} introduced the work successfully detecting IgG-antibodies complex in blood serum by SPR.\textsuperscript{109} In addition, Wu \textit{et al.} developed an immunoassay based on SERS with gold/silver core-shell nanorod, characterizing Raman spectra of IgG with high sensitivity.\textsuperscript{107} Based on previous work as reference, SPR-SERS spectroscopy can be applied to detect IgG in blood serum. This work is expected to be developed as one of the highly sensitive analytical tools to screen specific antibody in matrixes.
4.3 Future Directions of Capturing Nanoparticles in NEAs by Simultaneous Amperometry and SERS

Our work in Chap 3 shows that concurrent Amperometry and SERS detection of nanoparticles transferred into nanopores electrode is a promising method to examine the particle-pore collision. In the work, the nanopores are consisted of single gold electrode where external voltage is applied.

Fu et al. researched dual-ring electrode system to transfer AgNPs and discovered the voltage threshold range holding minimum voltages where particles start to avalanche into the nanopores (Figure 4.2). By doing so, this work provides a precise insight to monitor nanoparticle transport and redox reaction within attoliter confined space.

Figure 4.2 Capturing AgNPs with a dual-ring nanopore system (left) and the threshold voltage detection (right). Reprinted from ref 76. Copyright 2018 American Chemical Society.
As the detection was only conducted by an amperometry, we suggest that SERS tandem spectroscopy with amperometry by using our completed work. Simultaneous detection of capturing nanoparticles related to voltage-gated NEAs may demonstrate the possibility to examine chemical identification of the nanoparticle-nanopore interface as well as redox reaction by their collision, which further resembles cell membrane environment.


