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Development of Raman Spectroscopic Techniques for the Detection of Biomarkers of Neurological Disease

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To the Graduate Council:

I am submitting herewith a dissertation written by Amber Shea Moody entitled "Development of Raman Spectroscopic Techniques for the Detection of Biomarkers of Neurological Disease." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Chemistry.

Bhavya Sharma, Major Professor

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**Development of Raman Spectroscopic Techniques for the
Detection of Biomarkers of Neurological Disease**

**A Dissertation Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville**

**Amber Shea Moody
December 2018**

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I would like to thank my advisor, Dr. Bhavya Sharma for agreeing to take me into her group and giving me the absolute best graduate school experience. You have always been patient, encouraging, and enthusiastic every step of the way. You have motivated me to improve every day by the example you set and pushed me to be a better scientist by constantly challenging me with a new task. I could never thank you enough for the things you have taught me.

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Abstract

The goal of the research in this dissertation is to develop detection methods for neurochemicals towards achieving early neurological disease diagnosis. Ideally, the detection methods in physiologically relevant ranges are rapid, selective, label-free, and sensitive. Our approach is Raman spectroscopy, a light scattering technique that provides information about the vibrational modes of a system through interaction with a monochromatic light source. Raman scattering results in a molecular fingerprint for each species, however it is inherently weak. To enhance the Raman signal, we use the localized surface plasmon resonance (LSPR), which arises from the collective oscillation of surface conduction electrons of a metal nanoparticle when excited with a laser, resulting in an enhanced electric field. When a molecule is adsorbed on the nanoparticle surface, the excited Raman scattering is enhanced by the LSPR, and referred to as surface-enhanced Raman spectroscopy (SERS). SERS permits the detection of low concentration analytes. Here we discuss optimization of SERS substrates and excitation wavelength for low concentration detection of neurochemicals. This comprehensive study involved detection of neurotransmitters on both silver and gold nanoparticles at 3 different excitation wavelengths to determine ideal conditions for detection.

SERS can also be combined with spatially offset Raman spectroscopy (SORS), termed SESORS, to detect low concentration analytes in subsurface layers of diffusely scattering media. SORS takes advantage of the spatial component of light in which the photons that travel deeper into the material traverse laterally before being scattered out at the surface. By collecting the Raman scattered light at a point that is spatially offset from the incident illumination point, signal can be obtained from the deep layers. SESORS was used to detect neurotransmitters in a brain tissue mimic through an animal skull. Initial experiments involved optimization of the volume and order of the addition of the SERS probe as well as determining the optimal offset needed for the skull. These experiments were followed by finding limits of detection of various

neurotransmitters through the skull including melatonin, serotonin, dopamine, epinephrine, and norepinephrine, as well as determining the feasibility of this technique for an *in vivo* detection technique using a mouse model.

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Abbreviations & Symbols

α	Polarizability
α_0	Polarizability at equilibrium position
aCSF	Artificial cerebral spinal fluid
ADU	Analog-to-digital converter units
AEF	Analytical enhancement factor
AgNPs	Silver nanoparticles
ATP	Adenosine triphosphate
AuNPs	Gold nanoparticles
c	Speed of light
CCD	Charge coupled device
CW	Continuous wave
d	Groove spacing
D	Diameter
DI	Deionized
DOPAC	3,4-dihydroxyphenylacetic acid
E	Electric field strength
E_0	Laser amplitude
f	Focal length
$f/\#$	F number
FON	Film over nanosphere
FSCV	Fast scan cyclic voltammetry
g	g -factor
GABA	γ -aminobutyric acid

HPLC	High performance liquid chromatography
HVA	Homovanillic acid
I_0	Intensity of incoming flux
I_{RS}	Intensity of Raman scattered light
λ	Wavelength
LOD	Limit of detection
LSPR	Localized surface plasmon resonance
3-O-MD	3-O-Methyldopamine
MS	Mass spectrometry
n	Order of diffraction
P	Induced dipole moment
PCA	Principal component analysis
q	Nuclear displacement
q_0	Vibrational amplitude
RS	Raman spectroscopy
SEM	Scanning electron microscopy
SERS	Surface enhanced Raman spectroscopy
SESORS	Surface enhanced spatially offset Raman spectroscopy
SORS	Spatially offset Raman spectroscopy
σ_{RS}	Raman cross section
t	Time
Θ_d	Angle of diffraction
Θ_i	Angle of incidence
UV	Ultraviolet

UPLC	Ultra-performance liquid chromatography
ν_m	Frequency of the scattered radiation
ν_0	Laser frequency
ω	Frequency difference between incident and scattered radiation
ω_s	Frequency of incident excitation

Chapter 1

General Introduction

The ability to monitor neurological function and changes is critical in early diagnosis of neurological diseases, stress, or injury. Many neurological diseases are not diagnosed until a substantial amount of neurological damage has already occurred, with the only confirmation of the disease occurring after death through an autopsy.¹⁻² This late diagnosis leads to treatment methods that are ineffective, often due to an early misdiagnosis of the disease. Many diagnostic methods rely on the patient answering questions or body movements that evaluate their cognitive motor functions, however, this requires that the patient be able to perform these tasks.^{1,3} There is a need for a method which looks at the underlying chemical changes in the development of neurological disease through detection of biomarkers of the disease. A biomarker is a substance that can be measured and which is characteristic of an infection or disease. Biomarker detection can be used to provide point-of-care diagnosis for diseases that are difficult to detect including neurological disease. A known diagnostic biomarker for neurological disease could lead to earlier diagnosis and monitoring the progression of the disease. The purpose of this dissertation is to develop a detection method for biomarkers of neurological disease, specifically neurotransmitters. Monitoring fluctuations in neurotransmitter concentrations gives insight to the condition of the brain.

The U.S. BRAIN Initiative sparked research in this area resulting in the emergence of techniques investigating biomarkers of neurological disease.⁴ High performance liquid chromatography (HPLC), ultra-performance liquid chromatography (UPLC), and mass spectrometry (MS) are commonly used in the detection of neurotransmitters⁵⁻⁷, however, these methods involve laborious and extensive sample preparation, complex and expensive instrumentation and long collection times. Electrochemical detection methods are very sensitive for use with detection of neurotransmitters⁸⁻⁹, however, these methods can be invasive involving drilling holes in or cutting out portions of the skull to directly detect neurotransmitters in the

brain. Electrochemistry can also lack specificity when investigating neurotransmitters with very similar oxidation potentials. Fluorescence methods have also been used for neurotransmitter detection¹⁰⁻¹² but also require extensive sample preparation and fluorescent labeling of the sample. Imaging techniques can be used non-invasively, such as magnetic resonance imaging (MRI) and functional MRI (fMRI), to give excellent spatial resolution, however, these techniques give no chemical information.¹³ Raman spectroscopy has the ability to overcome these limitations with the extreme selectivity to target molecules using simple instrumentation.¹⁴

When the vibrational modes of a molecule interact with a monochromatic light source, or laser, in Raman spectroscopy, the resulting Raman scattered light give a “molecular fingerprint” for each analyte. The Raman scattered light, however, is very weak. In order to achieve the detection of low concentration analytes similar to what is present in the physiological environment, surface enhanced Raman spectroscopy (SERS) is used. SERS results in an enhanced Raman signal by adsorbing the analyte of interest to a metal nanoparticle. The interaction of the metal nanoparticle with the laser drives the collective oscillation of the surface conduction electrons, producing an enhanced electric field. When an analyte is within 1-2 nm of, or adsorbed to the nanoparticle surface an enhanced Raman signal from the molecule is observed.¹⁵⁻¹⁶

After the initial discovery of SERS there was considerable interest in the 1980s-1990s in the SERS detection of neurotransmitters¹⁷⁻¹⁹, however, the interest quickly declined until the commencement of the BRAIN Initiative in 2013⁴. There are conflicting results in the literature of the SERS detection of neurotransmitters where there is no comprehensive study of the detection of multiple neurotransmitters known to be associated with neurological disease. The details of SERS detection of neurotransmitters reported in the literature is elucidated in Chapter 3.

In order to develop a non-invasive method for the detection of neurotransmitters in the body, SERS can be combined with spatially offset Raman spectroscopy (SORS) in a technique termed SESORS. SORS allows detection of subsurface layers of turbid media through a surface layer by taking advantage of the spatial component of light. The Raman light is collected at a point that is spatially offset from the incident illumination point to collect the subsurface photons that have migrated laterally before being scattered from the surface.²⁰ All techniques are discussed in detail in Chapter 2.

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Chapter 2

Raman Spectroscopy and Instrumentation

2.1 Raman Spectroscopy

2.1.1 Introduction

Raman spectroscopy is a vibrational spectroscopy that gives information about the vibrational modes of a system through the interaction with a monochromatic light source, or a laser.¹ The Raman Effect was discovered in 1928 by Sir C. V. Raman while passing the violet light of the solar spectrum through a liquid sample and observing that some of the scattered light was a different color than the incident light. Due to the inherent weakness of Raman scattered light, sunlight proved to be an inefficient excitation source. The development of the laser in 1960 stimulated an abundance of research applications using Raman spectroscopy.² When monochromatic light from a laser interacts with a molecule, the light is scattered from the molecule both elastically and inelastically. Elastic scattering, also referred to as Rayleigh scattering, describes the process where there is no interaction between the light and the molecule and the light is scattered out at the same wavelength as the incident radiation. With inelastic scattering, the light interacts with the molecule resulting in an energy shift of the scattered light from the incident wavelength. This inelastic scattering is referred to as Raman scattering and involves the absorption of energy by the photon or the loss of energy by the photon. The absorption of energy by the photon from a vibrational mode results in anti-Stokes scattering, where the light is scattered at a higher energy. When the energy is transferred from the photon to the vibrational mode, the light is scattered back out at a lower energy and is referred to as Stokes scattering. This energy shift is unique to the vibrational modes present in a molecule, which results in a “molecular fingerprint” for each species.³

2.1.2 Theory

An electromagnetic wave, or laser beam, has an electric field strength $\mathbf{E}$ which fluctuates with time t . This is represented by equation 2.1 where E_0 and ν_0 represent the vibrational amplitude and frequency of the laser, respectively.

$$E = E_0 \cos 2\pi \nu_0 t \quad 2.1$$

When a molecule is excited by a laser, or placed in an electric field, $\mathbf{E}$, an induced electrical dipole moment, $\mathbf{P}$ is experienced (equation 2.2).

$$P = \alpha E = \alpha E_0 \cos 2\pi \nu_0 t \quad 2.2$$

In equation 2.2, α represents the polarizability of a molecule. The nuclear displacement $\mathbf{q}$, of a molecule vibrating with a frequency of ν_m can be written as

$$q = q_0 \cos 2\pi \nu_m t \quad 2.3$$

where q_0 is the vibrational amplitude (equation 2.3). We can then write equation 2.4 in which α is a linear function of $\mathbf{q}$ for a vibration with a small amplitude and α_0 and $(\delta\alpha/\delta q)_0$ is the polarizability and the rate of change of α with respect to change in q , respectively, both evaluated at the equilibrium position.

$$\alpha = \alpha_0 + \left(\frac{\delta\alpha}{\delta q}\right)_0 q_0 + \dots \quad 2.4$$

We can then combine equation 2.2 with equation 2.4 to obtain equation 2.5

$$\begin{aligned} P &= \alpha E_0 \cos 2\pi \nu_0 t \\ &= \alpha_0 E_0 \cos 2\pi \nu_0 t + \left(\frac{\delta\alpha}{\delta q}\right)_0 q E_0 \cos 2\pi \nu_0 t \end{aligned} \quad 2.5$$

$$= \alpha_0 E_0 \cos 2\pi \nu_0 t + \left(\frac{\delta \alpha}{\delta q}\right)_0 q_0 E_0 \cos 2\pi \nu_0 t \cos 2\pi \nu_m t$$

We can apply the trigonometric identity of $\cos \alpha \cos \beta = \frac{1}{2} \cos(\alpha + \beta) + \cos(\alpha - \beta)$ to yield equation 2.6

$$P = \alpha_0 E_0 \cos 2\pi \nu_0 t + \frac{1}{2} \left(\frac{\delta \alpha}{\delta q}\right)_0 q_0 E_0 [\cos\{2\pi(\nu_0 + \nu_m)t\} + \cos\{2\pi(\nu_0 - \nu_m)t\}] \quad 2.6$$

where the first term represents Rayleigh scattering, the second term represents anti-Stokes scattering, and the third term represents Stokes scattering. For a vibration to be Raman active, there must be a change in polarizability, specifically $(\delta \alpha / \delta q)_0 \neq 0$. If $(\delta \alpha / \delta q)_0 = 0$, the vibration is not Raman active.²⁻³

In normal Raman spectroscopy, the energy of the scattered photon does not correspond to a difference between two energy levels of a molecule. The excitation occurs by a change in the polarizability from the distortion of the electron cloud inducing a dipole. The energy is then promoted from the ground state to a “virtual state” which is below the energy of the first excited state (Figure 2.1). For anti-Stokes Raman scattering, the molecule must be in a higher energy state ($v=1$), which can be induced by thermal excitation, where Stokes scattering can occur from the ground state ($v=0$). The population of the molecules in the $v=0$ state is much higher than the population of the $v=1$ state, explained by the Maxwell-Boltzmann distribution, resulting in much more intensity from Stokes scattering versus anti-Stokes scattering. For this reason, the Stokes Raman scattering is typically measured as opposed to the anti-Stokes scattering.³

For Raman spectroscopy, the intensity of scattered light is measured as a function of wavelength when a monochromatic light source, or laser is focused onto a sample. A Raman

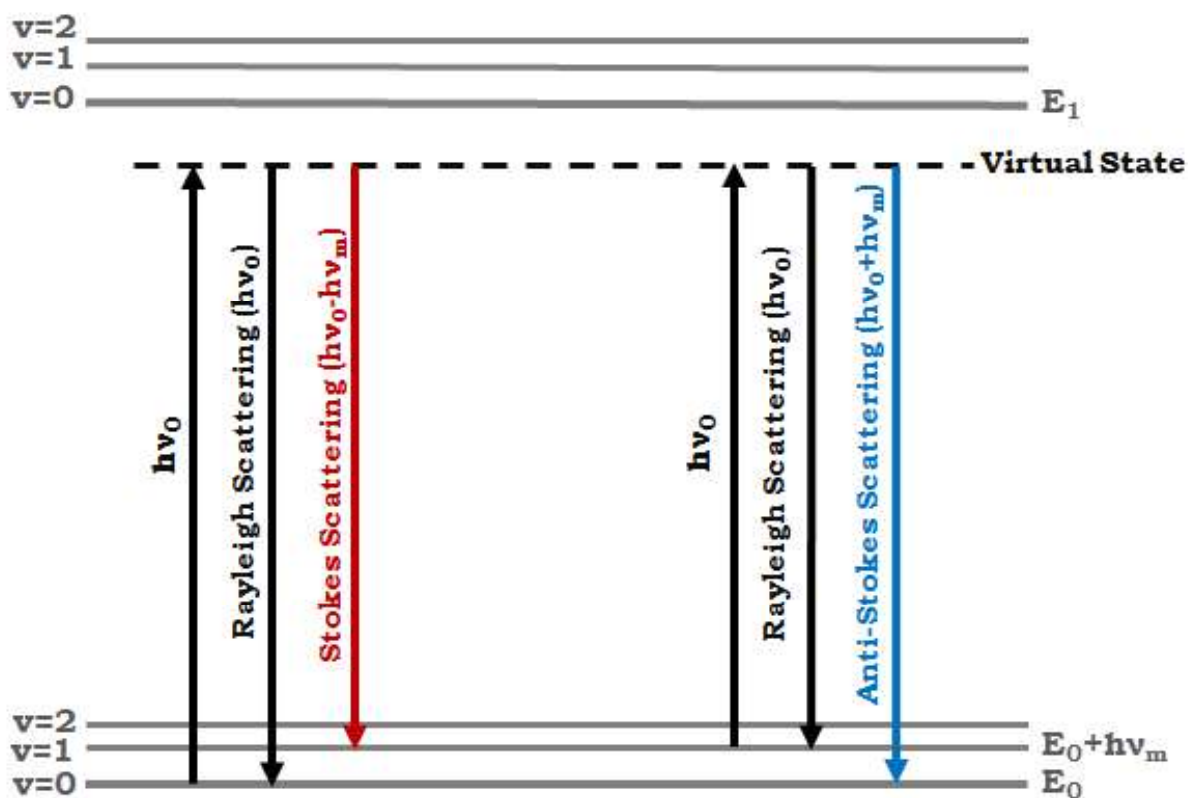


Figure 2.1: Jablonski diagram representing Rayleigh and Raman scattering. The vibrational energy levels are represented by v , E_0 and E_1 are the ground state and first excited electronic state energies, respectively, h is Planck's constant, ν_0 represents the frequency of the ground vibrational state, and ν_m represents the frequency of a vibrational mode.

spectrum has axes that represent the intensity of scattered light versus the Raman wavenumber. The Raman wavenumber ω denotes the difference in frequency between the incident electromagnetic radiation and the scattered light (equation 2.7).

$$\omega = \overline{\nu_m} - \overline{\nu_0} = \frac{\nu_m}{c} - \frac{\nu_0}{c} \quad 2.7$$

In equation 2.7, ν_m is the frequency of the scattered radiation, ν_0 is the frequency of the incident radiation, c is the speed of light and wavenumbers, ω are typically expressed in cm^{-1} . A typical Raman spectrum of benzenethiol is shown in figure 2.2.

The intensity of the Stokes Raman scattered light I_{RS} is proportional to the Raman cross section σ_{RS} and the incoming photon flux I_0 (equation 2.8).⁴

$$I_{RS} = \sigma_{RS} I_0 \quad 2.8$$

The incoming photon flux is reported in units of photons $\text{s}^{-1} \text{cm}^{-1}$ where the Stokes scattered light is reported in units of photons s^{-1} . The Raman cross section, σ_{RS} , is significant of the available area of a molecule for scattering. This cross section has units of cm^2 and is dependent upon the frequency of the incident excitation, ω_s to the fourth power and the square of the polarizability derivative α' for a vibrational transition, shown in equation 2.9.⁴

$$\sigma_{RS} \propto \omega_s^4 |\alpha'_{m \rightarrow n}|^2 \quad 2.9$$

For normal Raman spectroscopy, the approximate order of magnitude for a Raman cross section of a molecule ranges from 10^{-30}cm^2 – 10^{-26}cm^2 .⁴ The small Raman scattering cross sections require a large number of molecules to be present (i.e. high concentrations of analyte) in order to achieve a detectable Raman signal. While Raman spectroscopy provides excellent specificity, it gives an inherently weak signal, only permitting detection of analytes in high concentrations.

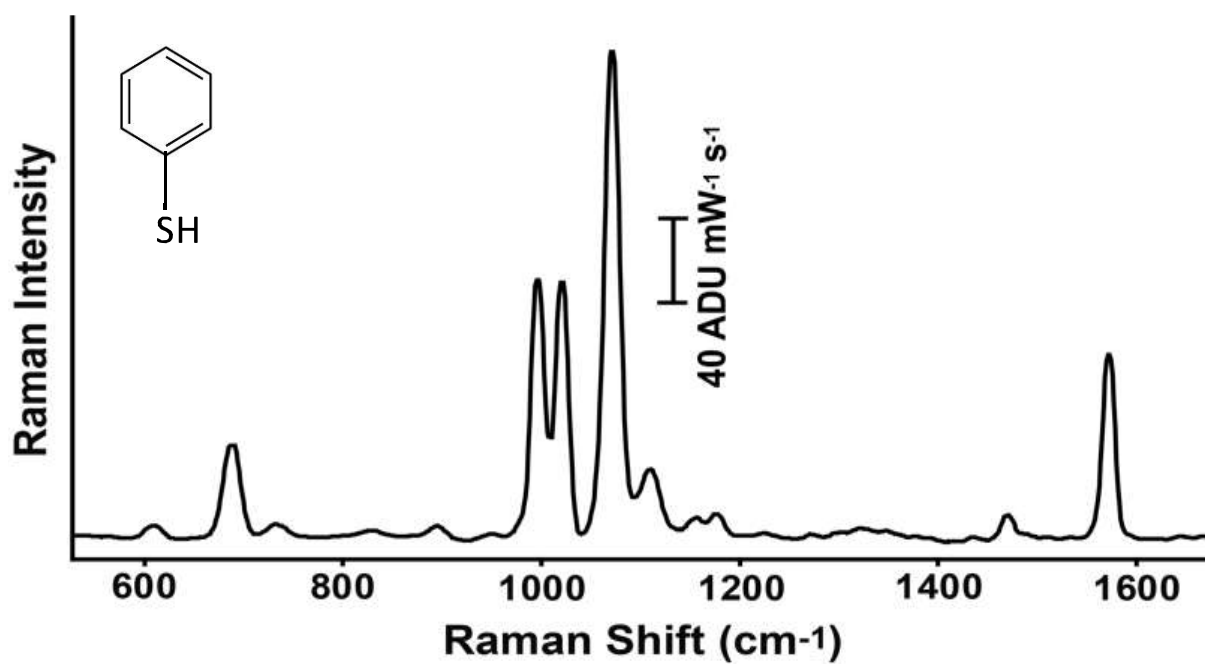


Figure 2.2: Typical Raman spectrum of a strongly scattering Raman molecule, benzenethiol. The intensity of the Raman peaks is plotted with respect to the Raman shift in energy relative to the excitation wavelength.

2.2 Surface Enhanced Raman Spectroscopy (SERS)

2.2.1 Introduction

Normal Raman spectroscopy does not allow detection of very low concentrations of analyte similar to what is seen in the physiological environment. Based on equation 2.2, increasing the electric field will enhance the Raman signal acquired and lead to decreased limits of detection (LOD). One way to achieve an increased electric field is through surface enhanced Raman spectroscopy (SERS). SERS was discovered in 1977 by Jeanmaire and Van Duyne⁵ who theorized that the greatly enhanced signal from a monolayer of pyridine adsorbed on a roughened silver electrode observed by Fleischman *et al.*⁶ was due to enhanced electric fields. During this same time, Albrecht and Creighton⁷ proposed that the increased signal was due to a resonance enhancement, where the excitation occurs within the molecular electronic states.

2.2.2 Mechanism of SERS

Generally, it is viewed that both electromagnetic and chemical interactions between the analyte and substrate contribute to the enhanced signal obtained with SERS. Chemical enhancement is said to be due to a resonant charge-transfer state between the analyte and substrate. Electromagnetic enhancement is dependent on the roughness and dielectric properties of the substrate and has been more thoroughly studied. The electromagnetic enhancement dominates the SERS enhancement mechanism with it being responsible for 10^5 - 10^6 of the overall SERS enhancement of 10^6 - 10^8 , while the chemical enhancement only contributes factors of 10-100.⁸

The enhanced electric field of SERS occurs through a phenomenon known as the localized surface plasmon resonance of conductive metal nanoparticles (Figure 2.3).⁸⁻¹² The LSPR is the electric field produced through the collective oscillation of conduction band

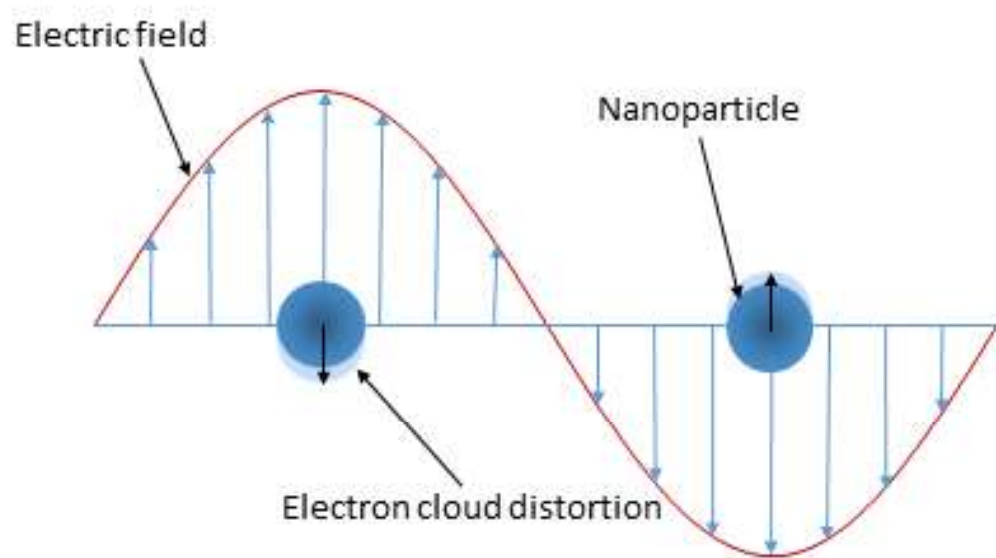


Figure 2.3: Electric field produced surrounding metallic nanoparticles during the collective oscillation of surface conduction electrons when excited with a laser.

electrons of a metal nanoparticle when excited with a laser which increases the polarizability of the surface and localized electric field. The increased SERS Raman cross section contributes to the enhanced signal obtained where the SERS cross section for a molecule has an approximate order of magnitude of 10^{-16} cm^2 . Various experiments have been conducted to determine the experimental parameters that influence the LSPR of the nanoparticle and thus the enhancement of SERS nanoparticles.⁸ The results show that the LSPR is dependent upon the size, shape, roughness, and dielectric environment of the nanoparticle.

For particles that are smaller than the wavelength of incident illumination, all interactions are characterized as surface interactions, where bulk properties are not observed. A simple model for the LSPR is demonstrated for a single particle.¹³ The electrons travel freely throughout the material and are influenced by an electromagnetic field. The incident electric field results in a collective oscillation of the electrons only at particular frequencies that are within or close to within the field frequency which results in a standing wave. This resonance field frequency is what is dependent on the size, shape, and dielectric function of the particle. The method used to measure these resonant excitations or LSPR of the nanoparticles is with extinction spectroscopy (absorption + scattering).¹⁴

Theoretical calculations of extinction spectra have been performed for spherical nanoparticles by Mie by solving Maxwell's equations and for ellipsoidal particles by Gan.^{13, 15} Using the discrete dipole approximation (DDA), the LSPR can be numerically calculated for any geometry where the particle is represented as N polarizable points in a cubic lattice and the induced polarization of each point, $\mathbf{P}_i$, is given by equation 2.10.

$$P_i = \alpha_i * E_{loc}(r_i) \quad 2.10$$

In equation 2.10, α_i is the polarizability of the i^{th} element which is centered at r_i and $E_{\text{loc}}(r_i)$ represents the sum of the incident electric field and all other dipoles in the particle at position i . Thus, as the particle size increases, the LSPR is shifted to longer wavelengths.

The shapes and number of nanoparticles change the position and shape of the LSPR such as a nanoparticle rod. When a rod forms, two LSPR peaks are observed in the extinction spectrum, one for the longitudinal oscillation, and the other for the transverse. When the nanoparticles aggregate, or when more than one particle approaches another to within a few diameters, the LSPR is significantly redshifted and broadened.¹⁴ This represents a coupling of the induced dipoles in each particle, creating a SERS “hot spot” with an increased electric field. The dependence of the LSPR on these factors demonstrates the ability to develop a tunable substrate in order to optimize detection conditions.¹⁶

Calculations of the localized electric field at the surface begins with a simple model system, consisting of a single metal spheroid which is small relative to the incident wavelength of light and is coated with analyte molecules. The polarizability of the sphere is proportional to the volume of the sphere and a g factor found through analytical solutions to Maxwell’s equations. (Equation 2.11)

$$\alpha = 4\pi a^3 g_m$$

$$\alpha = 4\pi a^3 \frac{\varepsilon(\omega) - \varepsilon_m}{\varepsilon(\omega) + 2\varepsilon_m} \quad 2.11$$

The electric field at the analyte, $E(\omega)$, can be calculated at the incident frequency, ω , and at the frequency of Raman scattering, ω' , with Maxwell’s equations resulting in a SERS enhancement factor that is proportional to $[E(\omega)]^2[E(\omega')]^2$.

The most common way to calculate enhancement factors for SERS is through the analytical enhancement factor (AEF). The AEF gives the amount of signal that can be obtained for a specific type of nanoparticle.¹⁷

$$AEF = \frac{d\sigma_{SERS}/d\Omega}{d\sigma_{Raman}/d\Omega} * \frac{t_{a,Raman}}{t_{a,SERS}} \quad 2.12$$

In equation 2.12, $d\sigma/d\Omega$ is the differential Raman cross section and t_a is the acquisition time. Equation 2.13 can be used to find the differential Raman cross section.

$$\frac{d\sigma_{SERS}/d\Omega}{d\sigma_{Raman}/d\Omega} = \frac{IA_{SERS}/c_{SERS}}{IA_{Raman}/c_{Raman}} \quad 2.13$$

IA represents the integrated area under a peak of interest in the normal Raman or SERS spectrum and c is the concentration of the analyte. By substituting equation 2.13 into equation 2.12, we obtain equation 2.14.¹⁸

$$AEF = \frac{IA_{SERS}/c_{SERS}}{IA_{Raman}/c_{Raman}} * \frac{t_{a,Raman}}{t_{a,SERS}} \quad 2.14$$

2.2.3 Nanoparticle Synthesis

Materials commonly used for SERS substrates for excitation in the visible and near infrared range are noble or coinage metals including silver (Ag), gold (Au), and copper (Cu). These metals can be used to synthesize nanoparticles in a variety of shapes including but not limited to spheres, rods, prisms, cubes, and nanoraspberries.¹³ SERS substrates can also be synthesized in a 2-dimensional (2D) morphology which have proven to be more reproducible. Two criteria must be met for the complex dielectric function of a metal for the nanoparticle to exhibit plasmonic properties. The first is that the real component of the dielectric constant must be large and negative and the second is that the imaginary component must be small and

positive. For Ag, Au, and Cu, these conditions are met in the visible and NIR regions of the electromagnetic spectrum, demonstrating their usefulness for SERS substrates in these regions.¹⁹

For the projects outlined in this dissertation, both Au and Ag nanoparticles (AuNPs, AgNPs, respectively) have been synthesized in various morphologies and sizes. Citrate reduced Au spherical colloids have been synthesized using well-established methods.²⁰⁻²¹ First, the methods established by Frens²¹ were used, who outlined procedures for making citrate reduced AuNPs of varying sizes. For the purposes of our research, we have found that particles averaging in size of 60-80 nm give the best SERS signal and are the most reproducible. To synthesize particles of this size, a 0.01% by weight solution of HAuCl₄ and a 1% by weight solution of Na₃-citrate are made. A volume of 45 mL of the HAuCl₄ solution is brought to a boil where 0.3 mL of the Na₃-citrate solution is added and boiled for an additional 5 minutes.

The methods established by B.V. Enüstün and J. Turkevich²⁰ are a modified version of the Frens synthesis and are used to synthesize larger volumes of citrate reduced AuNPs. We have also used this method, where 850 mL of deionized H₂O were boiled in a 1L flask. Once the solution was boiling, 100mL of a 0.05% by weight solution of HAuCl₄ was added. The resulting solution was, again, brought to a boil where 50 mL of 1% by weight Na₃-citrate was added. After addition of the Na₃-citrate, the solution was boiled and stirred continuously for 30 minutes. The synthesized AuNPs were 60 nm on average with an LSPR max of 525 nm. (Figure 2.4)

Ag spherical colloids were prepared using two different reducing agents. The first method was established by Lee and Miesel²² and involves a reduction of silver nitrate with sodium citrate. Briefly, 90 mg of silver nitrate was dissolved in 500 mL of deionized H₂O. This solution was brought to a boil where 10 mL of 1% by weight Na₃-citrate was added and subsequently allowed to boil for 1 hour. The second method described by Creighton *et al.*²³

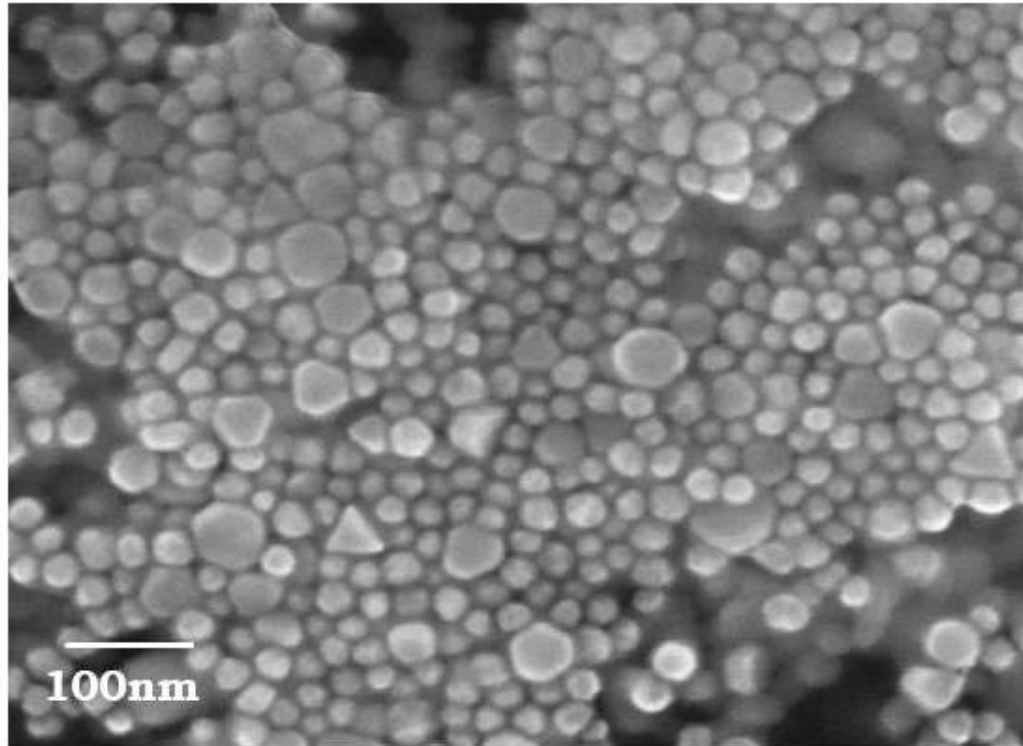
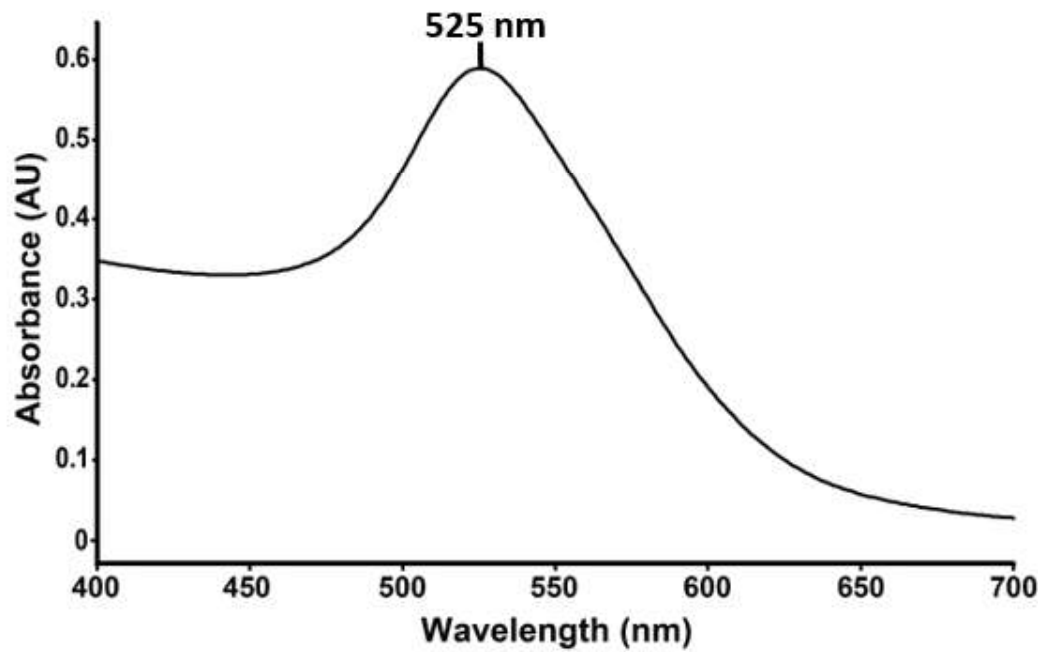
A**B**

Figure 2.4: SEM image (A) and extinction spectrum (B) of AuNPs synthesized with the methods established by Eüstün and Turkevich.

involves a reduction of silver nitrate with sodium borohydride. Briefly, 1 mM sodium borohydride was prepared and allowed to cool to 5°C and 2 mM silver nitrate was prepared at room temperature. Under strong stirring, 3 volumes of the 1 mM sodium borohydride at 5°C was added dropwise to 1 volume of the 2 mM silver nitrate and allowed to stir for 30 minutes.

Au nanoraspberries have also been synthesized according to methods established by Masson²⁴ which uses HEPES as a reducing agent. Nanoraspberries, which are a nanoparticle with a raspberry-like configuration, are known to be more enhancing than spherical colloids. A 100mM solution of HEPES buffer, pH 7.4 and a 250 mM HAuCl₄ solution were made. 6.25 mL of HEPES buffer was added to 100 mL of deionized H₂O followed by addition of 100 µL of the HAuCl₄ solution. The solution was allowed to stir overnight at room temperature.

2D substrates are known to give a more reproducible enhancement than colloidal solutions.²⁵ One type of these 2D substrates that has been used during this project is a Au film over nanospheres (Au FONs). These substrates were synthesized by dropcasting polystyrene spheres on glass coverslips followed by sputtering of a 200 nm layer of Au. The glass substrates were cleaned in a piranha solution (3:1 H₂SO₄:H₂O₂) for 30 minutes then rinsed with copious amounts of deionized H₂O. The glass substrates were then sonicated for 1 hr in a 5:1:1 H₂O:H₂O₂:NH₄OH solution to make the surface hydrophilic which promotes self assembly of the polystyrene nanospheres. The substrates were, again, rinsed with copious amounts of deionized H₂O and. Polystyrene beads (390 nm) were then drop-casted on the surface of the treated glass substrates and allowed to dry in ambient conditions. A 200 nm layer of Au was then deposited with an AJA Magnetron Sputtering System at a deposition rate of 2 Å/s.

2.3 Spatially Offset Raman Spectroscopy (SORS)

2.3.1 Mechanism of SORS

A disadvantage of normal Raman spectroscopy when investigating materials with multiple layers is the strong bias towards the surface layer. Probing the deeper layers of diffusely scattering media can be achieved with spatially offset Raman spectroscopy (SORS). SORS is a nondestructive, noninvasive technique that takes advantage of the spatial component of the photon migration process through the collection of the Raman scattered light at a point that is spatially offset from the incident illumination point. The technique was developed as a subsurface detection technique following the use of ultrafast temporal gating. When photons travel deeper into the subsurface layers of a material, the Raman scattered photons are temporally separated from the Rayleigh scattered photons, i.e. the Raman signal decays more slowly than Rayleigh signal. By gating the signal with ultrafast temporal gating, the Raman light that has emerged from the deeper layers of the material can be selectively captured. Temporal gating, however, requires the use of high intensity laser pulses leading to very complex and costly instrumentation. Instead, by utilizing the spatial component of light, subsurface layers can be probed using simple instrumentation and laser powers that remain within the safe exposure limits.²⁶

Photons that emerge from these subsurface layers travel longer distances to the surface and diffuse laterally during this process resulting in the signal being in a larger spatial area than the surface signal. (Figure 2.5) Collecting the Raman scattered light at a point that is laterally offset from the incident illumination point, the signal from the deeper layers of a material can be obtained. The size of the spatial offset used is dependent upon the thickness of the surface layer of the sample and the desired detection depth. The contribution from the surface layer should

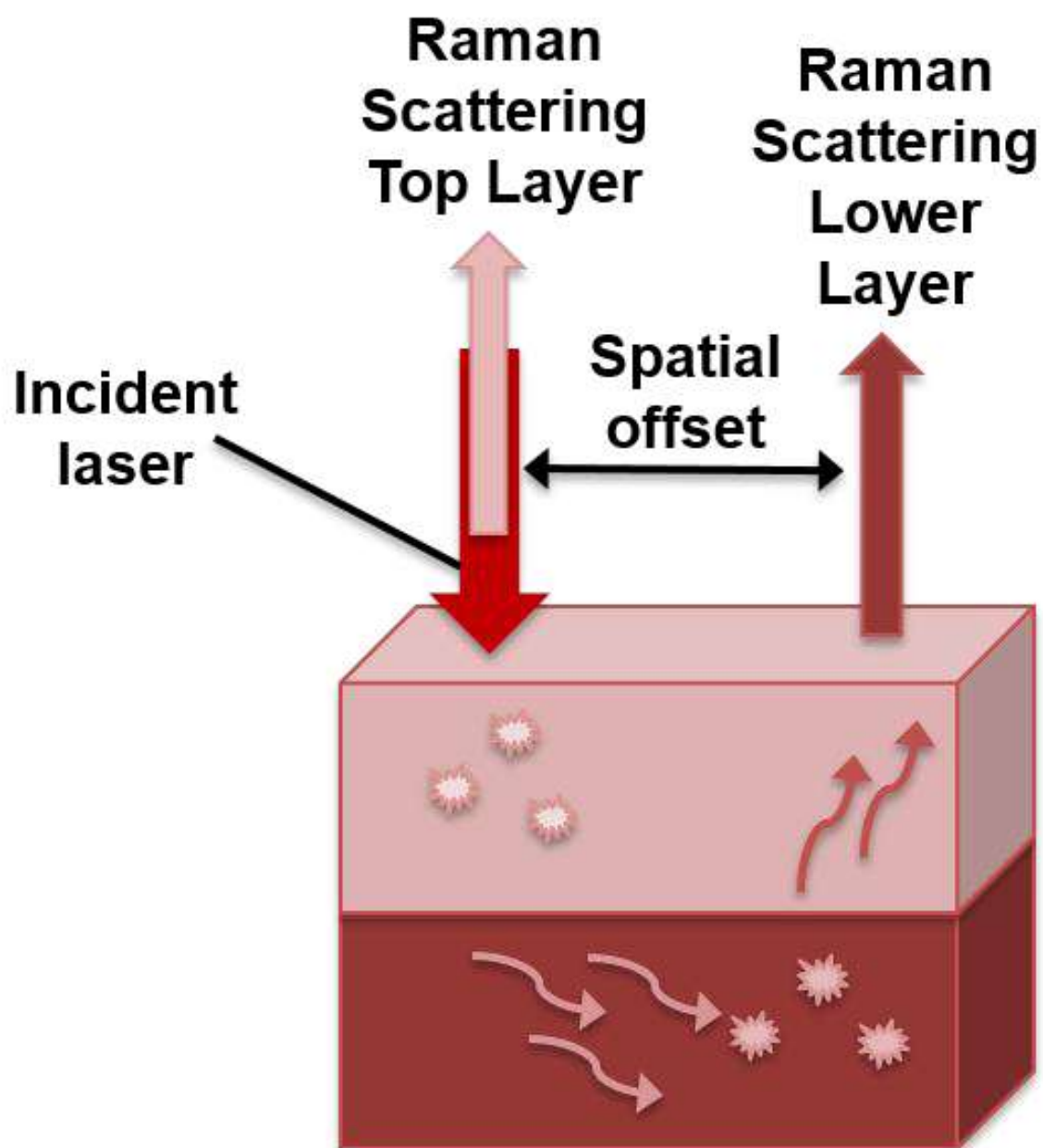


Figure 2.5: Schematic of SORS describing the pathway of photons deep in the material before scattering from the surface.

decrease more rapidly than the contribution from the subsurface layer.²⁶ The Raman spectra of the subsurface layer in a two-layered material can be separated from the surface layer through a scaled subtraction of the spectrum collected with no spatial offset from the spectrum collected with a spatial offset. If a sample contains more than two layers, multivariate analysis techniques can be utilized to separate the individual layers.²⁷

SORS is excellent for applications with biological and organic materials as well as *in vivo* applications where it has the ability to suppress the fluorescence associated with the surface layers often involved in these types of samples.²⁶ The technique is limited, however, in working with metallic and highly absorbing samples where there is a reduction in the distance of photon migration, resulting in a smaller depth of penetration.

2.3.2 Collection Geometry

Illumination of the sample and collection of Raman scattered light in SORS can be accomplished in a number of geometries. The simplest collection geometry is a single point collection, which permits any normal Raman set-up to be easily adapted to obtain SORS data. In a point collection geometry, simple optics such as a dichroic mirror are used to move the illumination point and achieve the spatial offset. (Figure 2.6A) The scattered light is subsequently collected in a 180° backscattering geometry and directed into the spectrometer. Although a point collection provides simplicity in the set-up, it results in a limited sensitivity.²⁶

Fiber optic probes can be used in SORS for an increased sensitivity in two different geometries. The first geometry, conventional SORS, involves the collection fibers being arranged in a ring pattern where each ring represents a different spatial offset. The laser spot illuminates the sample through a small spot centered in the rings. (Figure 2.6B) Conventional SORS allows probing of biological tissue while maintaining safe illumination powers, however,

spectral distortions arise from multiple Raman spectra being collected on different tracks of a charge coupled device (CCD). This leads to decreased sensitivity where artefacts arise in the spectra during post processing.²⁶ The second geometry is referred to as inverse SORS and switches the illumination and collection fibers. (Figure 2.6C) Inverse SORS allows for use of overall higher laser powers where sample illumination occurs through a ring and the Raman signal is collected through a group of fibers in the center. This permits the binning of the signal onto the CCD in a single spectrum, eliminating artefacts apparent in processing with conventional SORS.²⁸ Inverse SORS can also be achieved through use of optics such as a conical lens, or axicon where the size of the spatial offset is changed by modifying the distance between the axicon and the sample.²⁹

When the goal is to identify a known signal at an unknown location by investigating the bulk of a sample, transmission Raman is most useful. Transmission Raman separates the illumination and collection points to the extreme by illuminating on one side of the sample and collecting the Raman scattered light through the other side of the sample. (Figure 2.6D) This permits probing of the entire volume of a sample and is insensitive to the depth of the target analyte.³⁰ Conversely, when looking at very thin layers of turbid media, microscopy can be combined with SORS. The spatial offset is achieved by defocusing the laser from the sample. Initially, the spectrum should be taken with the laser focused on the sample, followed by a spectrum with the laser defocused from the sample. A scaled subtraction can reveal the spectrum of the subsurface layers (Figure 2.6E).³¹

In order to probe analytes in low concentrations in subsurface layers of diffusely scattering media, SORS can be combined with SERS in a technique termed SESORS. To obtain SESORS data, SERS active nanoparticles or a SERS nanotag are implemented into deep layers

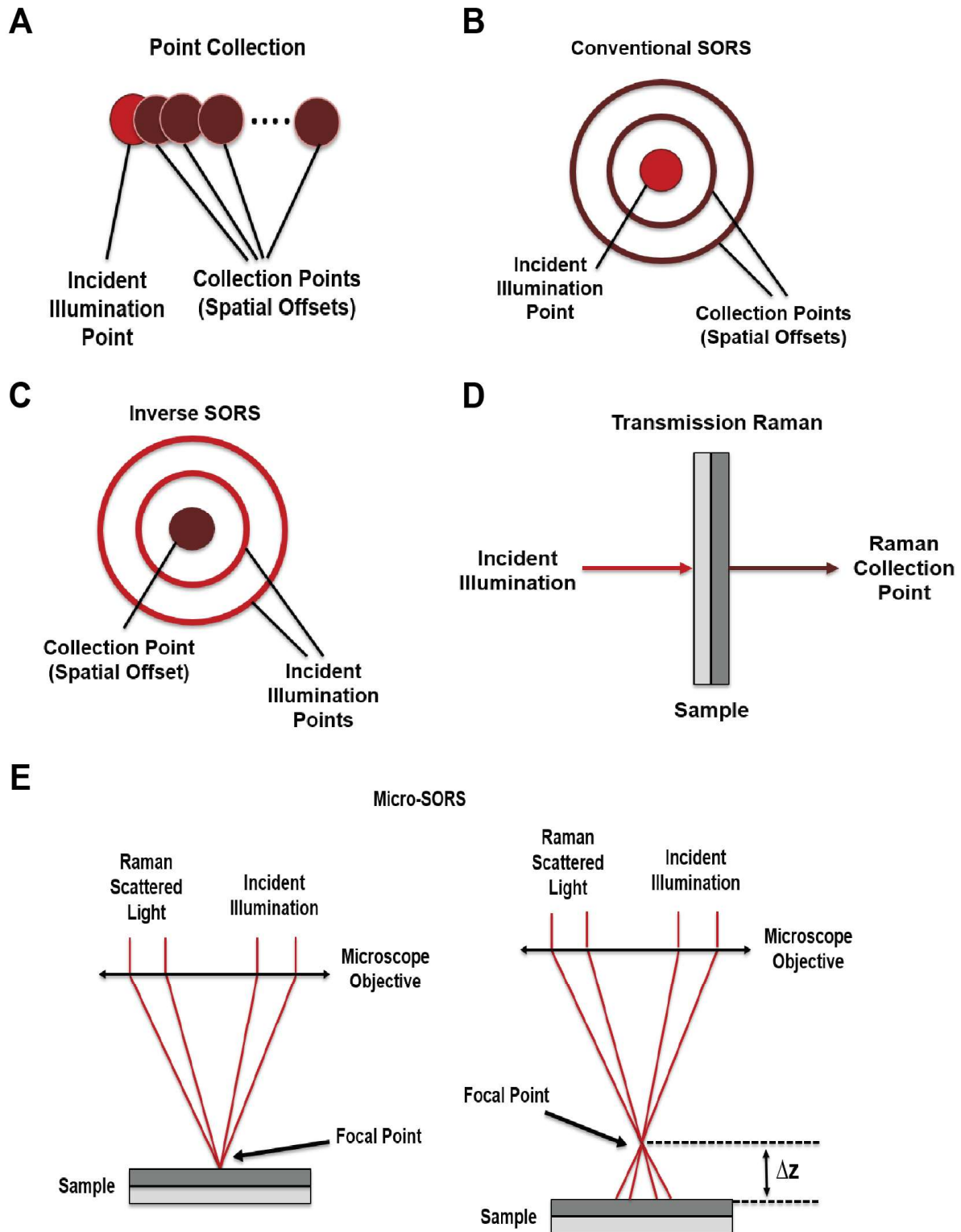


Figure 2.6: SORS geometries of a point collection (A), conventional SORS (B), inverse SORS (C), transmission Raman (D), and micro-SORS (E).

of turbid media followed by SORS measurements to reveal an enhanced signal of the subsurface layers.³²

2.4 Instrumentation

2.4.1 Introduction

There are four major components that make up a Raman optical system including the excitation source, sample delivery and collection system, spectrograph, and detection and computer system. There are commercially available Raman systems, or they can be home-built. All of the experiments in this dissertation were conducted on home-built Raman systems.

2.4.2 Excitation Sources

Before the development of the laser, the primary excitation source used for Raman spectroscopy was typically a mercury lamp. Filters were used in combination with the lamps in order to select a single or small wavelength range. These lamps required a great amount of time to warm up and ignite and were riddled with problems of shutting down intermittently. Lasers are an ideal excitation source for Raman spectroscopy due to several factors; they have high powers, narrow line widths, small beam diameters, are linearly polarized, and can be tunable. The smaller, more focused diameters of lasers made it possible to decrease the sample volume used as well as increase the power of the excitation source. Most of the early lasers were gas lasers which emitted light in the visible region. Today, the lasers used are continuous wave (CW) or pulsed. Pulsed lasers are typically used for nonlinear studies that require higher powers. CW lasers are more commonly used and allow for a stable, constant illumination source at lower powers. Materials used for the most commonly used Raman lasers are Argon, Krypton, Helium-neon (He-Ne), Titanium-Sapphire, yttrium aluminum garnet (YAG), and diode.^{2-3, 33}

For the purposes of this dissertation, in the visible and NIR range two diode lasers were used at wavelengths of 532 nm and 785 nm as well as a He-Ne gas laser at 633 nm. Diode lasers are advantageous due to their stability, low cost, small footprint, and reduced interference from fluorescence background.³⁴ He-Ne lasers were the first gas lasers demonstrated and have historically been chosen for applications in the visible range of the electromagnetic spectrum. It has better coherence and beam quality than that of diode lasers at similar wavelengths and is very cost effective.³⁵ In the UV range of the electromagnetic spectrum, a tunable kilohertz (kHz) Ti:Sapphire laser (Photonics Industries). This tunable laser can be operated at wavelengths ranging from 193 nm to 960 nm.

2.4.3 Sample Delivery and Collection System

As previously mentioned, Raman scattering is inherently weak, thus to obtain the most intense signal, the laser must be tightly focused onto the sample. The subsequent scattered radiation must also be collected efficiently. This can be controlled with optics, specifically focusing and collecting lenses. The laser source can be directed toward the sample and aligned using mirrors and a set of irises. A focusing lens should then be placed before the sample to achieve a tight focus of the laser and an increased power density. To collect the light and direct it into the spectrometer a set of collection lenses is used which include a collimating lens to collect and direct the light onto a focusing lens which focuses the collected light into the slit of the spectrometer. It is very important here to match the F number ($f/\#$) of these collection lenses with the $f/\#$ of the spectrometer to get maximum efficiency.³ The appropriate focal length for the collection lenses can be calculated by using the lens equation

$$f/\# = \frac{f}{D} \tag{2.15}$$

where the $f/\#$ should match the $f/\#$ of the spectrometer, f is the focal length of the lens needed, and D is the diameter of the lens. An adjustable sample stage should also be used that permits movement of the sample to achieve maximum focus.

2.4.4 Spectrograph

A spectrograph is a dispersive instrument that is used to spatially disperse light so that a detector can record the photons at different Raman shifts. Spectrographs consist of a dispersive element such as a grating or prism and an image transfer system, which includes entrance and exit slits, mirrors, and lenses. A diffraction grating is a plane or concave plate that is ruled with closely spaced grooves, which disperse polychromatic light into its constituent wavelengths. They provide a linear dispersion of wavelengths where the dispersion of the grating is given by

2.16

$$n \cdot \lambda = d \cdot (\sin\theta_i + \sin\theta_d)$$

n is the order of diffraction, λ is the wavelength diffracted, d represents the groove spacing, θ_i is the angle of incidence (measured from the normal) and θ_d is the angle of diffraction (measured from normal). The resolution of the spectrograph is ultimately determined by the resolution of the grating, which is directly proportional to the number of grooves the grating contains.³⁶

The most commonly used monochromator is the Czerny-Turner model monochromator (Figure 2.7). This is the monochromator design used for the experiments of this dissertation. The F number of a spectrometer is described by the focal length of the collimator mirror divided by D , which represents the size of the grating. To maintain good resolution, D must be large and the focal length must also be large where most monochromators have an F number between 5-10.³

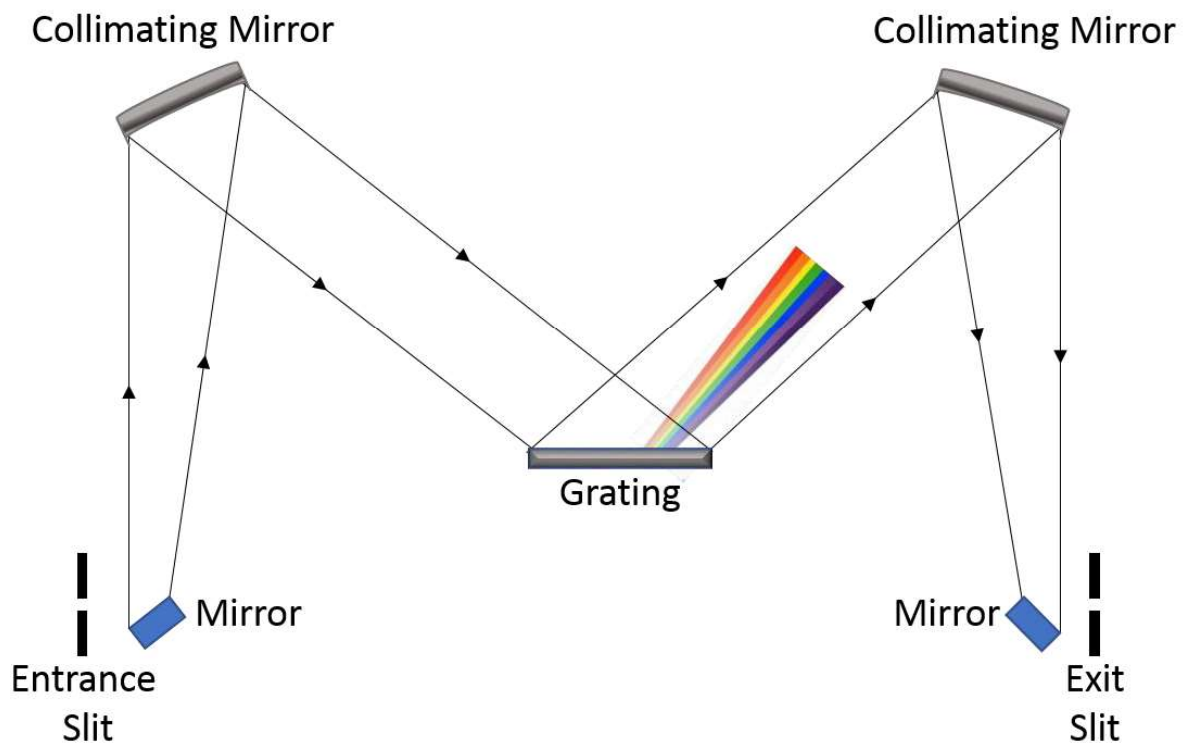


Figure 2.7: Schematic of a Czerny-Turner Monochromator with an entrance and exit slit, directing and collimating mirrors, and a grating.

2.4.5 Detectors

After the dispersion of light by the spectrometer, a detector is used to measure the intensity of photons at specific wavelengths. Recently, CCDs have become more popular over the traditionally used photomultiplier tubes or photodiodes. To determine the type of detector which is most useful, quality is measured by the background noise, sensitivity, and frequency range.³ While photomultipliers have a high sensitivity and low background noise, they do not have a linear response over the full range of wavelengths needed for Raman spectroscopy and lack the desired speed in recording measurements.^{3, 33} Photodiode detectors have the ability to cover a range of wavelengths, however they deteriorate without uniformity over time due to their sensitivity to high photon counts such as those seen with Raman spectroscopy. A CCD has the ability to cover a range of wavelengths with low readout noise and high quantum efficiency.^{3, 33} For the work in this dissertation, a CCD was used as the detector.

A CCD is a silicon wafer that has a geometrically regular array of photosensitive pixels that capture and store information in the form of localized electrons that vary with incident light intensity. This array of pixels is a 2D array also known as the focal plane array (FPA). The horizontal location is related to wavelength, where the wavelength position is determined by a calibration and the vertical axis related to intensity. The CCD technology is based on doped semiconductors where impurities are intentionally introduced into an extremely pure material to change its electrical properties. Typically, a CCD is made with a p-doped metal-oxide semiconductor so that electrons are stored. The incoming photons strike the surface and an electron-hole pair is created described by the photoelectric effect. The electrons are then collected and counted and fed into an output amplifier to produce the image.³⁷

2.4.6 Raman Systems Built in the Sharma Lab

In the Sharma lab, Dr. Sharma and I have built 3 different Raman systems. The first of these is a confocal micro-Raman system for use in the visible and NIR range. (Figure 2.8) The system consists of excitation wavelengths at 532 nm, 633 nm, and 785 nm. The laser light is directed toward the microscope using a set of mirrors. With Raman spectroscopy, it is imperative that the laser light be perfectly aligned to achieve the optimal signal. This can be achieved by using a set of irises, which are movable and close down to a pinhole, so the laser can be aligned in the center of the pinholes. After the light is aligned with a set of irises, it is focused onto a set of periscope optics which consists of a set of stacked mirrors and is used to raise the level of the laser. The purpose of the periscope is to raise the level of the laser to reach the entrance of the microscope. The light then enters the Nikon TIU inverted microscope and is focused onto the sample through an objective. The Raman scattered light then travels back through the objective and exits the microscope to pass through a focusing lens and a long pass filter before entering the IsoPlane SCT 320 spectrometer (Princeton Instruments). The long pass filter allows all of the Raman scattered light, at wavelengths longer than the excitation wavelength to pass through as a method to filter out the Rayleigh light before entering the spectrometer. The light is then dispersed onto the PIXIS 400 CCD camera (Princeton Instruments). Optics are all uncoated to permit use at various wavelengths and have been purchased from ThorLabs. This system is excellent for SERS measurements where the laser spot can be focused to micrometer size, increasing the power density and allowing the use of ultra-low powers. The use of a microscope is also imperative when analyzing biological samples to investigate only the spots of interest.

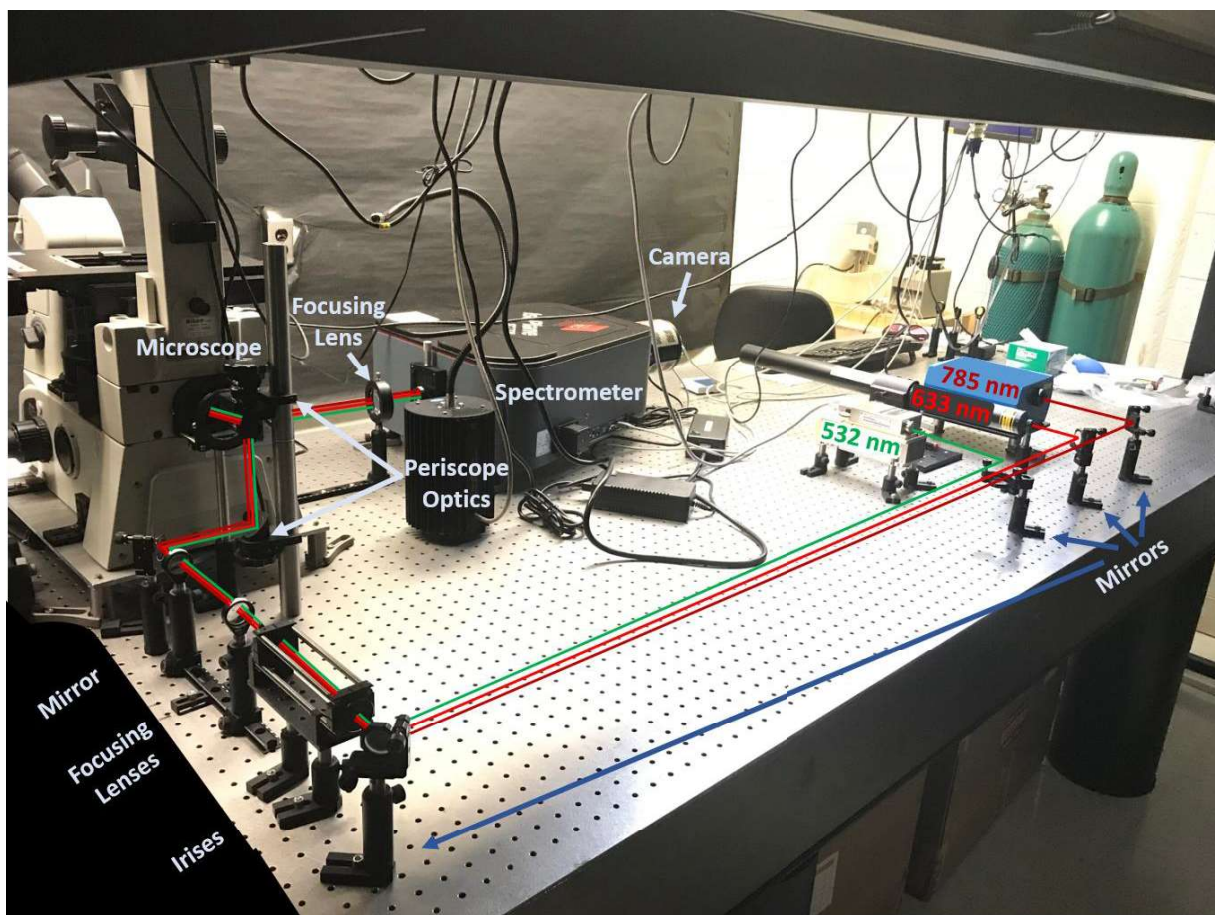


Figure 2.8: Macroscopic Raman system built in the Sharma Lab.

Another Raman system used in the Sharma Lab is a macroscopic Raman system. This system consists of only one laser wavelength of 785 nm. This system is most useful when examining samples too large for investigation with a microscope, SORS measurements, or biological samples. Biological tissue exhibits less fluorescence at NIR wavelengths, thus using an excitation wavelength of 785 nm allows more information to be acquired versus other visible wavelengths. The laser is directed onto the sample using a dichroic mirror, which acts similar to long pass filter. This dichroic mirror reflects the light at the excitation wavelength (785 nm) and allows all of the longer wavelengths to pass through (Raman scattered light). The Raman scattered light is then passed through a collimating lens to collimate the cone of scattered light, then through a focusing lens and long pass filter before entering the slit. The Acton LS785 spectrometer and PIXIS 400 camera were both purchased from Princeton instruments. (Figure 2.9) To achieve the spatial offset needed for SORS measurements, an optical element called an axicon is used. An axicon transforms the laser spot into a ring of illumination, where the radius of the circle is the size of the spatial offset (Figure 2.10).

The last system that was built in the Sharma Lab is for use in the ultraviolet range of the electromagnetic spectrum. This system consists of a tunable k-Hz Ti:Sapphire laser purchased from Photonics Industries. This uses a pulsed pump laser at an original wavelength of 527 nm which is subsequently passed through a series of harmonic crystals to be tuned to wavelengths ranging from 193 nm to 960 nm. With the ability to operate from the deep-UV to the infrared, this robust laser gives excellent functionality to the Sharma Lab. The use of harmonic crystals produces a laser spot that is often in the shape of a horizontal or vertical line. Cylindrical lenses can be used to convert a non-spherical laser spot to a sphere and have been implemented into the UV system. (Figure 2.11) The light is directed through a set of irises for alignment and up

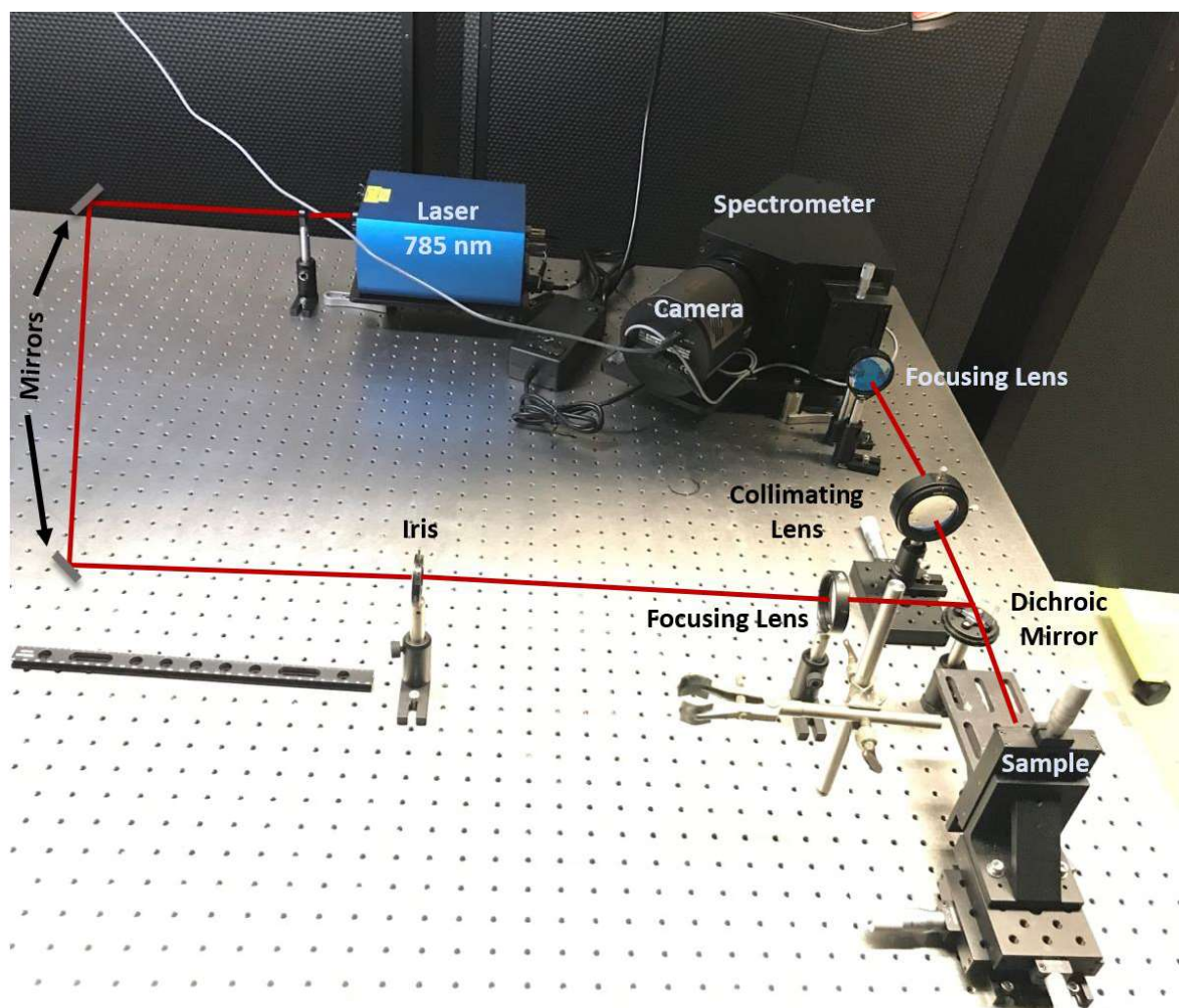


Figure 2.9: Macroscopic Raman system built in the Sharma Lab.

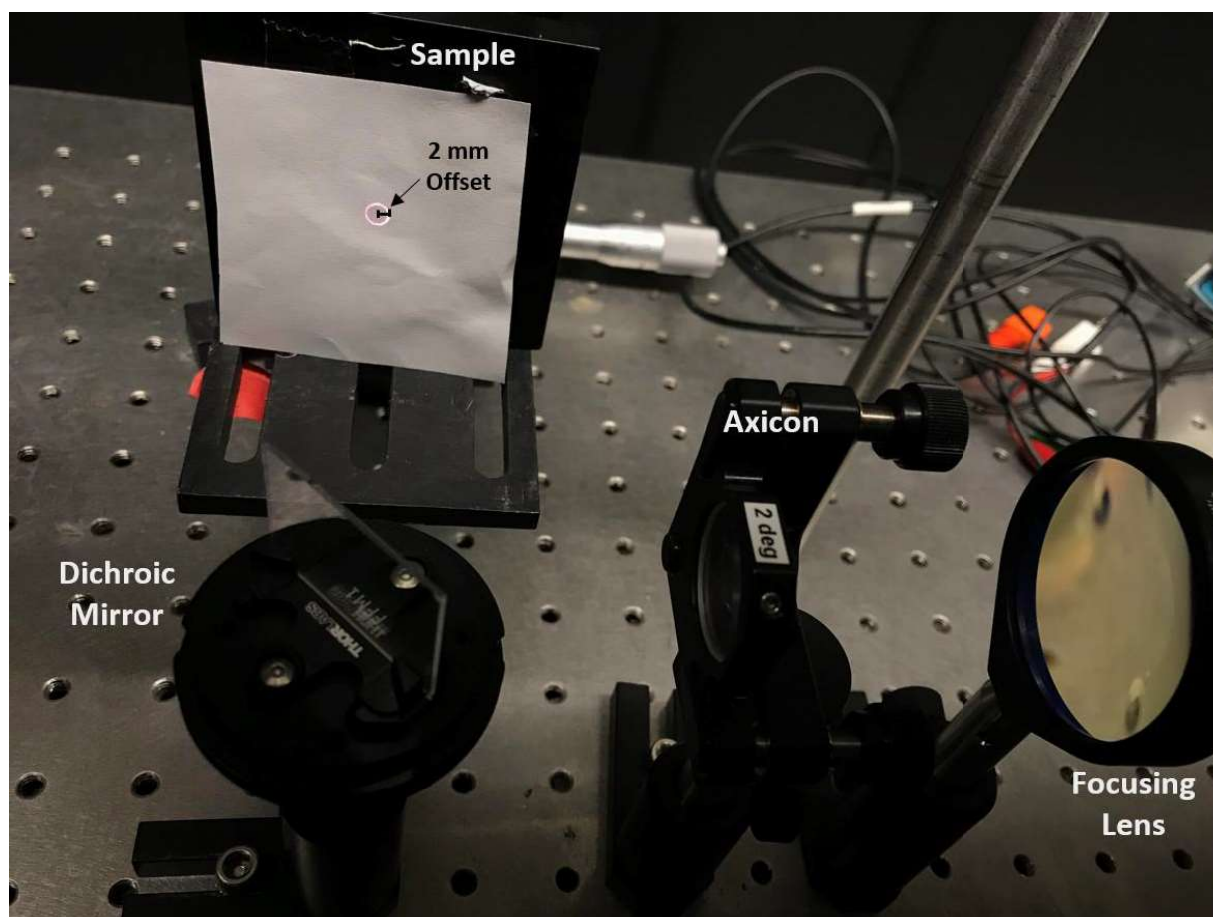


Figure 2.10: Placement of the axicon into the optical set-up of the macroscopic Raman system to create a spatial offset for SORS measurements.

through a focusing lens to a prism, where the prism redirects the light onto the sample. (Figure 2.12) Raman scattered light is then collected through a collimating lens and focused into a prism monochromator before entering the spectrometer (McPherson). (Figure 2.13)

A single long pass filter cannot be used for all of the possible wavelengths of the UV system. In order to filter out the Rayleigh light and send the Raman scattered light into the spectrometer to be dispersed, a prism monochromator can be used. This prism is on a turnable stage, which disperses the Rayleigh and Raman scattered light so that it can be turned to simply move the Rayleigh light off of the entrance slit of the spectrometer and only allow the Raman scattered light to pass through. The Raman scattered light is then dispersed onto a PyLoN camera purchased from Princeton instruments.

2.5 Spectral Analysis

Often with large datasets that contain more variables than can be easily labeled, unsupervised multivariate analysis techniques can be applied to reveal relationships between samples. For this dissertation, we have used principal components analysis (PCA) to reduce the dimensionality of the acquired SERS and SESORS spectra of neurotransmitters and reveal relationships between the samples. This has aided in determining limits of detection (LOD) for each neurotransmitter. PCA is widely used to reduce the dimensionality of the dataset through an orthogonal transformation. This decomposes the data into a series of principal components where each principal component contains a spectral pattern, or loading, and a score that describes the relationship between the sample and principal component. By plotting the scores of the different principal components, one can demonstrate clustering of the sample into classes that explain major trends, groups or patterns in the data set.³⁸⁻³⁹ The PCA has aided in the decomposition of the complex spectra and helped reveal spectral patterns not easily discernible by eye.

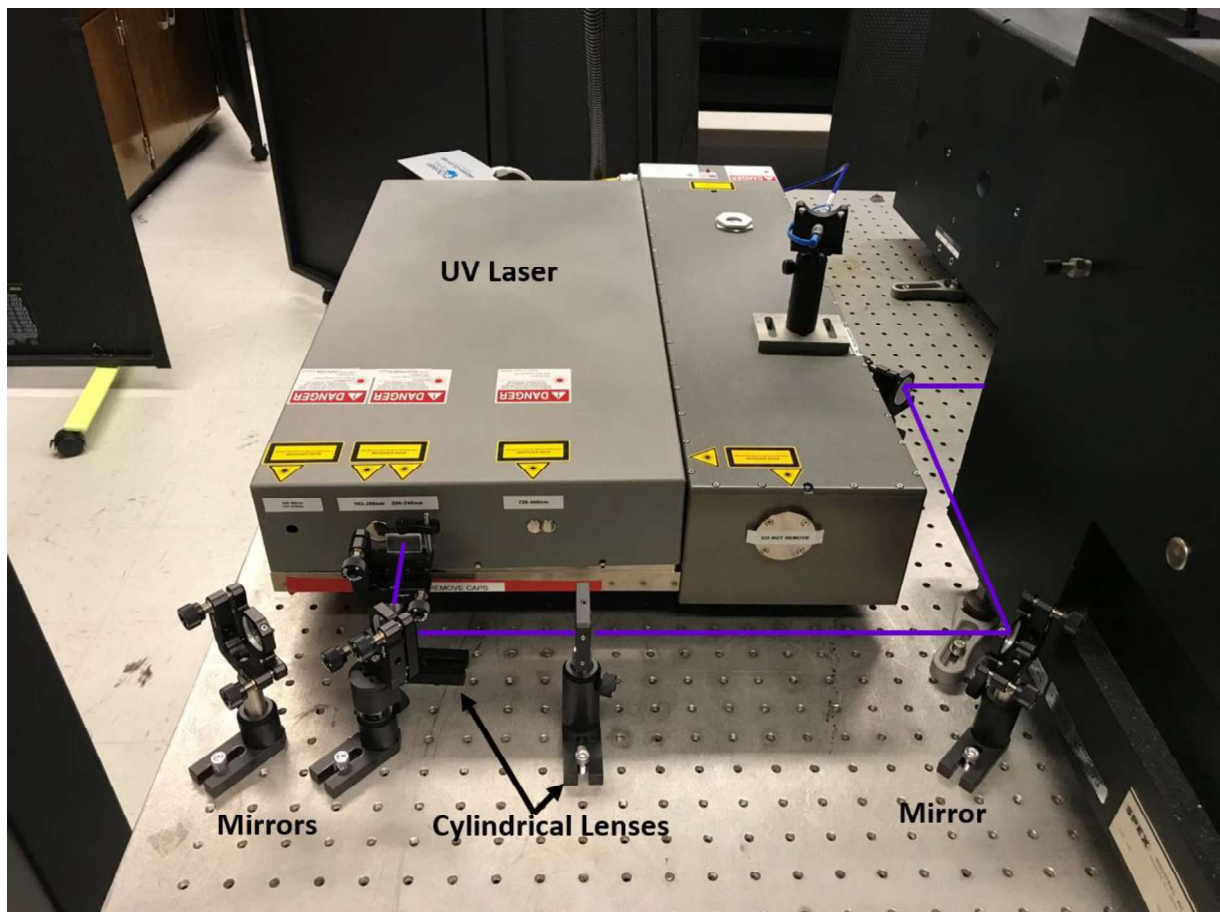


Figure 2.11: UV Laser purchased from photonics industries. Light is directed through a set of cylindrical lenses to obtain a spherical laser spot and subsequently directed toward the sample with a set of mirrors.

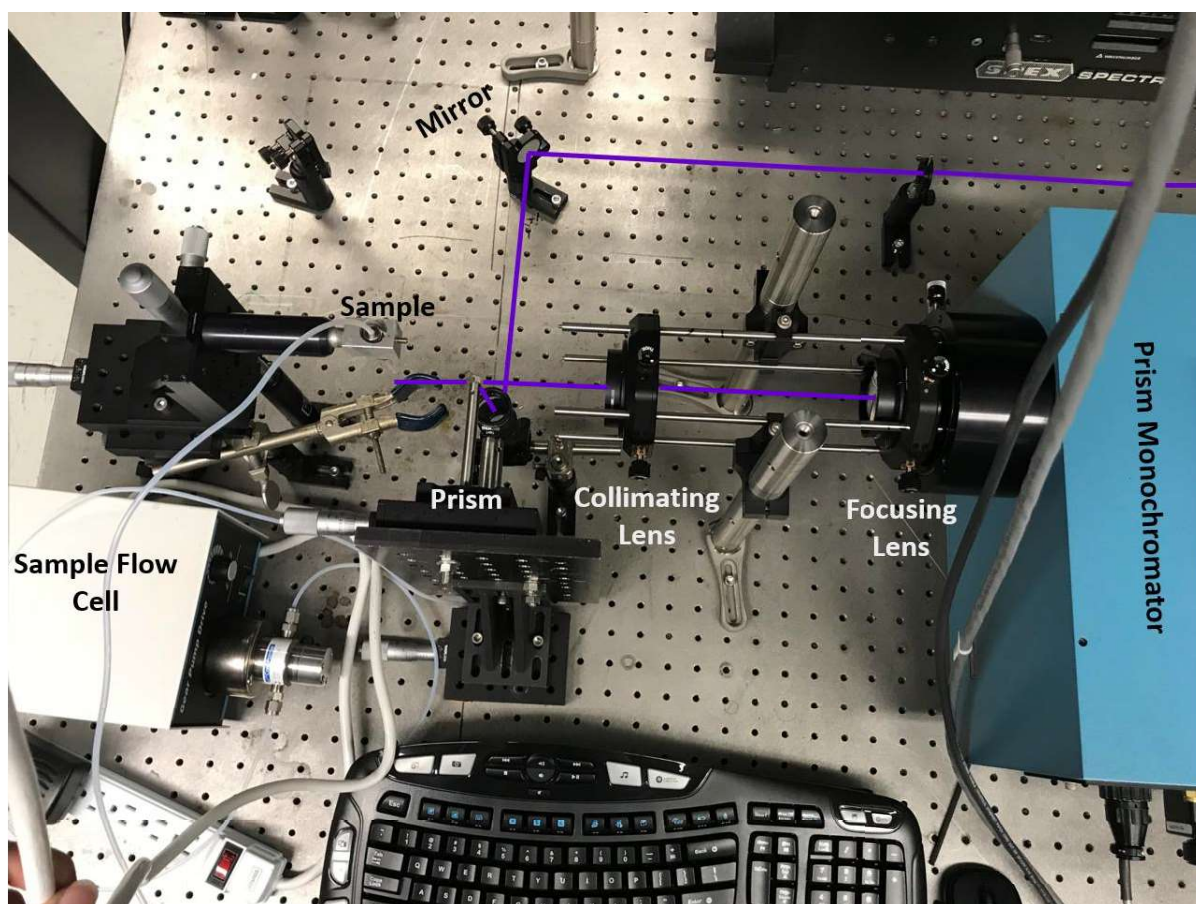


Figure 2.12: UV optics used to direct the light onto the sample. Laser light is directed from the laser (from the right) and up through a focusing lens and a prism to redirect the light onto the sample. Raman light is then scattered back and a set of collection lenses are used to direct the light into the spectrometer.

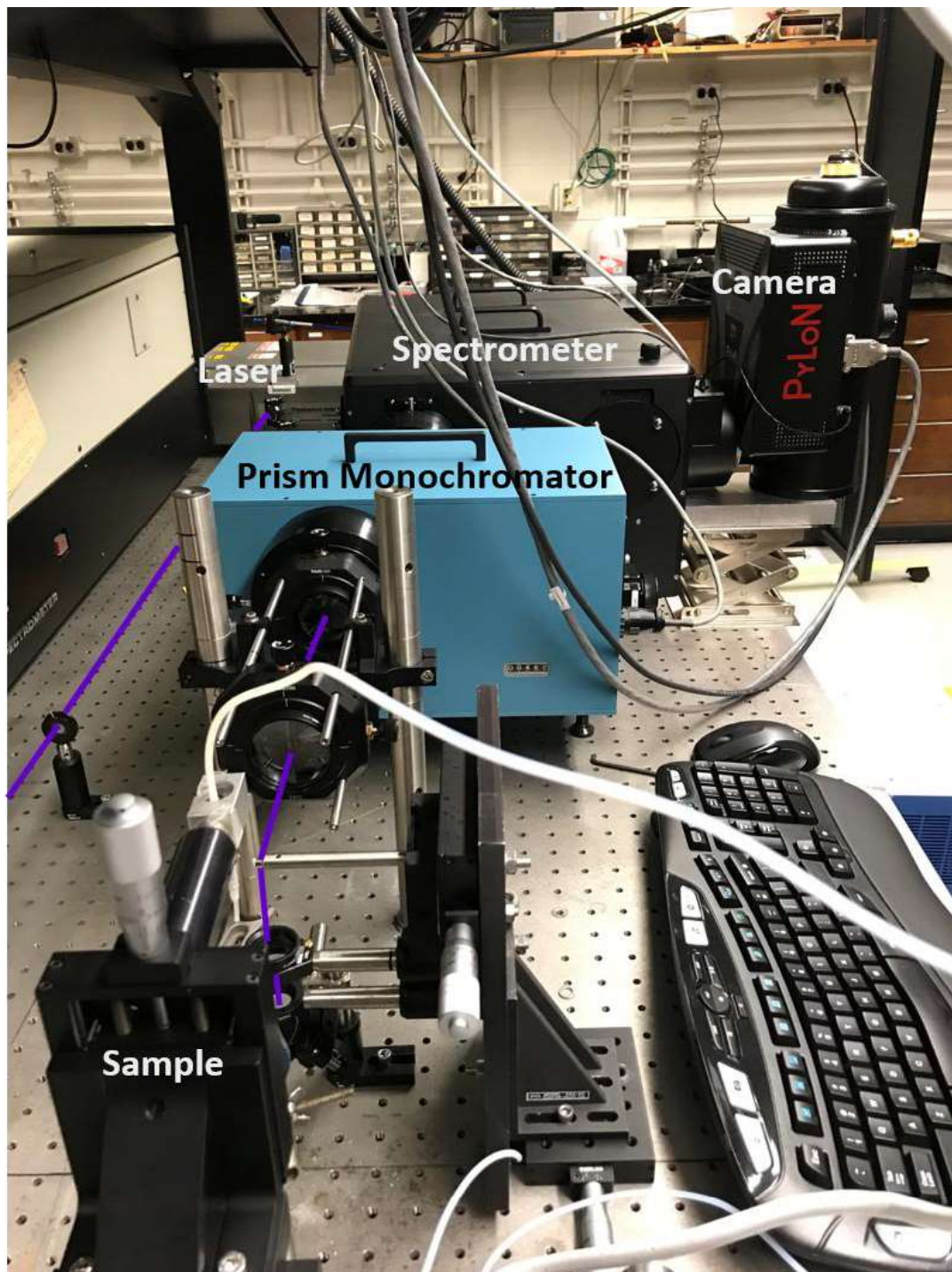


Figure 2.13: Pathway of Raman scattered light into the spectrometer. The Raman scattered light is collimated and sent through a focusing lens to enter the slit of the prism monochromator where the Rayleigh light can be filtered and the Raman light is allowed to pass to the spectrometer where it is dispersed onto the camera.

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Chapter 3

Multi-metal, multi-wavelength surface-enhanced Raman spectroscopy detection of neurotransmitters

Chapter 3 is an adaption of a research article in ACS Chemical Neuroscience. Moody, A.S. and Sharma, B., Multi-metal, Multi-wavelength Surface-Enhanced Raman Spectroscopy Detection of Neurotransmitters. *ACS Chem. Neurosci.* **2018.** 9, 1380-1387. The article describes the characterization of optimal SERS detection parameters for seven neurotransmitters. The SERS detection was optimized on both silver and gold nanoparticles at three excitation wavelengths.

3.1 Abstract

The development of a sensor for the rapid and sensitive detection of neurotransmitters could provide a pathway for the diagnosis of neurological diseases, leading to the discovery of more effective treatment methods. We investigate the use of surface enhanced Raman spectroscopy (SERS) based sensors for the rapid detection of melatonin, serotonin, glutamate, dopamine, GABA, norepinephrine, and epinephrine. Previous studies have demonstrated SERS detection of neurotransmitters, however, there has been no comprehensive study on the effect of the metal used as the SERS substrate or the excitation wavelength used for detection. Here, we present the detection of seven neurotransmitters using both silver and gold nanoparticles at excitation wavelengths of 532 nm, 633 nm, and 785 nm. Over the range of wavelengths investigated, the SERS enhancement on the silver and gold nanoparticles varies, with an average enhancement factor of 10^5 - 10^6 . The maximum SERS enhancement occurs at an excitation wavelength of 785 nm for the gold nanoparticles and at 633 nm for the silver nanoparticles.

3.2 Introduction

Monitoring changes in neurological function is key in diagnosis of various diseases, stress, or injury. These changes could be monitored through the detection of neurotransmitters.¹⁻² For many neurological diseases, the only confirmation of the disease is with an autopsy after death which leads to many incidences of misdiagnosis and ineffective treatment methods.³ There is a need for earlier diagnosis where treatment would be most beneficial. Commonly used

detection techniques for neurotransmitters are high performance liquid chromatography (HPLC)⁴⁻⁵, electrochemical detection⁶⁻⁸, or fluorescent labeling⁹⁻¹¹. These techniques, however, require extensive sample preparation, long run times, electroactive samples, or are destructive to the sample. Raman Spectroscopy (RS) has gained much interest as a biological sensing technique due to the excellent chemical specificity with simple instrumentation.¹² It provides a fingerprint-like spectrum without interferences from water and requires little to no sample preparation. A limitation of RS is the inherently weak signal obtained. This can be overcome with surface enhanced Raman spectroscopy (SERS), which provides an enhancement of the weak Raman signal through adsorption of the analyte to the surface of metal nanoparticles. SERS utilizes a property of plasmonic metal nanoparticles known as the localized surface plasmon resonance (LSPR), where an oscillating electric field is generated at the surface of the nanoparticle when excited with a laser, which results in enhancement of the Raman signal by up to 8 orders of magnitude.¹³⁻¹⁴ SERS is sensitive, rapid, label-free, and has multiplexing capabilities.

SERS measurements have previously been reported using Au nanoparticles for the detection of serotonin¹⁵ and melatonin¹⁶ and using Au nanoraspberry coated nanopipettes for the detection of ATP, glutamate, acetylcholine, GABA, and dopamine¹⁷. SERS detection of catecholamines using silver electrodes¹⁸⁻¹⁹ and silver colloids,²⁰ as well as detection of glutamate and GABA²¹ using silver colloids, have been reported²². The role of surface adsorption on SERS measurements of neurotransmitters has been studied with dopamine, epinephrine, serotonin, norepinephrine, and catechol on Ag electrodes²³. There has, however, been no comprehensive study of the SERS detection of neurotransmitters across a range of wavelengths. The SERS effect, due to an enhanced electric field, occurs when the nanoparticles are excited using a wavelength within the range of their LSPR. By studying molecules across a range of

wavelengths, the greatest enhancement for a particular substrate can be determined to obtain the lowest limit of detection¹⁴.

Here, we present the SERS detection of seven neurotransmitters: melatonin, serotonin, glutamate, dopamine, GABA, norepinephrine, and epinephrine. The spectra have been collected at three commonly used laser wavelengths for Raman spectroscopy: 532 nm, 633 nm, and 785 nm on both silver nanoparticles (AgNPs) and gold nanoparticles (AuNPs). While there are a variety of SERS substrates available²⁴, we chose Ag and Au colloids because they are easily synthesized SERS substrates, commonly used, and cost-effective. Additionally, we are interested in developing *in vivo* and *in vitro* SERS-based sensors. In general, AuNPs are more biologically compatible than AgNPs, because Au is inert while Ag is easily oxidized; therefore, AuNPs are preferred for *in vivo* detection. For *in vitro* detection methods, either AuNPs or AgNPs could be used, with the choice of metal based on the target analyte and its affinity for the metal surface. We report on the optimal parameters (wavelength and metal) for detection of the seven neurotransmitters.

3.3 Experimental Section

3.3.1 Materials and Reagents

Hydrogen tetrachloroaurate(III) trihydrate (HAuCl₄), 3-hydroxytyramine hydrochloride (Dopamine, 99%), trisodium salt dehydrate (citric acid, 99%), and sodium borohydride (NaBH₄) were purchased from ACROS Organics. Silver nitrate (AgNO₃) and hydrochloric acid were purchased from Fisher Scientific. 5-hydroxytryptamine hydrochloride (Serotonin), (-)-epinephrine, L-glutamic acid monosodium salt monohydrate, gamma-amino-n-butyric acid crystalline, and (-)-Norepinephrine were purchased from Sigma-Aldrich. Melatonin (99+%) was purchased from Alfa Aesar. For measurements at various concentrations, the neurotransmitters were diluted in DI water at five different concentrations.

3.3.2 Preparation of silver and gold nanoparticles

Silver nanoparticles (AgNPs) were prepared by reduction of silver nitrate with sodium borohydride, according to the method described by Creighton et al.²⁵. Briefly, in a 3 to 1 volume ratio, 1 mM sodium borohydride at 5° C (3 volumes) was added dropwise to 2mM silver nitrate (1 volume) at room temperature under strong stirring.

Gold nanoparticles (AuNPs) were prepared according to the method described by B. V. Enüstün and J. Turkevich³³. Briefly, 850mL of distilled H₂O was brought to a boil in a 1L flask where a 100mL of a 0.05% by weight solution of H₂AuCl₄ was added. Once the solution was boiling again, 50mL of a 1% by weight solution of Na₃-citrate was added. The resulting solution was boiled continuously for 30 minutes. The prepared AgNPs and AuNPs were characterized using an Agilent Cary 5000 UV/Vis/NIR spectrometer and scanning electron microscopy (SEM).

3.3.3 SERS Sample Preparation

Normal Raman spectra were taken of each neurotransmitter solid. Solution spectra could not be obtained below a concentration of 100mM for any of the neurotransmitters. To collect SERS spectra using Au nanoparticles, 4 mL aliquots of the Au nanoparticles were centrifuged, decanted, and 200 µL of the neurotransmitter solution were added. The pH of the neurotransmitter/nanoparticle solution was adjusted to pH 2 by addition of 0.75 µL of 3 M HCl. To collect SERS spectra using Ag nanoparticles, 1 mL aliquots of the Ag nanoparticles were centrifuged and decanted, and 200 µL of the neurotransmitter solution were added. For both Ag and Au NPs, SERS spectra were collected immediately after adding the neurotransmitter solution.

3.3.4 Raman Spectroscopy

Raman and SERS spectra were collected in a 180° backscattering geometry and directed into a IsoPlane 320 spectrometer (Princeton Instruments), equipped with a PIXIS 400 camera

(Princeton Instruments). The laser illuminated the sample through a Nikon Ti-U microscope where a 20x objective (Nikon) was used to focus the laser on the sample. Excitation wavelengths of 532 nm (Coherent, Inc.), 633 nm (Newport), and 785 nm (Innovative Photonic Solutions) were used. For normal Raman, powers of 10 mW, 6 mW, and 20 mW were used for wavelengths of 532 nm, 633 nm, and 785 nm, respectively. For SERS spectra, powers of 1 mW, 3 mW, and 5 mW were used for wavelengths of 532 nm, 633 nm, and 785 nm, respectively. Acquisition times of 60 s were used for SERS spectra and 10 s for Raman solid spectra.

3.4 Results and Discussion

The extinction spectrum of the borohydride reduced AgNPs shows an LSPR maximum at 389 nm which is in good agreement with the literature²⁵. The position of the LSPR maximum allows the use of the AgNPs for SERS experiments with wavelengths of 532 nm, 633 nm, and 785 nm. For the citrate reduced AuNPs, an LSPR maximum of 525 nm was observed. Due to the interband transition of gold, the AuNPs are only feasible for use at wavelengths of 633 nm and 785 nm. Both AgNPs and AuNPs were also characterized with scanning electron microscopy (SEM) which revealed that the particles are mostly spherical in shape with an average diameter of 35 nm and 61 nm, respectively. (Figure 3.1)

Initially, the solid spectra of each neurotransmitter were recorded at wavelengths of 532 nm, (Figure 3.2A) 633 nm, (Figure 3.2B) and 785 nm (Figure 3.2C). The solid spectra provide a reference for peaks associated with each neurotransmitter for the SERS spectra.

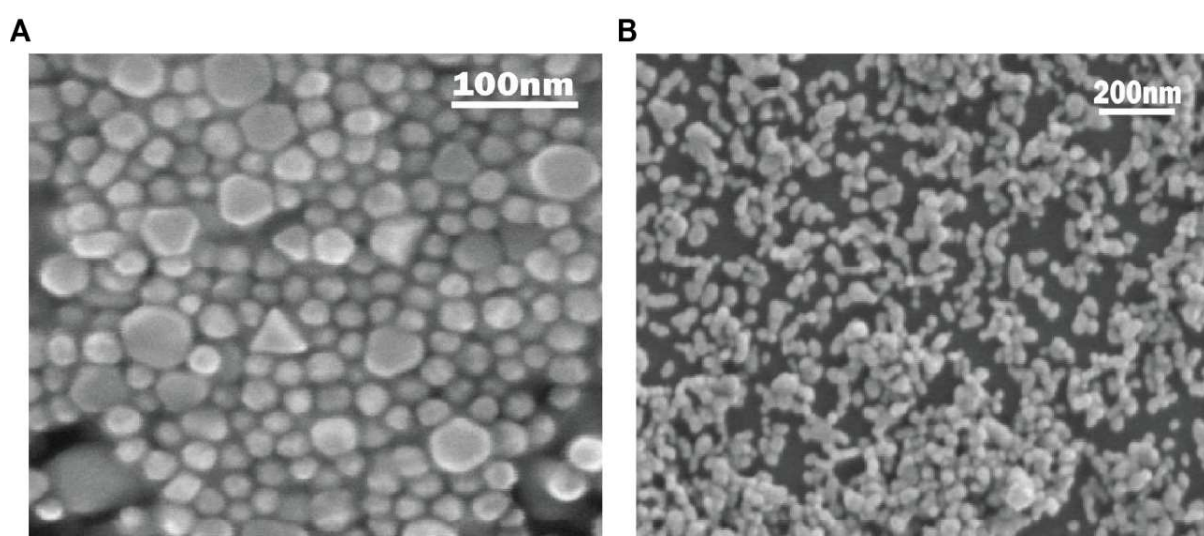


Figure 3.1: SEM images of AuNPs (A) and AgNPs (B) used for SERS detection.

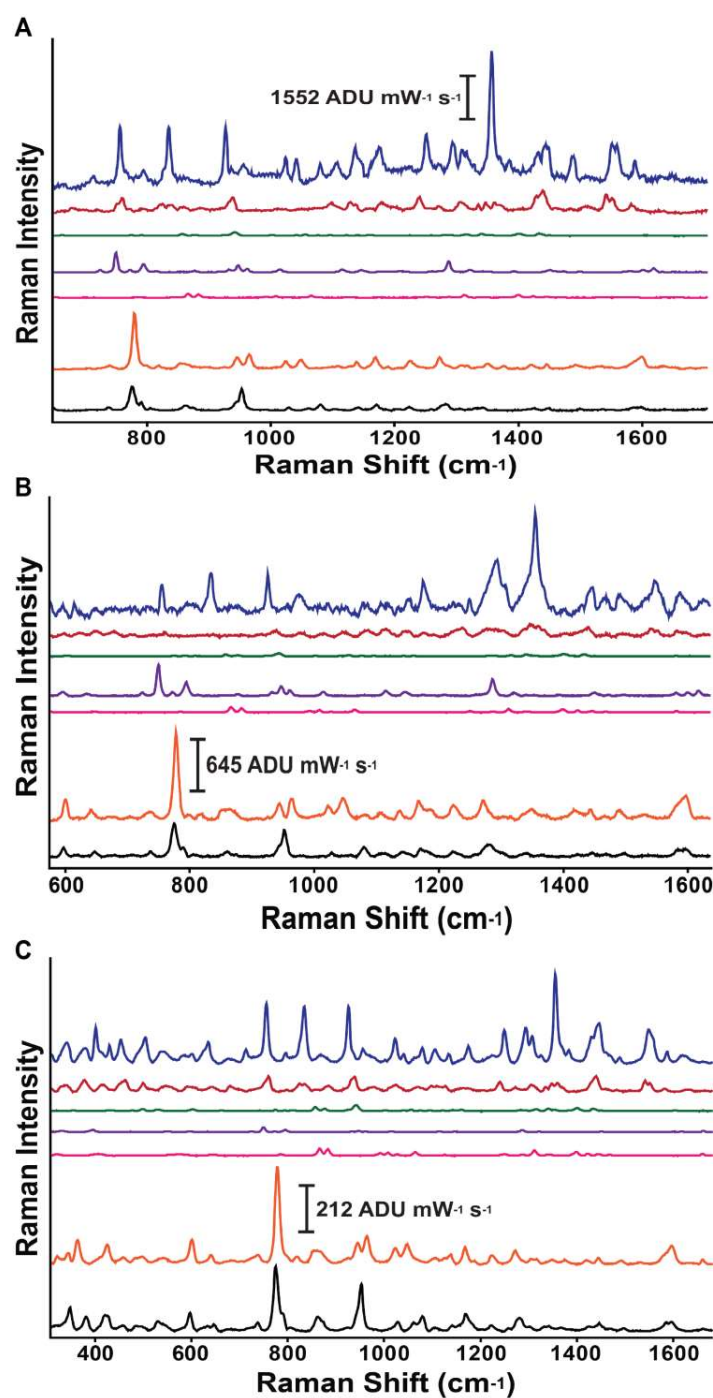


Figure 3.2: Solid spectra of melatonin (blue), serotonin (red), glutamate (green), dopamine (purple), GABA (pink), norepinephrine (orange), and epinephrine (black) at (A) $\lambda_{\text{ex}} = 532 \text{ nm}$, $P = 10 \text{ mW}$, $t = 10 \text{ sec}$, (B) $\lambda_{\text{ex}} = 633 \text{ nm}$, $P = 6 \text{ mW}$, $t = 10 \text{ sec}$, and (C) $\lambda_{\text{ex}} = 785 \text{ nm}$, $P = 20 \text{ mW}$, $t = 10 \text{ sec}$.

The SERS spectra of melatonin, serotonin, glutamate, dopamine, GABA, norepinephrine, and epinephrine were then collected using AuNPs and AgNPs at wavelengths of 532 nm, 633 nm, and 785 nm. SERS spectra for each neurotransmitter were recorded at concentrations of 500 μ M, 100 μ M, 50 μ M, 10 μ M, 1 μ M, 800 nM, 600 nM, 400 nM, 200 nM, 100 nM, 75 nM, 50 nM, 25 nM, and 1 nM. The purpose was to identify the SERS spectral features, which can be shifted in wavenumber position with respect to the normal Raman peak positions²⁶⁻²⁷, as well as determine a limit of detection (LOD) associated with each neurotransmitter on AuNPs and AgNPs.

Principal components analysis (PCA) was also performed (Solo analysis software, Eigenvector) at each wavelength and metal to determine our LODs. PCA is an unsupervised multivariate analysis method, which is often applied for large datasets that contain more variables than can be easily labeled. PCA is widely used to reduce the dimensionality of the dataset through an orthogonal transformation. This decomposes the data into a series of principal components where each principal component contains a spectral pattern, or loading, and a score that describes the relationship between the sample and principal component. By plotting the scores of the different principal components, one can demonstrate clustering of the sample into classes that explain major trends, groups or patterns in the data set.²⁸⁻²⁹ Since we have large data sets with seven neurotransmitters at different concentration ranges and on different metals, we chose PCA to aid in the decomposition of the complex spectra and to help reveal spectral patterns not easily discernible by eye. Each spectrum was first zapped for cosmic rays, baselined and subtracted (home written software in Python), then smoothed with a Savitzky-Golay function (Solo).

Figure 3.3A shows a representative SERS spectrum for each neurotransmitter on AgNPs with an excitation wavelength of 532 nm and at a concentration of 50 μ M. The PCA results from

the neurotransmitter detection with AgNPs at 532 nm are displayed in Figure 3.3B. From these results we see a good separation of serotonin, melatonin, glutamate, and GABA down to their individual LODs, whereas dopamine, epinephrine, and norepinephrine do not exhibit good enhancement with AgNPs and therefore do not show the same separation. For melatonin, dopamine, norepinephrine, and epinephrine a LOD of 10 μ M is observed. A LOD of 50 μ M is observed for GABA, 600 nM for glutamate, and 100 nM for serotonin. We have outlined peaks associated with each neurotransmitter as well as their chemical structures and LODs in Table 3.1.

The SERS spectra of the seven neurotransmitters were then recorded at a wavelength of 633 nm on both AuNPs and AgNPs. At 633 nm with AgNPs, a larger enhancement is observed in comparison to the enhancement observed at 532 nm, in part due to suppression of the fluorescence exhibited by the neurotransmitters at 532 nm. A representative SERS spectrum of each neurotransmitter at a concentration of 50 μ M is shown in Figure 3.4A for AuNPs and in Figure 3.4B for AgNPs. The differences in the relative intensities of peaks with the AuNPs versus AgNPs can be explained with the binding sites of the neurotransmitter on the particles. SERS spectra at 633 nm on AuNPs exhibit the highest enhancement for the aromatic neurotransmitters (melatonin, serotonin, dopamine, epinephrine, and norepinephrine) and little to no SERS enhancement for the amino acid chain neurotransmitters (GABA and glutamate) where these chemical structures are shown in Table 3.1A-G. The surface of gold colloids has a high affinity for the indole ring³⁰ of the aromatic neurotransmitters which explains why the representative spectra show that the SERS effect for these neurotransmitters on AuNPs is greater than that of GABA and glutamate. The carboxyl groups on the amino acid chain neurotransmitters can be within a close proximity to the AuNP surface,

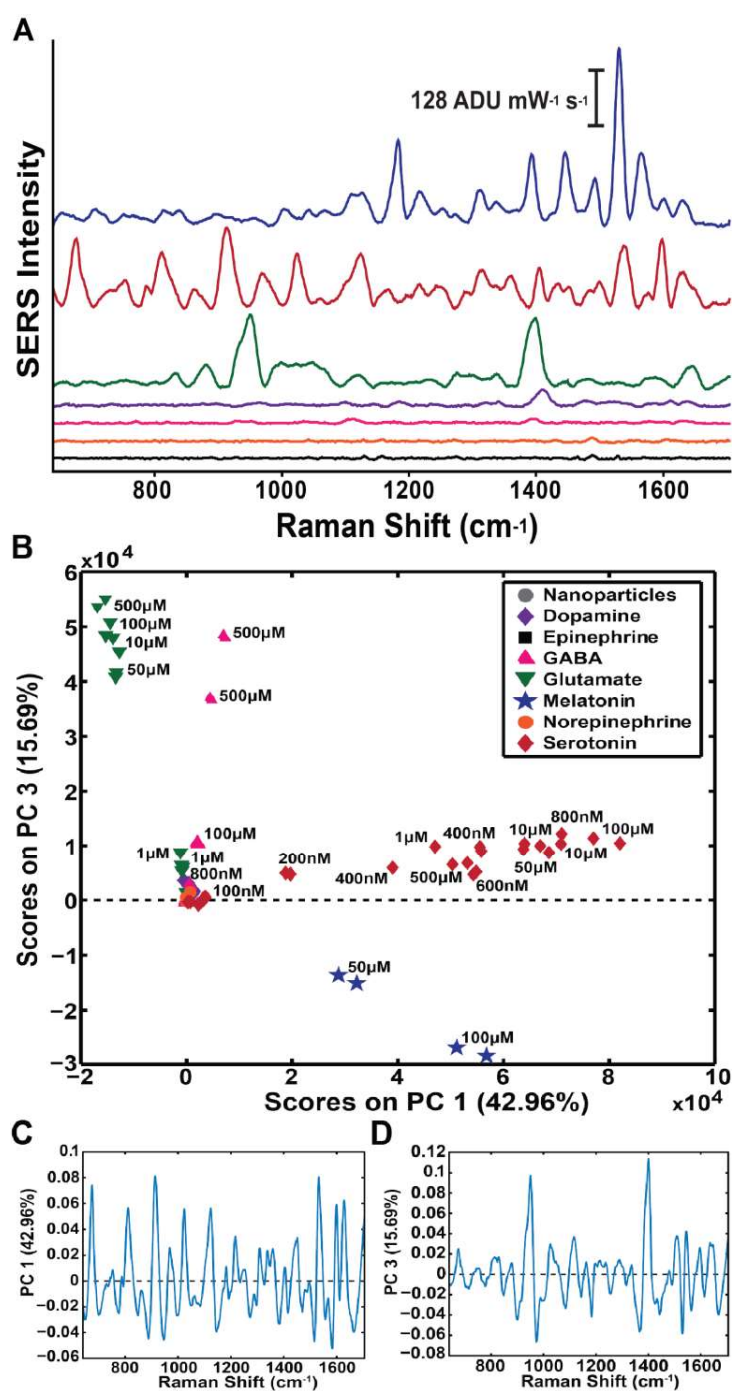


Figure 3.3: SERS spectra (A) of melatonin (blue), serotonin (red), glutamate (green), dopamine (purple), GABA (pink), norepinephrine (orange), and epinephrine (black) on AgNPs at 50 μM with a PCA of scores on PC1 plotted against scores on PC3 (B) of concentrations from 500 μM to 1 nM and their accompanying loadings (C, D). $\lambda_{\text{ex}} = 532 \text{ nm}$, $P = 1 \text{ mW}$, $t = 60 \text{ sec}$.

Table 3.1A: Peak positions, chemical structure, and LODs of melatonin on AuNPs and AgNPs at an excitation wavelength of 532 nm, 633 nm, and 785 nm.

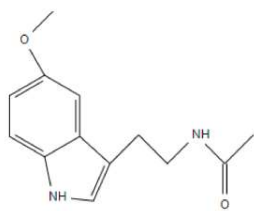
Neurotransmitter	532 nm AgNP	633 nm AgNP	633 nm AuNP	785 nm AgNP	785 nm AuNP	aCSF 785 nm AuNP	aUrine 785 nm AuNP
Melatonin 	653				636	636	641
	710						704
	751	748	747		744	744	749
	815		816		817	817	817
	838	837			840	840	852
			907		905	905	902
			942				
	1004		1008	1040	1009	1008	1010
	1110	1108				1090	1090
	1125	1125				1129	1118
	1183	1180				1180	1184
	1218		1220		1220	1220	1221
	1253						
	1309		1293	1315	1290	1290	1296
		1340	1355		1353	1353	1349
	1392	1390				1412	1410
	1444	1442					
	1491		1470		1469	1469	1462
	1529	1528	1541		1516	1516	1515
	1566	1566			1588	1590	1590
	1626	1624					
		1668		1663			
LOD	10 μ M	50 μ M	400 nM	1 μ M	100 nM		

Table 3.1B: Peak positions, chemical structure, and LODs of serotonin on AuNPs and AgNPs at an excitation wavelength of 532 nm, 633 nm, and 785 nm.

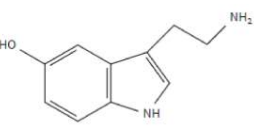
Neurotransmitter	532 nm AgNP	633 nm AgNP	633 nm AuNP	785 nm AgNP	785 nm AuNP	aCSF 785 nm AuNP	aUrine 785 nm AuNP
Serotonin 	674	675		635	640	635	635
		754	753	789	752	749	750
	810	810	816	809	820	812	821
	914	915	910	916	911	934	913
	972			967			
	1024	1024		1021		1018	
	1123	1118		1118	1110	1110	1106
	1216	1220	1221	1222	1221	1216	1223
	1308			1285	1297	1307	
	1335	1350	1342	1354	1345	1346	1338
	1403	1405		1404			1367
	1430			1426	1424	1420	1421
	1452	1452					1473
	1500						
	1538	1532		1526	1521	1515	1521
	1598	1600	1586	1598	1595	1597	1592
	1626	1634					
LOD	100 nM	100 nM	200 nM	200 nM	100 nM		

Table 3.1C: Peak positions, chemical structure, and LODs of glutamate on AuNPs and AgNPs at an excitation wavelength of 532 nm, 633 nm, and 785 nm.

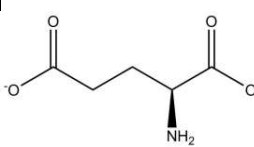
Neurotransmitter	532 nm AgNP	633 nm AgNP	633 nm AuNP	785 nm AgNP	785 nm AuNP
Glutamate 	832 881 951 1050 1117 1393	832 894 908 936 984 1043 1117 1292 1389		832 908 935 982 1038 1118 1290 1386	
LOD	600 nM	600 nM		600 nM	

Table 3.1D: Peak positions, chemical structure, and LODs of dopamine on AuNPs and AgNPs at an excitation wavelength of 532 nm, 633 nm, and 785 nm.

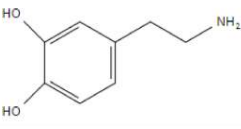
Neurotransmitter	532 nm AgNP	633 nm AgNP	633 nm AuNP	785 nm AgNP	785 nm AuNP	aCSF 785 nm AuNP	aUrine 785 nm AuNP
Dopamine 			677 711 819 956	452 947	469 674 710 951	463 669 709 951	467 676 707 889
	1097	890 965 1080	1113 1150 1285 1327		1110 1156 1286	1044 1120 1179 1276	1029 1109
	1183	1142	1285 1327				1281 1323
	1323	1392	1367	1388	1370	1365	1369
	1340		1431			1410	1420
	1410	1467					
	1477	1489	1492		1489	1476	1498
		1541	1568		1564	1549	1564
		1593	1591				
	1608		1610	1600		1611	
			1657		1659	1657	1653
LOD	10 μ M	10 μ M	600 nM	10 μ M	200 nM		

Table 3.1E: Peak positions, chemical structure, and LODs of GABA on AuNPs and AgNPs at an excitation wavelength of 532 nm, 633 nm, and 785 nm.

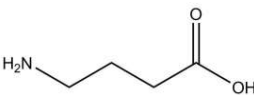
Neurotransmitter	532 nm AgNP	633 nm AgNP	633 nm AuNP	785 nm AgNP	785 nm AuNP
GABA 	818 858 931 1034 1108 1167 1217 1238 1394 1447 1483	687 813 858 898 937 1036 1109 1220 1395 1445		858 896 939 1029 1109 1216 1392 1446	
LOD	50 μ M	50 μ M		50 μ M	

Table 3.1F: Peak positions, chemical structure, and LODs of norepinephrine on AuNPs and AgNPs at an excitation wavelength of 532 nm, 633 nm, and 785 nm.

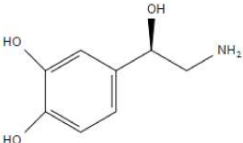
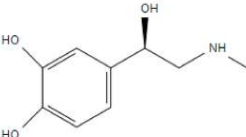
Neurotransmitter	532 nm AgNP	633 nm AgNP	633 nm AuNP	785 nm AgNP	785 nm AuNP	aCSF 785 nm AuNP	aUrine 785 nm AuNP
Norepinephrine 		639		640	677 723	641 768	755 847
		815 1042	788 1043	815		1050	
	1157		1157	1157	1161	1177	1163
	1291	1272	1283	1271	1281	1279	1281
	1364	1340	1368	1336	1370	1337	1372
	1437	1430		1422	1438	1433	1435
	1487	1489	1494	1486	1500	1485	1498
				1567	1566	1555	1576
	1590		1598		1605	1602	
					1663	1659	1663
LOD	10 μ M	10 μ M	1 μ M	10 μ M	1 μ M		

Table 3.1G: Peak positions, chemical structure, and LODs of epinephrine on AuNPs and AgNPs at an excitation wavelength of 532 nm, 633 nm, and 785 nm.

Neurotransmitter	532 nm AgNP	633 nm AgNP	633 nm AuNP	785 nm AgNP	785 nm AuNP	aCSF 785 nm AuNP	aUrine 785 nm AuNP
Epinephrine 	1041 1275 1318 1487	640 1041 1268 1318 1372 1465 1486	671 736 943 1059 1248 1281 1355 1456 1580 1692	 1040 1315 1486	674 735 942 1000 1245 1289 1356 1571 1694	740 929 1008 1044 1245 1278 1383 1433 1483 1583 1660	755 849 1064 1236 1280 1313 1460 1500 1661
LOD	10 μ M	10 μ M	10 μ M	500 μ M	10 μ M		

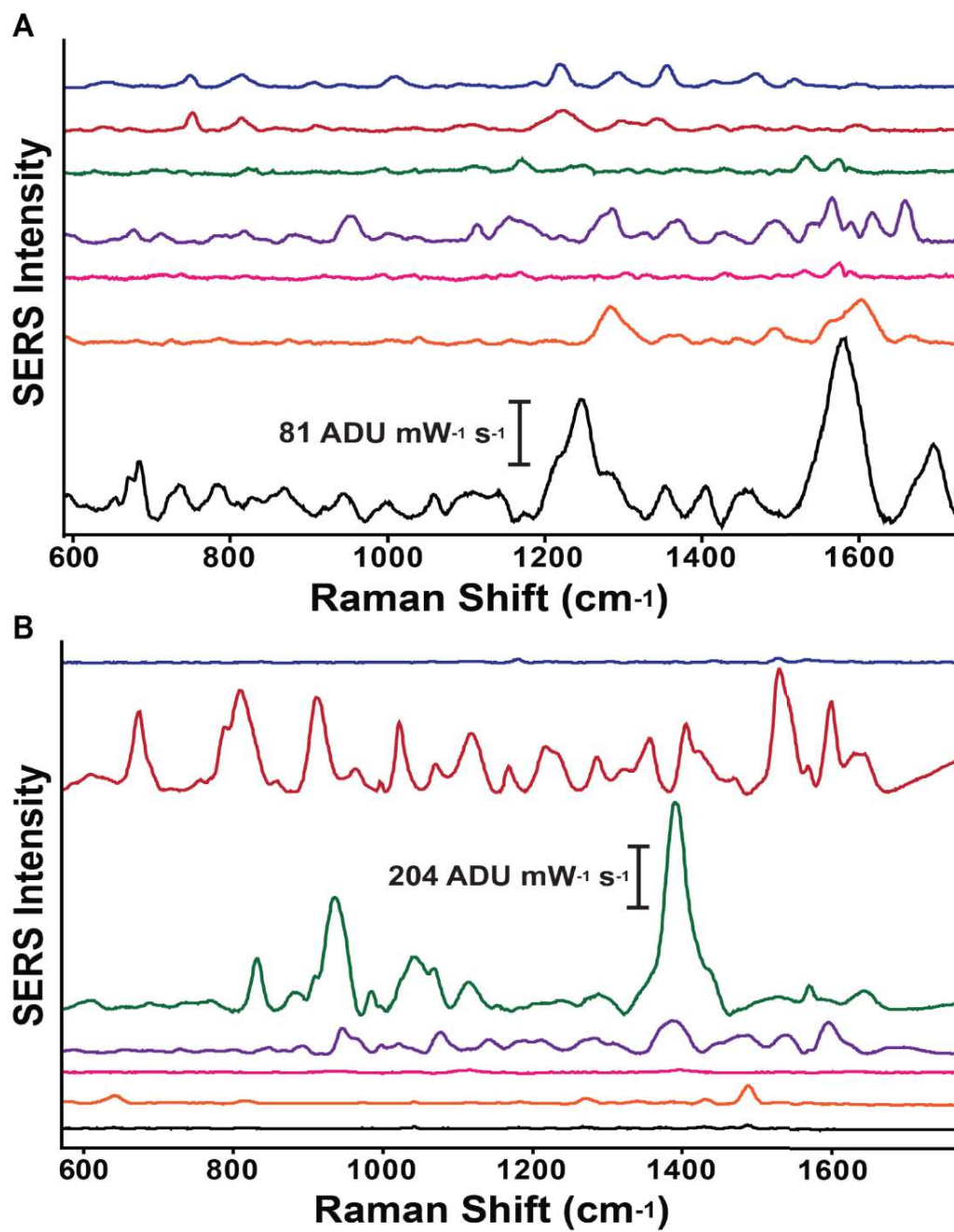


Figure 3.4: SERS spectra of melatonin (blue), serotonin (red), glutamate (green), dopamine (purple), GABA (pink), norepinephrine (orange), and epinephrine (black) on AuNPs (A) and AgNPs (B) at 50 μ M. λ_{ex} = 633 nm, P = 3 mW, t = 60 sec.

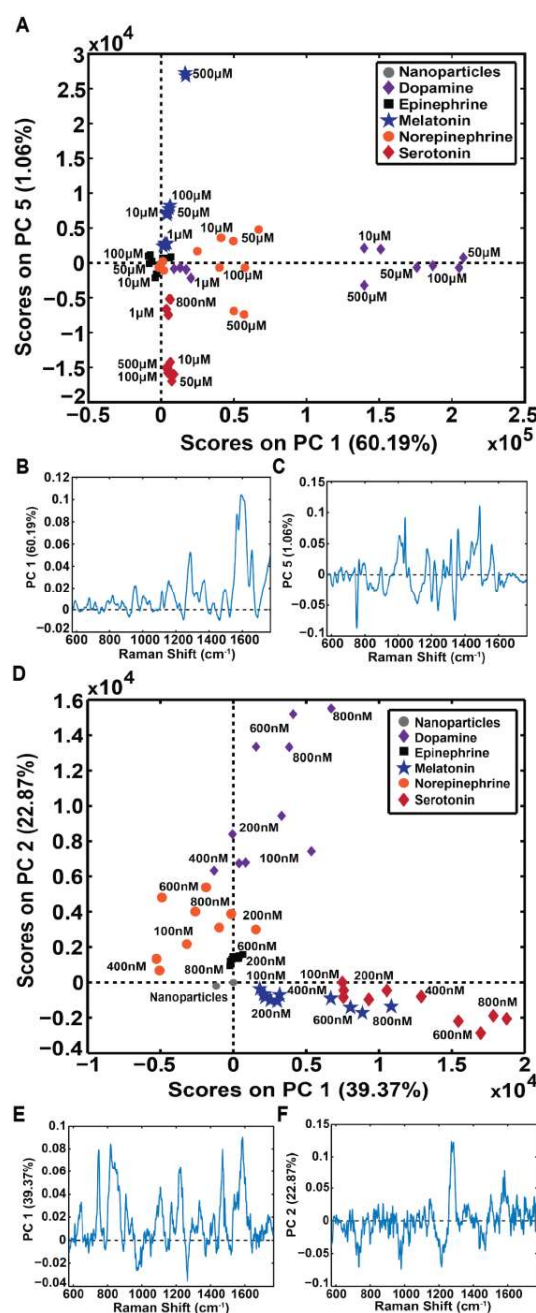


Figure 3.5: PCA plots of SERS spectra taken at 633 nm on AuNPs. Scores on PC1 plotted against scores on PC5 (A) of concentrations from 500 μ M to 800 nM and their accompanying loadings (B, C) and Scores on PC1 plotted against scores on PC2 (D) of concentrations from 800 nM to 100 nM and the accompanying loadings (E, F). $\lambda_{\text{ex}} = 633$ nm, $P = 3$ mW, $t = 60$ sec.

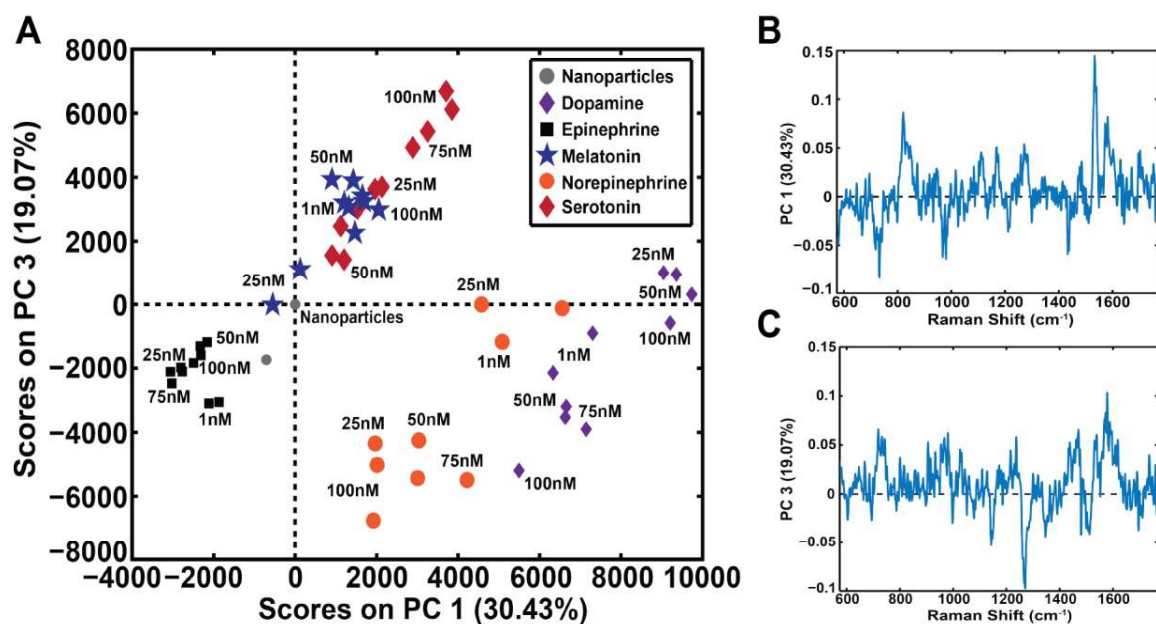


Figure 3.6: Scores on PC1 plotted against scores on PC2 (A) of concentrations from 100 nM to 1 nM and their accompanying loadings (B,C) for the SERS spectra of melatonin (blue), serotonin (red), dopamine (purple), norepinephrine (orange), and epinephrine (black) on AuNPs. $\lambda_{\text{ex}} = 633$ nm, $P = 3$ mW, $t = 60$ sec.

however they do not interact, which would lead to very weak or no signal and a very high LOD if the molecules are detected at all.³¹ The AgNPs interact strongly with the carboxyl and amine groups on the amino acids giving more intense signals and lower LODs for these neurotransmitters.³²

The PCA results for the SERS spectra at 633 nm on AuNPs are displayed in Figure 3.5. As previously stated, glutamate and GABA do not exhibit any SERS enhancement with AuNPs, and for this purpose were excluded from any PCA of SERS detection with AuNPs at both 633 nm and 785 nm. Any peaks observed in the GABA and glutamate SERS spectra arise from enhancement of the citrate on the surface of the AuNPs. The PCA was separated into three concentration ranges. When a PCA is performed on the entire concentration range studied, the neurotransmitters do not separate due to the large changes in intensity between high and low concentrations. The first concentration range (Figure 3.5A) includes the highest concentrations measured (500 μ M-800 nM). The middle concentration range for our PCA plots include concentrations from 800 nM-100 nM (Figure 3.5D). Figures 5A and 5D show melatonin, serotonin, and dopamine have the lowest LODs which are in the nanomolar range at 400 nM, 200 nM, and 600 nM, respectively. Epinephrine and norepinephrine have LODs in the micromolar range at 10 μ M and 1 μ M, respectively. The PCA for the low concentration range (100 nM-1 nM) shows no separation between the neurotransmitters below 100 nM demonstrating that the LODs for the individual neurotransmitters have been reached (Figure 3.6).

Figure 3.7 shows the PCA plots for SERS spectra acquired on AgNPs at an excitation wavelength of 633 nm. Again, it is observed that the highest concentrations show some separation while the lower concentrations cluster together (Figure 3.7A). The middle concentration range allows us to observe LODs (Figure 3.7D). Due to the vast difference in intensity between the SERS spectra of serotonin and glutamate and the SERS spectra of the other

neurotransmitters, we are not able to see LODs for the other neurotransmitters in this PCA. We can, however, visually observe LODs from the individual SERS spectra of the neurotransmitters. As shown in Table 3.1, for serotonin and glutamate LODs of 200 nM and 600 nM are observed, respectively whereas LODs of 10 μ M for dopamine and norepinephrine, 1 μ M for melatonin, 50 μ M for GABA, and 500 μ M for epinephrine are observed. The low concentration range (100 nM-1 nM) shows no further separation of the neurotransmitters confirming we have reached the LOD for each neurotransmitter (Figure 3.8).

Finally, SERS spectra were acquired for AuNPs and AgNPs at 785 nm. A representative SERS spectrum for each neurotransmitter at a concentration of 50 μ M on AuNPs is shown in Figure 3.9A and on AgNPs in Figure 3.9B. Similar to what is observed at an excitation wavelength of 633 nm, SERS spectra with the AuNPs at 785 nm show no enhancement of GABA and glutamate. SERS spectra acquired for AgNPs at 785 nm exhibit approximately the same enhancement as the SERS spectra with AgNPs at 532 nm. We observe that for AgNPs, serotonin and glutamate exhibit a measurably larger enhancement than the other neurotransmitters.

The PCA for the SERS spectra at 785 nm on AuNPs is displayed in Figure 3.10. Again, GABA and glutamate were excluded from PCA. We find LODs of 100 nM for melatonin and serotonin, 200 nM for dopamine, 1 μ M for norepinephrine, and 10 μ M for epinephrine (Figure 3.10A & 3.10D). The PCA of concentrations lower than 100 nM reveal no further separation, demonstrating that the LODs for each neurotransmitter have been reached (Figure 3.11).

The PCA plots for the SERS spectra at 785 nm on AgNPs are shown in Figure 3.12. The PCA reflects the large intensity difference in the SERS spectra of serotonin and glutamate where they are largely separated from the other neurotransmitters. When serotonin and glutamate are removed from the PCA, LODs for the other 5 neurotransmitters can be observed (Figure 3.13,

3.14). No further separation is observed in the PCA for concentrations below 100 nM (Figure 3.15). We find LODs of 1 μ M for melatonin, 10 μ M for dopamine and norepinephrine, 50 μ M for GABA, 500 μ M for epinephrine 600 nM for glutamate, and 200 nM for serotonin.

We find that for serotonin, as well as the amino acid neurotransmitters such as GABA and glutamate, the AgNPs act as the best substrate. For the catecholamines (eg. melatonin, dopamine, epinephrine, and norepinephrine) the AuNPs are the best substrate for achieving the lowest LODs, however, the gold nanoparticles also exhibit excellent detection capabilities for serotonin. To find an approximate enhancement factor for our nanoparticles, we used a common Raman reporter molecule, benzenethiol, and compared integrated peak intensities for three different Raman peaks at each wavelength. This provided us with an average enhancement factor for both AgNPs and AuNPs of 10^5 - 10^6 at all three excitation wavelengths. The neurotransmitters, however, behave differently than benzenethiol when adsorbed to the nanoparticles. To determine the best enhancement for each neurotransmitter, we compared peak areas for various peaks of each neurotransmitter, using peaks that were previously identified in the literature. Figure 3.16 shows the enhancement of each neurotransmitter on both AgNPs (Figure 3.16A) and AuNPs (Figure 3.16B), for a single peak from each neurotransmitter to simplify the figure, however all other peaks integrated for each neurotransmitter resulted similar trends. The integrated peak areas have been corrected for laser power and acquisition time. We find that the largest enhancement for the AgNPs occurs at a wavelength of 633 nm and the largest enhancement for AuNPs occurs at 785nm.

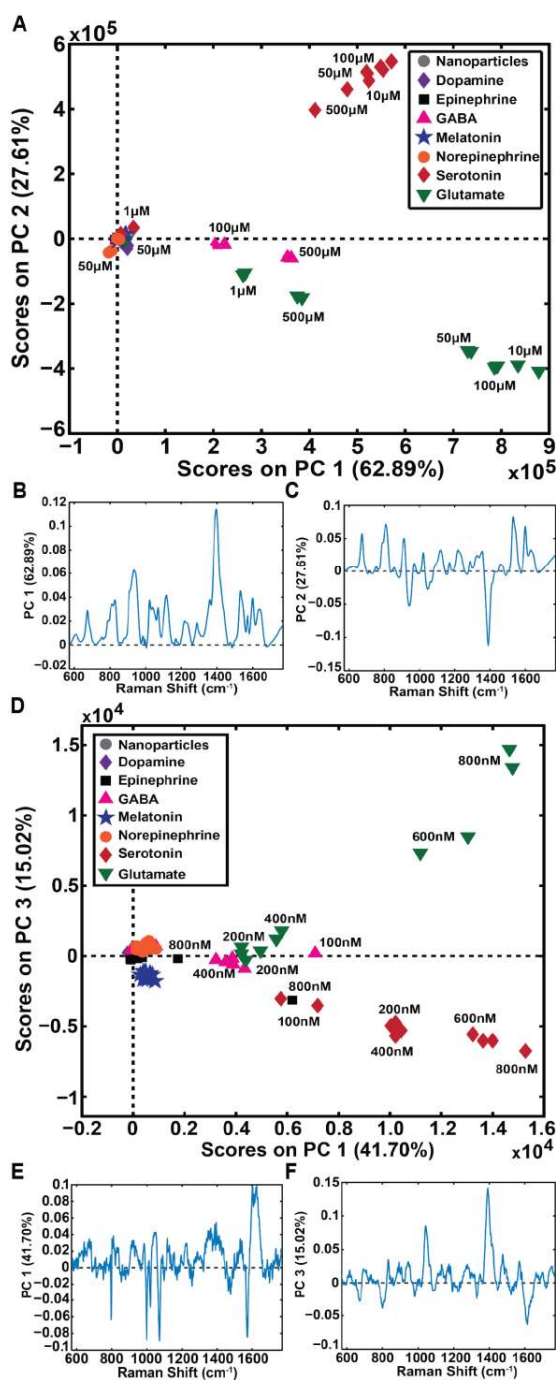


Figure 3.7: PCA plots for SERS spectra taken at 633 nm on AgNPs. Scores on PC1 plotted against scores on PC2 (A) at concentrations from 500 μ M to 800 nM and their accompanying loadings (B, C) and scores on PC1 plotted against scores on PC3 (D) of concentrations of 800 nM to 100 nM and their accompanying loadings (E, F). $\lambda_{\text{ex}} = 633$ nm, $P = 3$ mW, $t = 60$ sec.

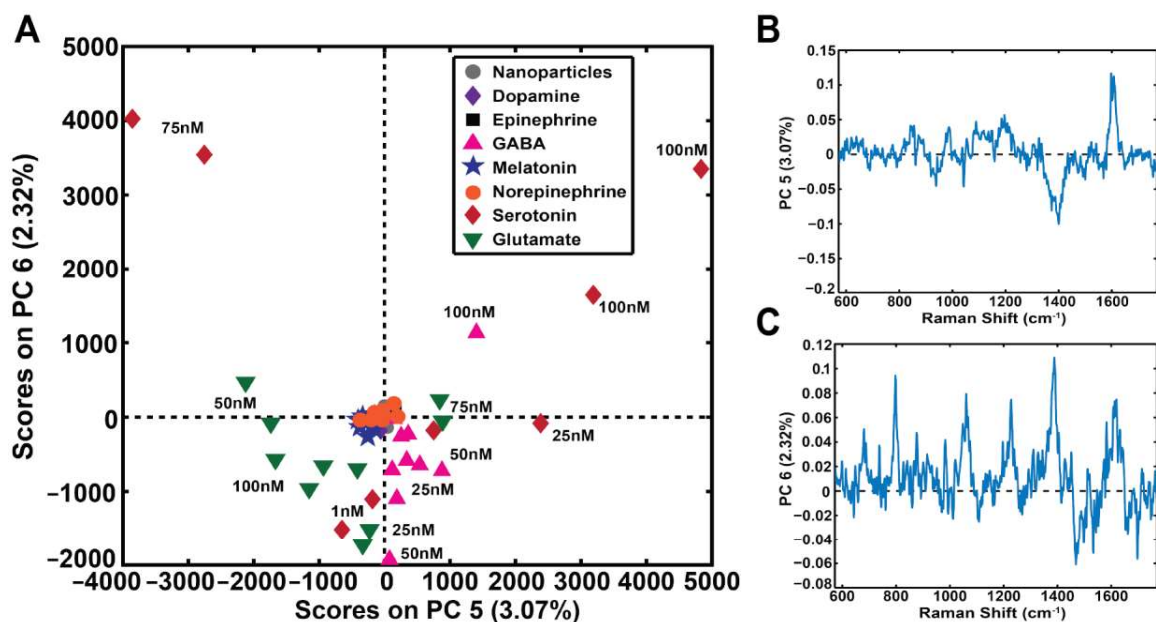


Figure 3.8: Scores on PC1 plotted against scores on PC2 (A) of concentrations from 100 nM to 1 nM and their accompanying loadings (B,C) for the SERS spectra of melatonin (blue), serotonin (red), glutamate (green), dopamine (purple), GABA (pink), norepinephrine (orange), and epinephrine (black) on AgNPs. $\lambda_{\text{ex}} = 633 \text{ nm}$, $P = 3 \text{ mW}$, $t = 60 \text{ sec}$.

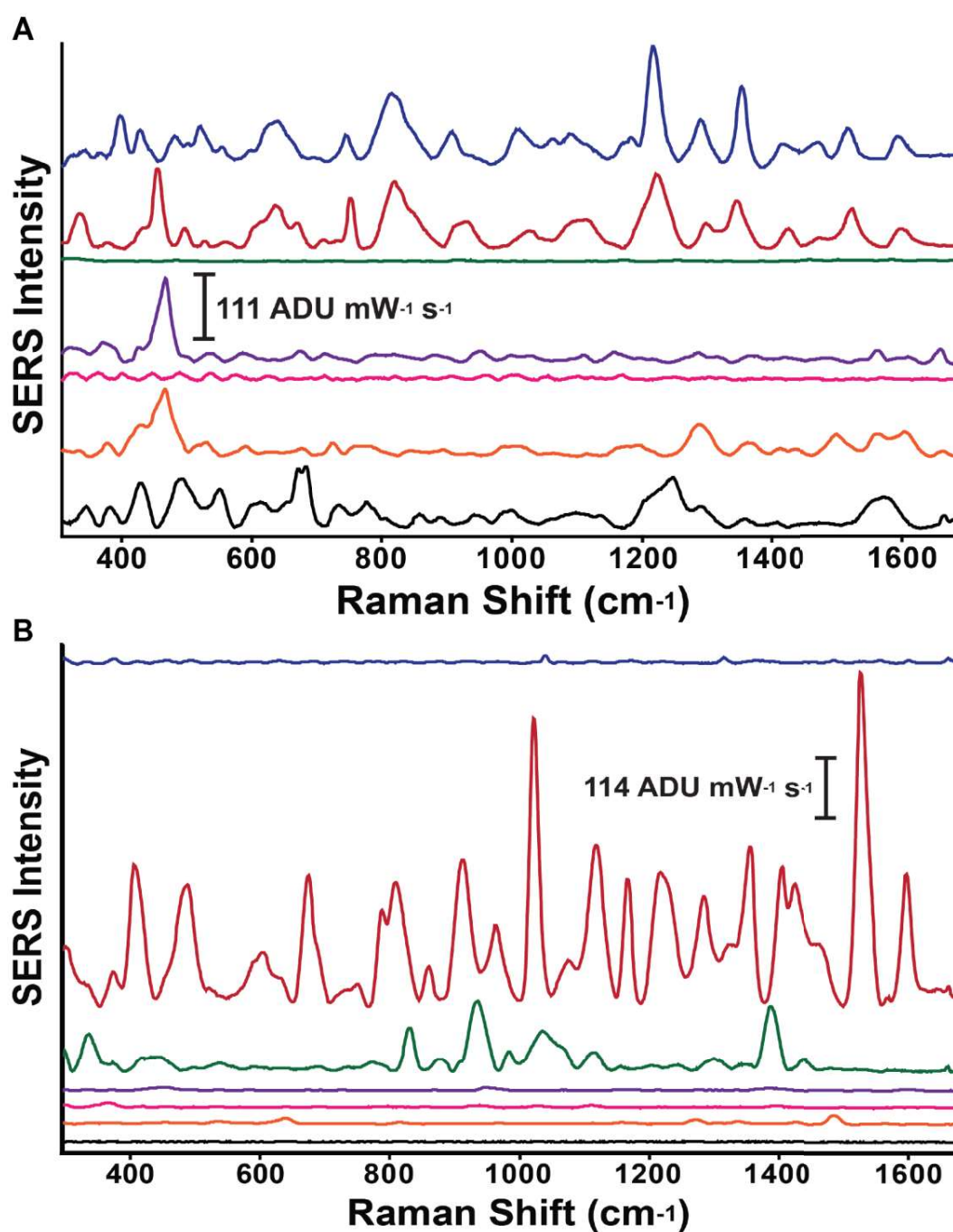


Figure 3.9: SERS spectra of melatonin (blue), serotonin (red), glutamate (green), dopamine (purple), GABA (pink), norepinephrine (orange), and epinephrine (black) on AuNPs (A) and AgNPs (B) at 50 μ M. $\lambda_{\text{ex}} = 785$ nm, $P = 5$ mW, $t = 60$ sec.

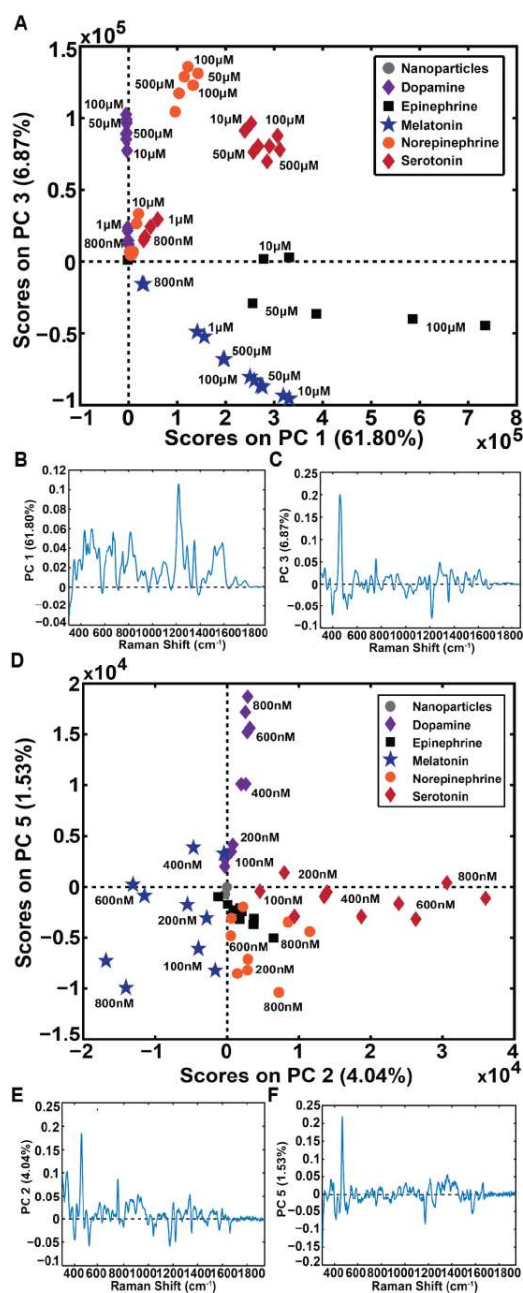


Figure 3.10: PCA plots for SERS spectra taken at 785 nm on AuNPs. Scores on PC1 plotted against scores on PC3 (B) of concentrations from 500 μM to 800 nM and their accompanying loadings (C, D) and Scores on PC2 plotted against scores on PC5 (E) of concentrations of 800 nM to 100 nM and their accompanying loadings (F, G). $\lambda_{\text{ex}} = 785 \text{ nm}$, $P = 5 \text{ mW}$, $t = 60 \text{ sec}$.

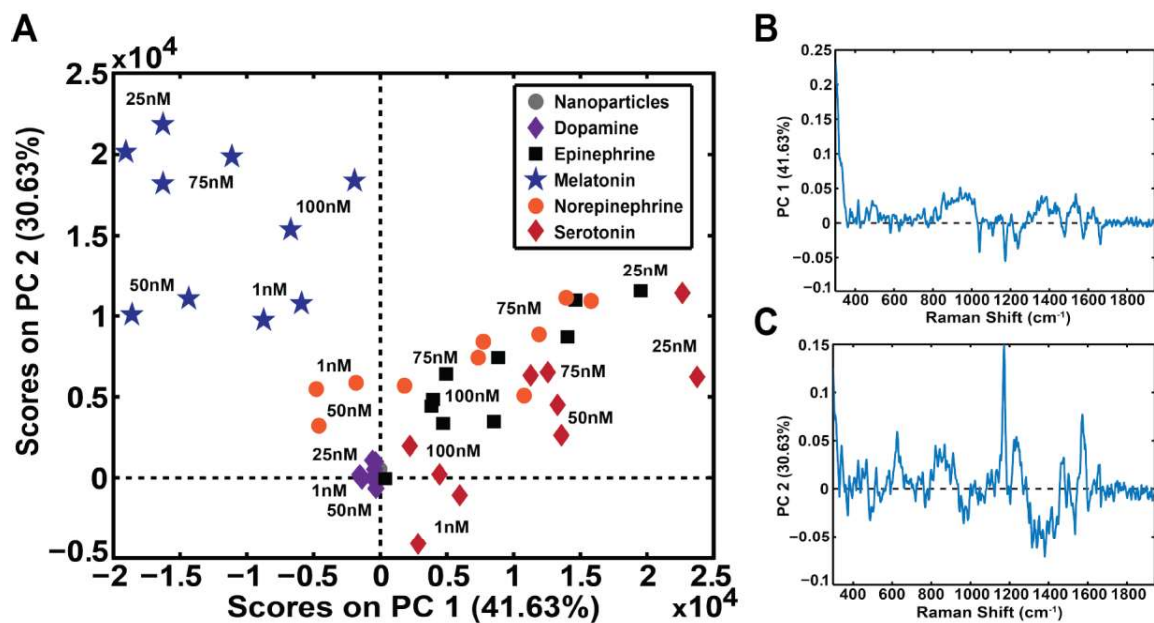


Figure 3.11: Scores on PC1 plotted against scores on PC2 (A) of concentrations from 100 nM to 1 nM and their accompanying loadings (B,C) for the SERS spectra of melatonin (blue), serotonin (red), dopamine (purple), norepinephrine (orange), and epinephrine (black) on AuNPs. $\lambda_{\text{ex}} = 785$ nm, $P = 5$ mW, $t = 60$ sec.

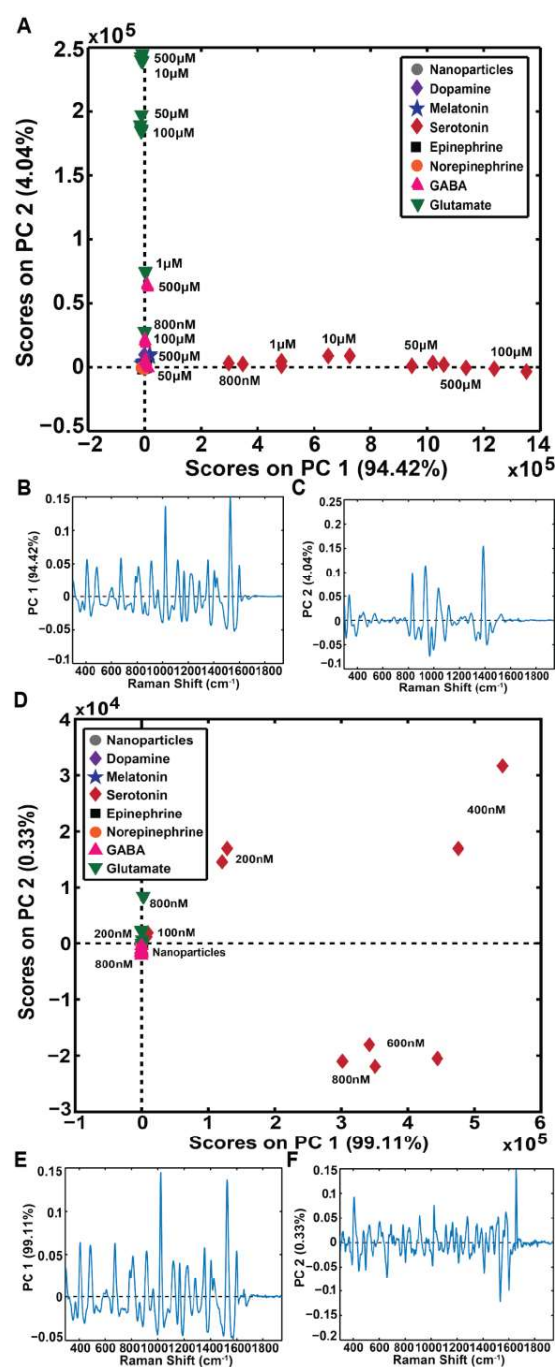


Figure 3.12: PCA plots for SERS spectra taken at 785 nm on AgNPs. Scores on PC1 plotted against scores on PC2 (B) of concentrations from 500 μM to 800 nM and their accompanying loadings (C, D) and Scores on PC1 plotted against scores on PC2 (E) of concentrations of 800 nM to 100 nM and their accompanying loadings (F, G). $\lambda_{\text{ex}} = 785 \text{ nm}$, $P = 5 \text{ mW}$, $t = 60 \text{ sec}$.

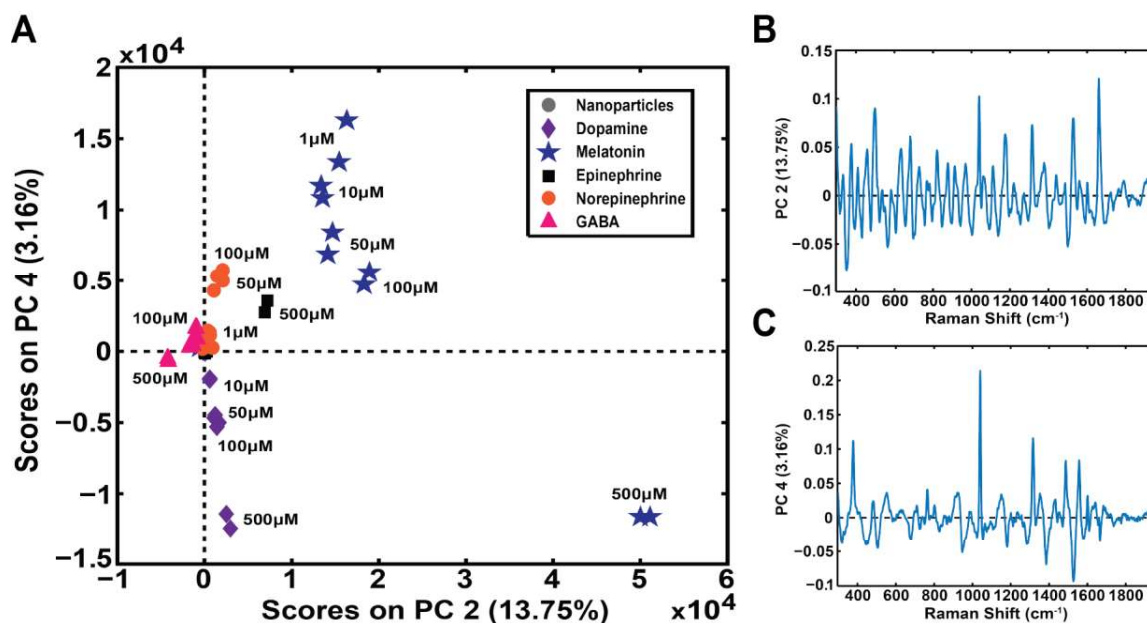


Figure 3.13: Scores on PC1 plotted against scores on PC2 (A) of concentrations from 500 μ M to 800 nM and their accompanying loadings (B,C) for the SERS spectra of melatonin (blue), dopamine (purple), GABA (pink), norepinephrine (orange), and epinephrine (black) on AgNPs.

$\lambda_{\text{ex}} = 785 \text{ nm}$, $P = 5 \text{ mW}$, $t = 60 \text{ sec}$.

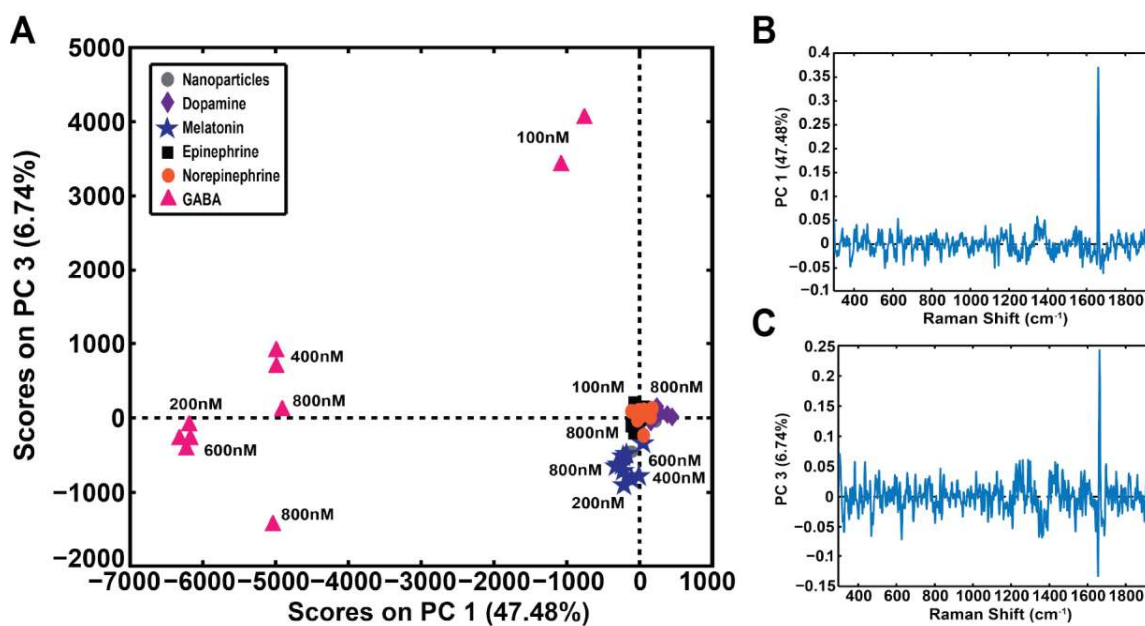


Figure 3.14: Scores on PC1 plotted against scores on PC2 (A) of concentrations from 800 nM to 100 nM and their accompanying loadings (B,C) for the SERS spectra of melatonin (blue), dopamine (purple), GABA (pink), norepinephrine (orange), and epinephrine (black) on AgNPs.

$\lambda_{\text{ex}} = 785 \text{ nm}$, $P = 5 \text{ mW}$, $t = 60 \text{ sec}$.

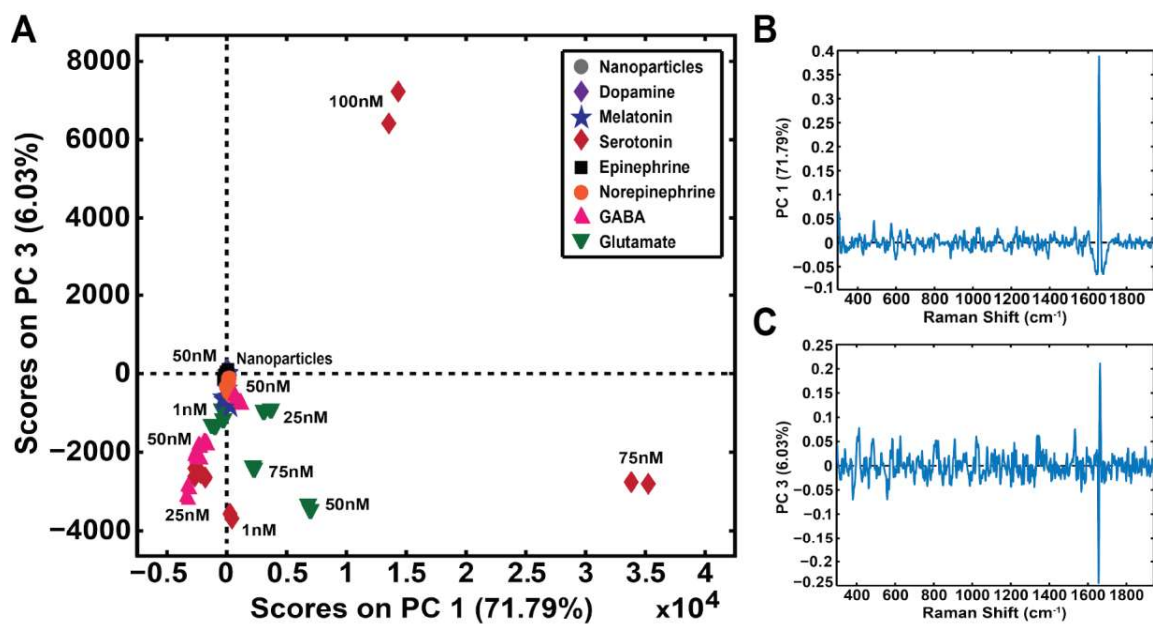


Figure 3.15: Scores on PC1 plotted against scores on PC2 (A) of concentrations from 100 nM to 1 nM and their accompanying loadings (B,C) for the SERS spectra of melatonin (blue), serotonin (red), glutamate (green), dopamine (purple), GABA (pink), norepinephrine (orange), and epinephrine (black) on AgNPs. $\lambda_{\text{ex}} = 785 \text{ nm}$, $P = 5 \text{ mW}$, $t = 60 \text{ sec}$.

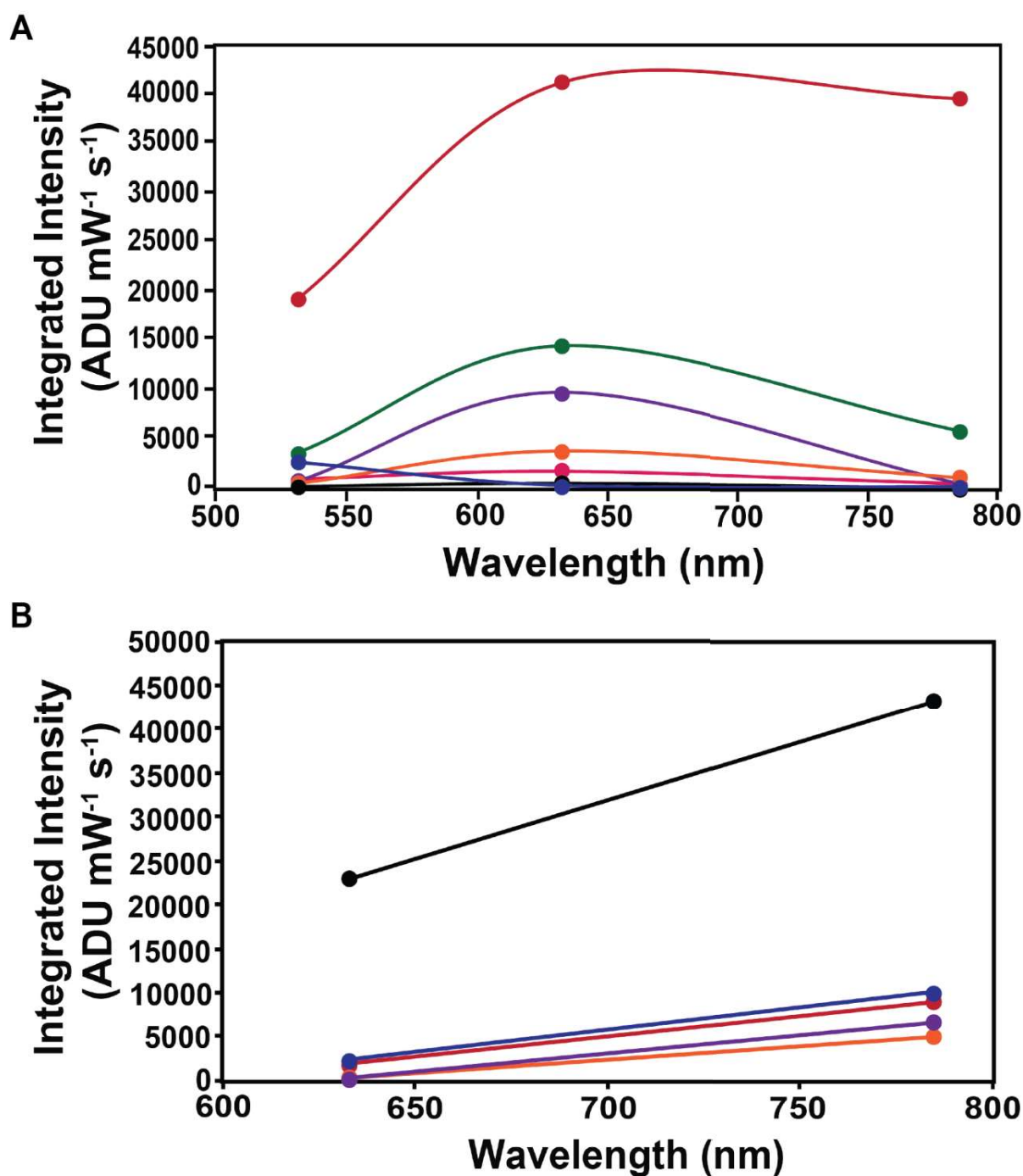


Figure 3.16: Integrated intensities, corrected for laser power and accumulation time, for melatonin (blue), serotonin (red), glutamate (green), dopamine (purple), GABA (pink), norepinephrine (orange), and epinephrine (black) on AgNPs (A) and AuNPs (B) at wavelengths of 532 nm, 633 nm, and 785 nm.

In order to test the viability of our SERS-based sensors in human biofluids, for future use in clinical lab applications for disease diagnosis, we collected SERS spectra of our neurotransmitters in artificial urine as well as artificial cerebral spinal fluid (aCSF) at a concentration of 50 μM on AuNPs at an excitation wavelength of 785 nm (Figure 3.17). The spectra for each neurotransmitter were baseline corrected and background subtracted to remove contributions from the bodily fluids and obtain the pure neurotransmitter spectra (home written software, Python). We find that in both urine and CSF, we obtain the pure spectra of the neurotransmitters with the same characteristic peaks listed for the neurotransmitters in aqueous solution, demonstrating their viability as biological sensors. The lower three spectra of Figure 3.17B are dopamine (purple), norepinephrine (orange), and epinephrine (black) which have very similar chemical structures: norepinephrine has one additional OH group than dopamine (represented at 334 cm^{-1}) and epinephrine adds a CH_3 group after the amine group of norepinephrine (represented at 1155 cm^{-1}). The power of SERS is demonstrated in the ability to distinguish between these neurotransmitters that differ by only one substituent group. As discussed above, AuNPs do not result in a SERS signal of amino acid chain neurotransmitters such as glutamate and GABA. These molecules were excluded from the representative spectra in Figure 3.17 due to this lack of SERS signal. While glutamate and GABA are among the most abundant neurotransmitters present in the brain, for an *in vitro* SERS sensor based on AuNPs, we would not have to be concerned about their interference in the detection of other neurotransmitters because their signal is not enhanced by the AuNPs.

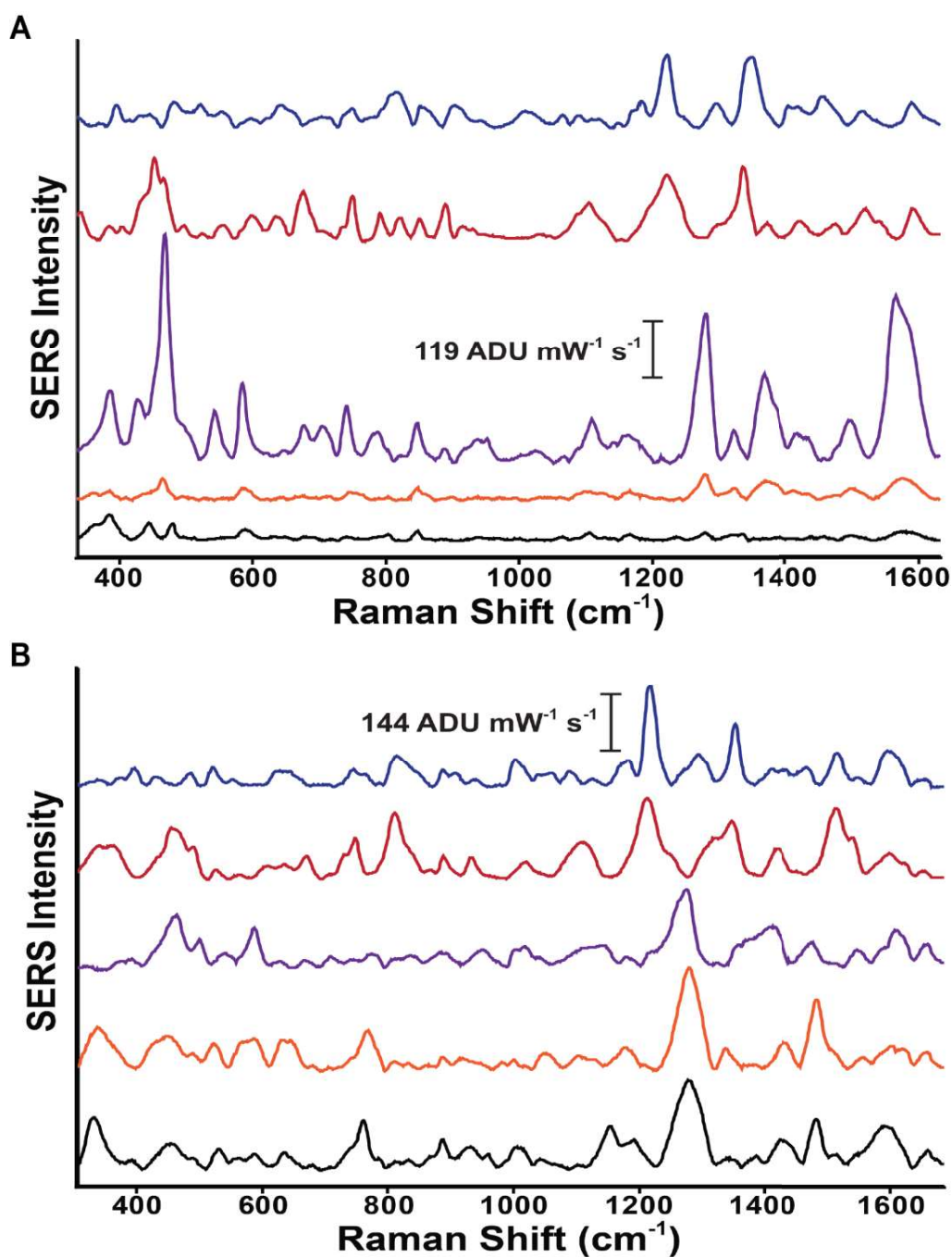


Figure 3.17: SERS spectra of melatonin (blue), serotonin (red), dopamine (purple), norepinephrine (orange), and epinephrine (black) on AuNPs at $50 \mu\text{M}$ in urine (A) and CSF (B).

$\lambda_{\text{ex}} = 785 \text{ nm}$, $P = 5 \text{ mW}$, $t = 60 \text{ sec}$.

3.5 Conclusions

We have presented a comprehensive study of the SERS detection of 7 neurotransmitters: melatonin, serotonin, glutamate, dopamine, GABA, norepinephrine, and epinephrine on bare AgNPs and AuNPs. Bare, colloidal nanoparticles, which are both cost-effective and easy to synthesize, were used to establish detection limits on non-functionalized nanoparticle surfaces. We investigated experimental parameters including which metal provides the highest intensity SERS signal, as well as the optimal wavelength for detection for each neurotransmitter. For serotonin, GABA, and glutamate, the lowest LODs are achieved using AgNPs as the SERS enhancing substrate at an excitation wavelength of 633 nm. For the indolic molecules including melatonin, dopamine, epinephrine, and norepinephrine the lowest LODs are achieved using AuNPs at an excitation wavelength of 785 nm, primarily due to the affinity of AuNPs to the indole ring. Choosing the appropriate metal and wavelength is useful in selectively enhancing the neurotransmitter of interest, while mitigating interferences from unwanted neurotransmitters. We have also demonstrated the ability to measure signal from neurotransmitters in biological media including urine and CSF. In the future, we aim to lower LODs through the use of capture molecules, such as antibodies or aptamers, to specifically bind the molecule of interest and increase both the selectivity and sensitivity of the SERS sensor.

3.6 Acknowledgements

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3.7 References

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Chapter 4

Surface Enhanced Spatially Offset Raman Spectroscopy detection of Neurochemicals Through the Skull

Chapter 4 is an adaption of a research letter in ACS Analytical Chemistry. Moody, A.S., Baghernejad, P. C., Webb, K. R., and Sharma, B., Surface Enhanced Spatially Offset Raman Spectroscopy Detection of Neurochemicals Through the Skull. *Anal. Chem.* **2017**, 89, 5688-5692. After characterization of the optimal detection parameters using SERS, we wanted to apply this method *in vivo*. This article describes the detection of neurochemicals through an animal skull in a brain mimic as a potential *in vivo* detection method for neurotransmitters that would be minimally invasive.

4.1 Abstract

The ability to noninvasively detect neurotransmitters through the skull would aid in understanding brain function and the development of neurological diseases. Surface enhanced spatially offset Raman spectroscopy (SESORS) is a powerful technique that combines the sensitivity of surface enhanced Raman spectroscopy (SERS) with the ability of spatially offset Raman spectroscopy (SORS) to probe subsurface layers. Here we present SERS measurements of neurotransmitters (melatonin, serotonin, and epinephrine) at various concentrations followed by the SESORS measurements of the neurotransmitters to a concentration as low as 100 μM in a brain tissue mimic through a cat skull. Principal components analysis was performed to distinguish between the surface bone layer and the subsurface layer, comprised of a brain tissue mimic modified with neurotransmitters, and to determine if each individual neurotransmitter could be accurately identified.

4.2 Introduction

Optical cerebral imaging is a rapidly expanding field in need of new techniques for gaining a greater understanding of brain function. Current noninvasive imaging techniques lack the ability to measure local concentrations of neurotransmitters, which are typically measured exogenously. Measurements of structural and functional information on neurochemicals have

been shown with techniques such as fast scan cyclic voltammetry (FSCV),¹⁻⁴ two-photon microscopy,⁵ and optogenetics.⁶ All three techniques are invasive, with FSCV and optogenetics involving drilling holes into the skull and two-photon microscopy requiring a craniotomy. Recent advances in combining bioresponsive imaging agents with functional MRI (fMRI) technology are now allowing for quantitative measurements to be made concurrent with imaging.⁷ Knowledge of properties and local concentrations of neurochemicals is critical to understanding neurological functions, fueling the need for a noninvasive procedure for measuring neurochemicals in the brain.

We present a method utilizing Raman spectroscopy to noninvasively detect low concentrations of neurotransmitters through the skull. Conventional Raman spectroscopy provides superb chemical specificity with simple instrumentation but has a strong bias toward surface layers and gives inherently weak signals. Our approach combines two types of Raman spectroscopy, surface enhanced Raman spectroscopy (SERS) and spatially offset Raman spectroscopy (SORS), to provide enhanced Raman signals from molecules in deep layers of turbid media.

SERS provides a greatly enhanced Raman signal from low concentration analytes that have been adsorbed to noble metal nanoparticles. The enhanced signal is a result of an intensified electric field due to the excitation of the localized surface plasmon resonances (LSPR) of the metal nanoparticles. Camden et al. have calculated Raman signal enhancements by factors up to 10^{10} – 10^{11} .⁸ SERS is surface selective and highly sensitive and has been applied in biological applications such as detection of glucose, various cancers, Alzheimer's disease, and Parkinson's disease.⁹

By collecting Raman spectra from a location at a set distance away from the incident illumination point, the Raman signal can be obtained from subsurface layers. This technique,

known as spatially offset Raman spectroscopy (SORS), is based on the concept that photons which interact with subsurface layers have to travel larger distances where they diffuse laterally through the material before scattering from the surface.^{10,11} SORS uses a continuous wave laser beam which allows illumination with higher powers, while remaining within maximum exposure limits. This method was initially described by Matousek et al. using a diffusely scattering, two-layer sample of trans-stilbene powder beneath a 1 mm thick layer of PMMA.¹² It has since been demonstrated in applications such as the noninvasive Raman spectroscopy of bones of animal and human samples by Morris et al.,¹³ in vivo observation of human tissue by Matousek et al.,¹⁴ and detecting contributions of breast tumors buried in breast tissue by Keller et al.¹⁵

The combined surface enhanced SORS, termed as SESORS, has been demonstrated for in vivo glucose sensing^{16,17} and for the measurements of nanoparticles embedded in tissue through ovine bone.¹⁸ Here we present SESORS measurements of neurotransmitters embedded in a brain tissue mimic through a cat skull.

4.3 Results and Discussion

Early experiments involved collection of SERS spectra of neurotransmitters, specifically melatonin (Figure 4.1A), epinephrine (Figure 4.1B), and serotonin (Figure 4.1C) on Au nanoparticles with enhancement factors of $\sim 10^5$ in aqueous solution. The spectra were obtained at various concentrations where representative peaks for each neurotransmitter can be identified down to nanomolar concentrations. Spectra were collected using a 785 nm spectrum stabilized laser (Innovative Photonic Solutions) with an average laser beam power at the sample of 50 mW. The light was directed onto the sample using a dichroic mirror, which was also utilized to achieve a spatial offset. The Raman light was then collected in an 180° backscattering geometry and directed into the spectrometer (Acton LS 785, Princeton Instruments) where it was dispersed onto a CCD detector (Pixis 400, Princeton Instruments).

We measured the optical properties of four animal skulls, including diffuse reflectance and transmittance measurements (Figure 4.2). In accordance with previous measurements of the skulls of both adult pigs¹⁹ and human cadavers,²⁰ our results show the skull is partially transparent at a wavelength of 785 nm. This is significant because it indicates that we should be able to both send 785 nm light through the skull as well as detect the light that is scattered back. To mimic brain tissue, we used a 0.6% agarose gel. Studies on the properties of agarose gel show that the poroelasticity and structure of the brain is analogous to that of a low concentration agarose gel. The agarose gel has a web-like structure formed by cross-linking as fibers overlap.^{21,22}

To test the capability of the 0.6% agarose gel to absorb the Au nanoparticles and still allow them to exhibit SER behavior, an agarose gel was incubated with the Au nanoparticles and benzenethiol (a SERS active molecule often used to validate SERS substrates) (Figure 4.3). Au nanoparticles synthesized according to a modified Turkevich method²³ were injected (volume = 200 μ L) into the agarose gel. After the nanoparticles incubated in the gel for 1 h, 100 μ M benzenethiol was injected (volume = 200 μ L) into the agarose gel and allowed to incubate for an additional hour.

First, we acquired a SERS spectrum of the benzenethiol adsorbed to the Au nanoparticles in the gel. A fragment of a cat skull was then placed in front of the gel to acquire a spectrum with no spatial offset followed by a spectrum at a spatial offset of 2 mm, which we previously determined to be the optimal offset position for the thickness of the cat skull fragment (Figure 4.3). All raw spectra were processed using GRAMS software (Thermo Fisher Scientific). Each spectrum was baseline corrected, since there is a visible fluorescence contribution in the uncorrected spectra involving the skull. Finally, the zero mm offset spectrum was subtracted from the 2 mm offset spectrum. The zero mm offset spectra (Figures 4.3 and 4.4) are dominated

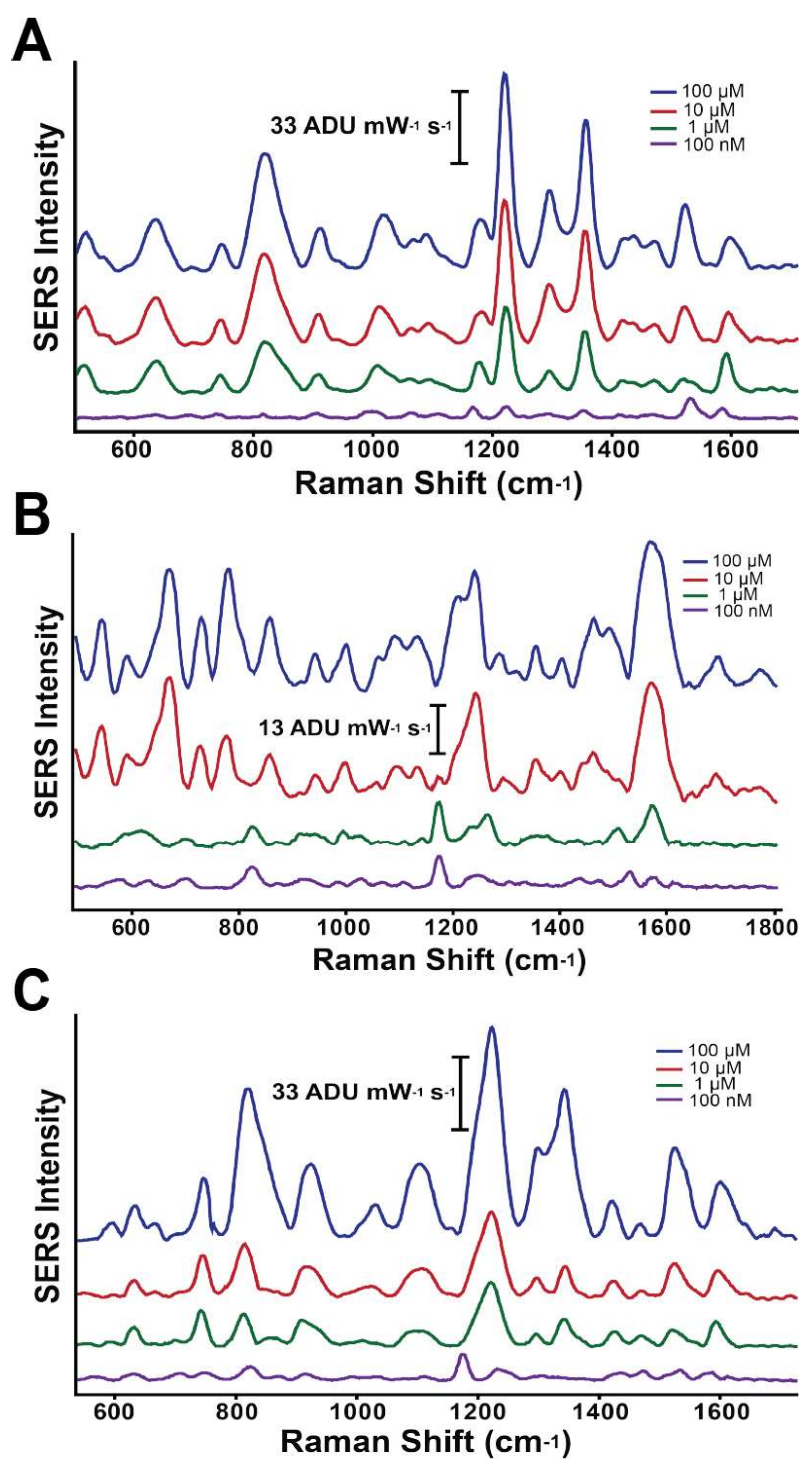


Figure 4.1: SERS spectra of (A) melatonin, (B) epinephrine, and (C) serotonin at concentrations of 100 μM (blue), 10 μM (red), 1 μM (green), and 100 nM (purple). $\lambda_{\text{ex}} = 785 \text{ nm}$, $P = 50 \text{ mW}$, $t = 30 \text{ sec}$.

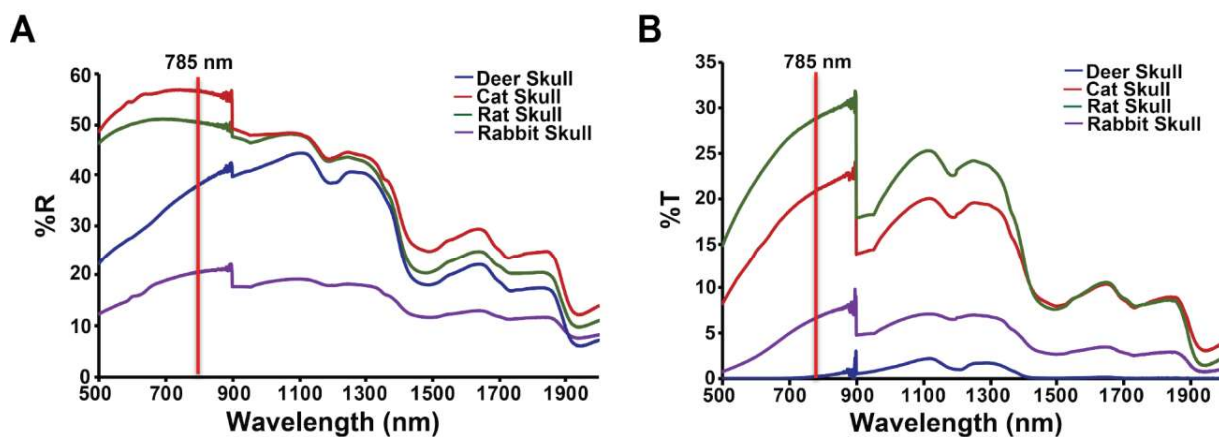


Figure 4.2: Diffuse reflectance (A) and transmittance (B) spectra of four animal skulls. Spectra were acquired with a Cary 5000 UV-Vis-NIR spectrophotometer (Agilent Technologies). The results demonstrate that 785 nm excitation (indicated by the vertical red line) is able to penetrate the skull. Based on these results, we determined the cat or rat skull would be the best candidates for the SESORS experiments. We decided to use the cat skull, primarily due to a less uneven structure of the surface.

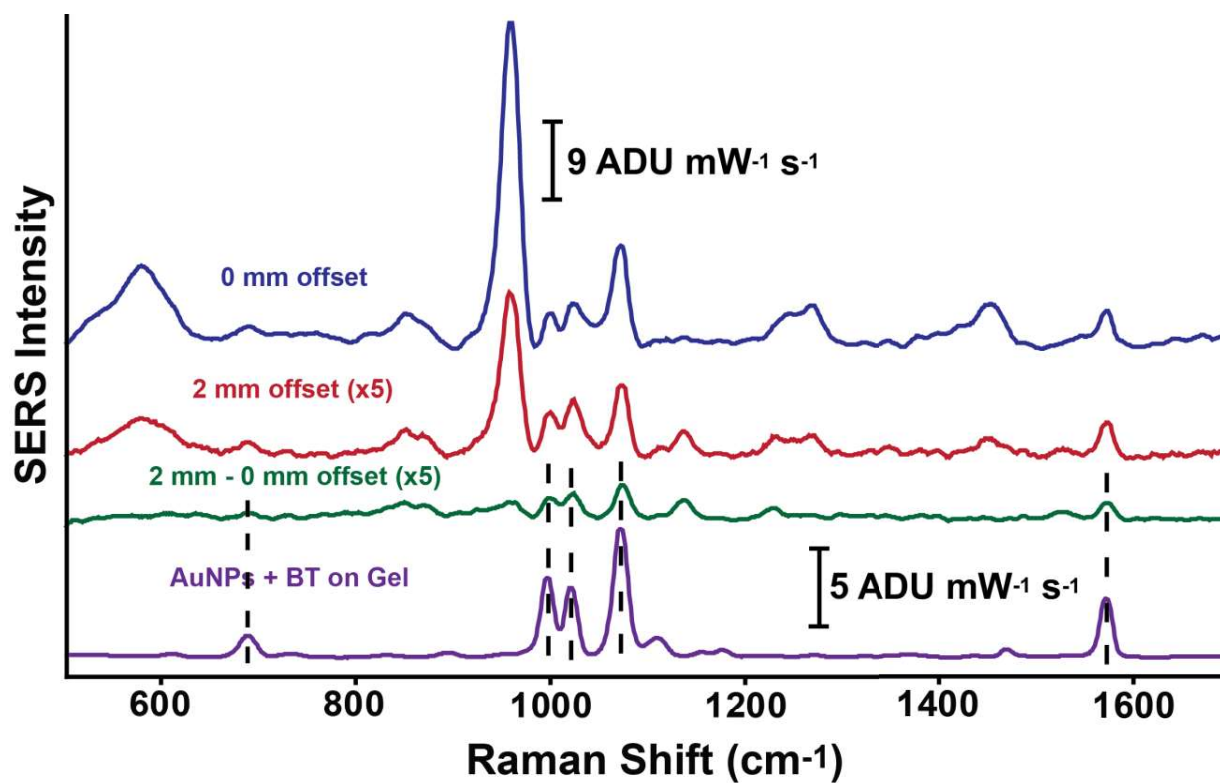


Figure 4.3: SESORS spectra of benzenethiol through the skull: 0 mm (purple), 2 mm offset (red), difference spectra of 2 mm – 0 mm (green) and AuNPs in agarose gel without the skull (purple). The black dashed lines indicate peaks in common between the subtracted spectrum (green) and the agarose-benzenethiol spectrum (purple). $\lambda_{\text{ex}} = 785 \text{ nm}$, $P = 50 \text{ mW}$, $t = 120 \text{ sec}$.

by the characteristic bone peaks, including the broad phosphate ν_4 mode at 582 cm^{-1} , $\nu(\text{C-C})$ mode at 872 cm^{-1} , strong bone phosphate ν_1 mode at 960 cm^{-1} , $\nu(\text{C-C})$ mode at 1000 cm^{-1} , phosphate ν_3 mode at 1035 cm^{-1} , the carbonate ν_1 mode at 1070 cm^{-1} , $\nu(\text{C-O-C})$ mode at 1160 cm^{-1} , and the $\delta(\text{CH}_2)$ mode at 1450 cm^{-1} .¹³ There is also a contribution in the surface layer (0 mm offset) from the benzenethiol (subsurface layer) due to the large Raman cross-section and enhanced Raman signal obtained from benzenethiol. The subtraction of the zero mm offset from the 2 mm offset reveals the contribution from the subsurface layer where the 995 cm^{-1} (γ_{CH}), 1023 cm^{-1} (β_{CH}), 1074 cm^{-1} (β_{CH}), and 1573 cm^{-1} (ν_{CC}) peaks of benzenethiol²⁴ are visible and match the SERS spectra of the benzenethiol functionalized Au nanoparticles in the gel alone.

SESORS spectra of melatonin, serotonin, and epinephrine were then acquired at concentrations of 50 mM and 100 μM (Figure 4.4). Using the same procedure as for the benzenethiol, the Au nanoparticles were injected into the gel (volume = 200 μL) and allowed to incubate for 1 hr. Following incubation of the nanoparticles, 200 μL of the neurotransmitter was injected into the agarose gel and incubated for an additional hour. The SERS spectra of the neurotransmitter with Au nanoparticles in the gel were taken, along with SESORS spectra through the skull with a zero mm offset and a 2 mm offset.

Common vibrations for indolic molecules include bending modes of the bond and the indole ring, stretching, breathing, wagging, and rocking. In Figure 4.4A and 4.4D, the subtracted SESORS spectra reveal characteristic peaks of melatonin. In particular, the modes at 926 cm^{-1} (b_{CCC}), 1226 cm^{-1} (tw_{CH_2}), 1351 cm^{-1} (b_{NH}), and 1537 cm^{-1} (b_{NH}) are visible.²⁵ In Figure 3B and 3E the 1029 cm^{-1} (b_{CH}), 1078 cm^{-1} (b_{CCH}), 1221 cm^{-1} (tw_{CH_2}), 1295 cm^{-1} (b_{NCH}), 1382 cm^{-1} (b_{CH_3}), 1487 cm^{-1} (st_{CC}), and 1537 cm^{-1} (b_{NH}) are representative of the SERS of epinephrine.²⁶ In Figure 3.4C and 3.4F characteristic peaks of serotonin are visible in the subtracted SESORS

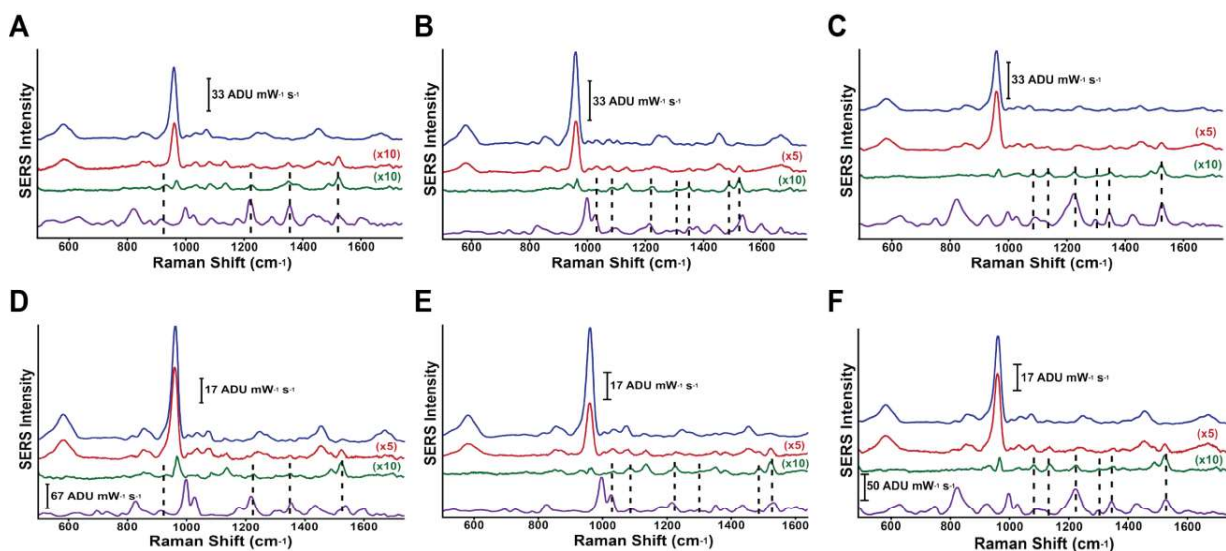


Figure 4.4: SESORS spectra of melatonin (A, D), epinephrine (B, E), and serotonin (C, F) at concentrations of 50 mM (top row) and 100 μ M (bottom row). The top spectrum for each panel (blue) is a spectrum of the bone surface with no spatial offset (zero mm). The red spectrum is the SESORS spectrum of the neurotransmitter embedded in agarose gel behind the bone at a 2 mm offset. The green spectrum is a subtraction of the surface bone spectrum from the SESORS spectrum at a 2 mm offset to reveal the subsurface contribution. The purple spectrum is the SERS spectrum of the neurotransmitter and Au nanoparticles embedded in the agarose gel. The black dashed lines indicate peaks in common between the subtracted spectra and the agarose-neurotransmitter spectra. $\lambda_{\text{ex}} = 785$ nm, $P = 50$ mW, $t = 120$ sec.

spectra, including 1085 cm^{-1} (b_{CCH}), 1224 cm^{-1} (tw_{CH_2}), 1130 cm^{-1} (br_{ring}), 1341 cm^{-1} (b_{CC}), 1374 cm^{-1} (w_{HCH}), 1522 cm^{-1} (b_{NH}).²⁷

The scattering efficiencies through the cat skull at the different concentrations were calculated by dividing the integrated intensities of the peaks through the bone by the integrated intensities of the peaks in the SERS of the individual neurotransmitters in the gel. All peak areas were normalized by the accumulation time and laser power. The scattering efficiency through bone at a concentration of 50 mM ranged from 3.4% to 5.7% and at 100 μM was 1.3% on average.

To confirm that our SESORS method can distinguish between the surface and subsurface layers and clearly identify individual neurotransmitters we applied multivariate analysis to the spectra. Specifically, principal components analysis (PCA) was performed. PCA identifies relationships between samples and reduces dimensionality of the data set through an orthogonal transformation to decompose the spectra into a series of principal components. Each principal component encompasses a spectral pattern, or loading, and a score describing the correlation between the sample and principal component. A plot of the scores of different principal components shows clustering of samples into similar or dissimilar classes and explains major trends in a data set.²⁸ Multivariate analysis techniques are often performed to determine spectral similarities and differences in Raman methods analyzing biological samples.²⁸⁻³¹

Before PCA was applied to the spectra, the data were preprocessed using GRAMS and Solo software (eigenvector Research) to baseline, subtract, and normalize the Raman spectra. The PCA was performed in Solo. When PCA was applied to the data with no spatial offset (surface layer) plotting PC1 versus PC2 reveals little to no separation between the spectra (Figure 4.5). The loadings for PC1 and PC2 (Figure 4.5B,C) indicate a large contribution from the bone peak at 960 cm^{-1} to PC1. Thus, plotting PC1 versus PC2 allows us to demonstrate the

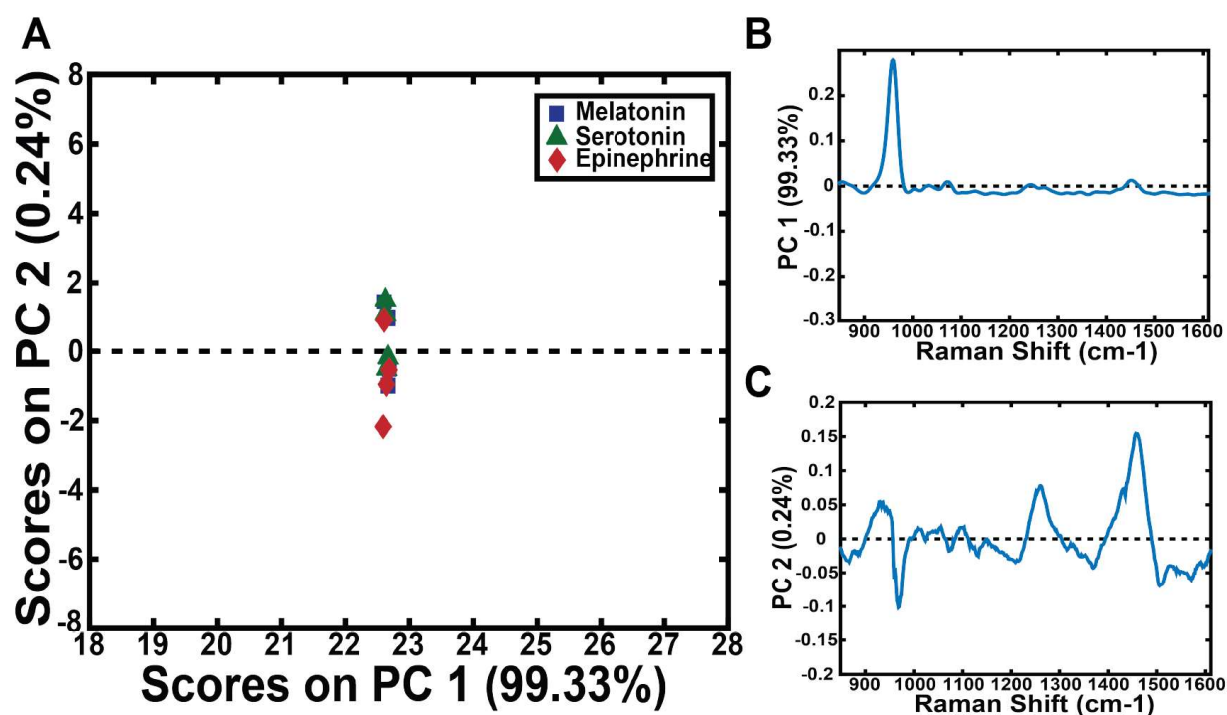
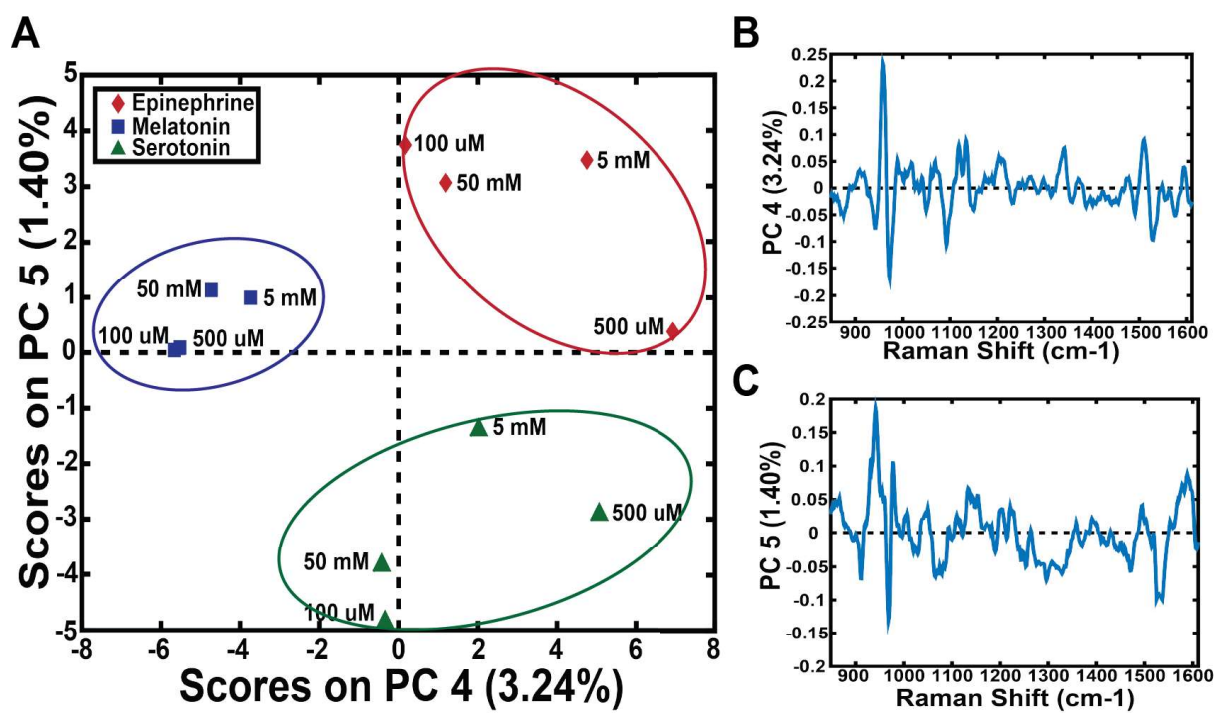


Figure 4.5: Scores of PC1 plotted against scores of PC2 with a PCA applied to the data with no spatial offset and a 2 mm spatial offset (A) along with the loadings from PC1 (B) and PC2 (C).

inability to detect the neurotransmitters without the use of SORS. We then applied the PCA model to the difference spectra (2 mm offset minus 0 mm offset). Plotting PC4 versus PC5 gives a separation of the neurotransmitters at different concentrations (Figure 4.6) into distinct areas of the PCA plot. The spread between different concentrations of each neurotransmitter varies and could be improved by increasing the number of data points in the PCA plot. Additionally, the loading for PC4 shows a residual peak from broadening of the bone phosphate vibration due to underlying neurotransmitter peaks.

4.4 Conclusion

In summary, we used Au nanoparticles coated with various neurotransmitters to demonstrate the first SESORS measurements of neurotransmitters through a skull. The Au nanoparticles have an enhancement factor of 10^5 . Characteristic peaks of each neurotransmitter were recognized in all spectra through the skull at concentrations of 50 mM and 100 μ M. Applying PCA to the data demonstrated our ability to identify melatonin, epinephrine, and serotonin through a cat skull. The cat skull is approximately 2 mm thick, which is slightly thinner than the thickness of the human skull, which averages from 3 to 14 mm depending on location on the skull and if the skull is male or female. SESORS demonstrates great potential for non-invasive *in vivo* neurochemical detection using rodent models for preclinical studies or through 3 mm thick regions of the human skull. In the future, studies will be done to extend the capability of this method by improving the enhancement of the SERS nanoparticles (to greater than 10^5) to increase signal to noise ratios. To quantitatively measure different concentrations of neurotransmitters through various skull thicknesses, we will modify the experimental set-up to increase collection efficiency of the SESORS signal, which has previously been demonstrated to depths of 20 mm in porcine tissue (without bone)³².



4.5 Acknowledgements

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Chapter 5

***In vivo* Detection of Neurochemicals in a Mouse Brain Through the Skull with Surface-Enhanced Spatially Offset Raman Spectroscopy**

Chapter 5 is an adaption of a research article in preparation for submission to ACS Nano by Moody, A.S., Payne, T. D., Barth, B. A., and Sharma, B. entitled “*In vivo* detection of neurochemicals in a mouse brain through the skull with surface-enhanced spatially offset Raman spectroscopy”. This article describes the applicability of SESORS as a potential *in vivo* detection method with the detection of serotonin in a mouse model. The detection of mixtures of neurotransmitters was also achieved and LODs were obtained for five different neurotransmitters.

5.1 Abstract

Detection techniques for neurotransmitters that are rapid, label-free, and non-invasive are needed to move towards earlier diagnosis of neurological disease. Surface-enhanced Raman spectroscopy (SERS) allows for sensitive and selective detection of target analytes. The combination of SERS with spatially offset Raman spectroscopy (SORS) in a technique termed surface enhanced spatially offset Raman spectroscopy (SESORS) permits a sensitive and selective detection of neurotransmitters through the skull. Here, we demonstrate the potential of SESORS for an *in vivo* detection method by demonstrating the detection of 100 μ M serotonin in the brain of a mouse model. We also present the SESORS detection of individual neurotransmitters and mixtures of neurotransmitters, while establishing limits of detection.

5.2 Introduction

Neurological disease is very difficult to detect in the early stages where treatment would be most beneficial. For diseases such as Parkinson’s disease, which is based on a degradation of dopaminergic neurons, diagnosis typically does not occur until about 70% of the neurons have already been lost, making treatment ineffective.¹⁻² The detection of varying concentrations of neurotransmitters can potentially be used to monitor and diagnose neurological disease earlier. Techniques such as electrochemistry, which are currently used for neurotransmitter detection, are

invasive and involve drilling holes in the skull or cutting out portions of the skull.³⁻⁴ Other commonly used techniques include high performance liquid chromatography (HPLC)⁵⁻⁶ and ultra-performance liquid chromatography combined with mass spectrometry (UPLC-MS)⁷ but these are destructive to the sample and include expensive instrumentation and long run times. Fluorescence detection has also successfully been used for neurotransmitter detection, however, this method requires labeling the sample with fluorescent tags.⁸⁻¹⁰ Surface-enhanced spatially-offset Raman spectroscopy (SESORS) offers a non-invasive, or minimally invasive, technique for detection of neurotransmitters through the skull, which is non-destructive and signal can be obtained in 2 minutes or less.

SESORS arises from a combination of surface-enhanced Raman spectroscopy (SERS) and spatially-offset Raman spectroscopy (SORS). Raman spectroscopy gives excellent chemical specificity by providing a molecular fingerprint for each molecule, however, it gives an inherently weak signal.¹¹ SERS is used to mitigate this weak signal with the use of noble metal nanoparticles. A localized surface plasmon resonance (LSPR) is produced when the metal nanoparticles are excited with a laser, causing a collective oscillation of surface conduction electrons, resulting in a surrounding electric field. This surrounding electric field contributes to the overall Raman signal, giving an enhanced signal by up to 8 orders of magnitude.¹²⁻¹³ SERS is a growing field for biomarker detection.¹⁴⁻¹⁷ A biomarker is defined as a measurable substance which is characteristic of a disease or infection.

SORS allows signal to be obtained from subsurface layers of a material with multiple layers through taking advantage of the spatial component of light by collecting the Raman signal at a point that is spatially offset from the incident illumination point. The theory is that the photons that travel deeper into the material have to traverse laterally before emerging from the

surface, so by collecting the Raman signal at a point that is spatially offset, one can collect the signal from the subsurface layers.¹⁸⁻¹⁹

SESORS allows for the use of nanoparticles to enhance the signal and achieve detection of low concentration analytes in these subsurface layers. SESORS has previously been used for detection of various diseases including breast cancer²⁰ and bone disease including osteogenesis imperfecta²¹. We have demonstrated proof-of-concept detection of neurotransmitters for the diagnosis of neurological disease with SESORS²². Many studies have used SERS for the detection of neurotransmitters in biological fluids, however, *in vivo* detection of neurotransmitters can greatly benefit from the use of SESORS.

Here, we present the applicability of SESORS as an *in vivo* technique through the detection of serotonin in a mouse model. We also demonstrate SESORS detection of melatonin, norepinephrine, serotonin, epinephrine, and dopamine in a brain mimic, through a rat skull. A limit of detection (LOD) was found for each neurotransmitter through as well as the detection of mixtures of these neurotransmitters.

5.3 Experimental Section

5.3.1 Materials

Citric Acid (trisodium salt dehydrate, 99%), dopamine (3-hydroxytyramine hydrochloride, 99%), sodium borohydride (NaBH_4), agarose, and hydrogen tetrachloraurate (III) trihydrate (HAuCl_4) were purchased from ACROS Organics. Serotonin (5-hydroxytryptamine hydrochloride), (-)-epinephrine, (-)-norepinephrine, gamma-amino-n-butyric acid, and L-glutamic acid were purchased from Sigma-Aldrich. Melatonin (99+%) was purchased from Alfa Aesar. Hydrochloric Acid and sodium chloride (NaCl) were purchased from Fisher Scientific.

5.3.2 Nanoparticle Synthesis

Gold nanoparticles (AuNPs) were synthesized according to a modified version of methods established by Frens. A 0.01% solution of HAuCl_4 was brought to a boil, then a 1% citrate solution was added and allowed to boil for 5 minutes, resulting in particles approximately 60 nm in diameter. The AuNPs were structurally characterized using scanning electron microscopy (SEM) and the LSPR was measured with an Agilent Cary 5000 UV/Vis/NIR spectrophotometer.

5.3.3 Preparation of Agarose Gels for SESORS Measurements

Agarose gel was used as a brain mimic in the SESORS experiments. A 0.6% agarose gel was prepared by combining agarose, NaCl, and DI H_2O , bringing the solution to a boil, then removing the solution from the hot plate and allowing it to set overnight. For the depth study, the agarose, NaCl, and DI H_2O were brought to a boil and quickly pipetted into a square mold to form gels with a thickness of 2 mm.

To prepare the gels for Raman spectra and dark field imaging, 1 mL of each neurotransmitter concentration was added to the gels and allowed to incubate for 1.5 hours. The excess liquid is then removed and 1 mL of AuNPs at pH 2 are added and allowed to incubate for an additional 1.5 hours. The SERS spectra are initially acquired with the gel followed by placing the gel behind a section of a rat skull where the SESORS spectra are obtained.

5.3.4 Raman Spectroscopy, SERS, & SESORS

The Raman, SERS, and SESORS scattered light were collimated and focused in a 180° backscattering geometry into an Acton-LS785 spectrometer (Princeton Instruments), where the light was dispersed onto a PIXIS 400 camera (Princeton Instruments). A 785 nm diode laser (Innovative Photonic Solutions) was used to illuminate the sample and was directed onto the sample using a dichroic mirror (Semrock). SERS spectra of the neurotransmitter, along with

AuNPs in the gel were taken of each sample initially. A section of a rat skull (donated by the University of Tennessee College of Veterinary Medicine) was then placed in front of the gel containing the neurotransmitters and AuNPs where a 0 mm offset was taken (no spatial offset). The SORS offset (2 mm offset) was then achieved using an axicon lens (Thorlabs) to transform the laser spot into a ring, permitting the use of overall higher laser powers. The size of the spatial offset could be changed by changing the distance of the axicon lens to the sample.

To find our SORS depth of penetration using a 2 mm offset, agarose gels were synthesized with a thickness of 2 mm. The bone was placed in front of the agarose gel, and a piece of Teflon was placed behind consecutive slices of agarose gel starting at 2 mm and ending at 22 mm until the Teflon signal could no longer be obtained.

5.3.5 Dark Field Imaging

Agarose gels were prepared similarly to those used for the SESORS spectra where the neurotransmitter is added and allowed to incubate followed by addition of AuNPs. The images were obtained using a dark field condenser for a Nikon-Ti-U inverted microscope. The gels were sliced to determine the uniformity of the AuNPs dispersed throughout the gel.

5.3.6 Mouse Model

Our collaborator donated an adult male BALB/c mouse post-mortem for the *in vivo* SESORS neurotransmitter detection experiment. The mouse was euthanized with an isoflurane injection followed by a cervical dislocation. The euthanization followed the National Institution of Health Guidelines for the Care and Use of Laboratory Animals, and the procedure was approved by the University of Tennessee Knoxville Institutional Animal Care and Use Committee. The head of the mouse was shaved to remove the hair. We initially acquired control spectra of the sacrificed mice, with no neurotransmitter or nanoparticles. A volume of 200 μL of 100 μM serotonin was then injected into the skull of the mouse and allowed to incubate for 1.5

hrs. After incubation with the neurotransmitter, 200 μ L of the AuNPs were injected into the brain and allowed to incubate for an additional 1.5 hrs. SESORS spectra of serotonin in the mouse model were then collected at a 0 mm offset, 1 mm offset, and 2 mm offset.

5.4 Results and Discussion

Dark-field microscopy imaging is commonly used to image metal nanoparticles and measure their extinction spectra.²³⁻²⁵ We used dark-field imaging to determine the dispersion of AuNPs infused in the agarose gels. The dark-field condenser produces a hollow cone of light which is focused at the sample. The objective must have a smaller numerical aperture than the condenser so only the light that is scattered out of this cone reaches the objective. The particles on the substrate then appear as bright spots on a dark background.²⁶ The neurotransmitter and nanoparticles were allowed to incubate in the agarose gel. The gel was then sliced into 3 pieces to determine the dispersion of nanoparticles throughout the agarose gel. The dark field images show uniform dispersion of AuNPs in the top and bottom layers of the gel (Figure 5.1A & 5.1C), with less AuNPs in the middle layer, however, there is still a significant number of particles dispersed evenly through the middle layer (Figure 5.1B). These dark-field images indicate that the method of nanoparticle incubation of the gel allows for uptake of the AuNPs into the gel and results in even distribution of the nanoparticles.

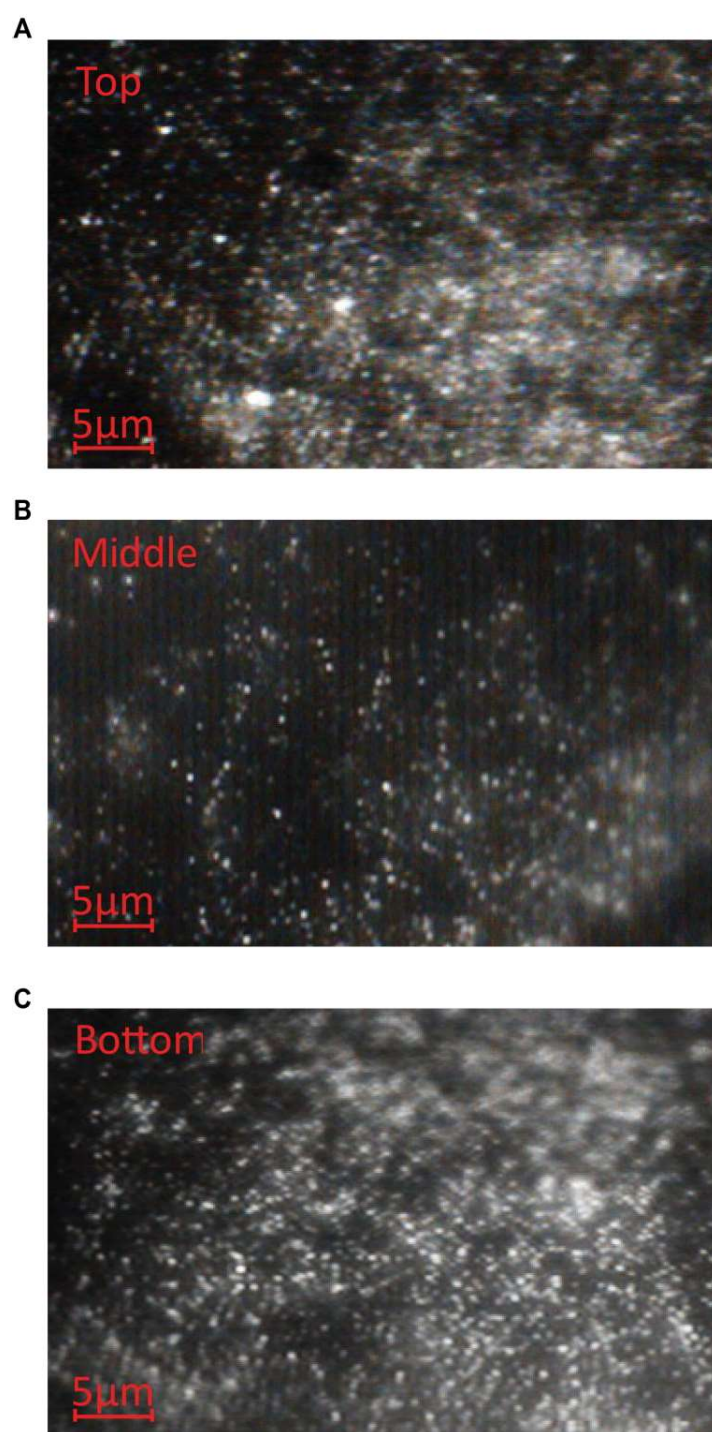


Figure 5.1. Dark field scattering images of the top, middle, and bottom of an agarose gel infused with AuNPs. The white spots are AuNPs, while the dark areas are the body of the gel.

We were also interested in determining the limits of our depth penetration beneath the skull. We used multiple layers of agarose gel behind the skull piece with Teflon inserted between the gel layers to create depths from 0 mm (no gel) to 22 mm depth at 2 mm increments (Figure 5.2A). SESORS spectra of Teflon behind the rat skull were acquired at a 0 mm offset followed by subsequent SESORS spectra obtained at a 2 mm offset behind the various thicknesses of agarose gel. A scaled subtraction was performed in GRAMS (Thermo-Fisher) of the 0 mm offset spectrum from the 2 mm offset SESORS spectra at the various thicknesses of agarose gel to remove the contribution of the bone spectrum (0 mm offset) from the lower layer spectra. The spectra were scaled based on the 960 cm^{-1} peak, which arises from the bone phosphate ν_1 mode.²⁷ Each spectrum was then baseline corrected using GRAMS software. The heat map for the depth study was plotted in MatLab using a home written code. In the MatLab code a Savitzky-Golay filter with order of 2 and frame length of 7 was applied to the spectra. The spectra were zeroed, so the minimum value in each spectrum was 0, to account for any baseline differences. Using the 2 mm depth spectrum, the location and widths of the four prominent Teflon peaks (734 cm^{-1} , 1218 cm^{-1} , 1302 cm^{-1} , 1382 cm^{-1}) were established to create a barcode for the Teflon spectrum. Barcoding has previously been demonstrated as a method for isolating relevant peaks in biological Raman spectra.²⁸⁻²⁹ Subsequent spectra for the increasing depths were barcoded with the peak boundaries determined by using the start and end points, which were symmetric about the max peak height location. We find that at the 2 mm depth, the Teflon spectrum is most intense where it decreases as we increase the layers of agarose to create greater depths. We see that the 734 cm^{-1} peak of Teflon is still visible at depths up to 18 mm, which is our limit for detection depth for a strongly scattering molecule through a rat skull using a spatial offset of 2 mm. (Figure 5.2B)

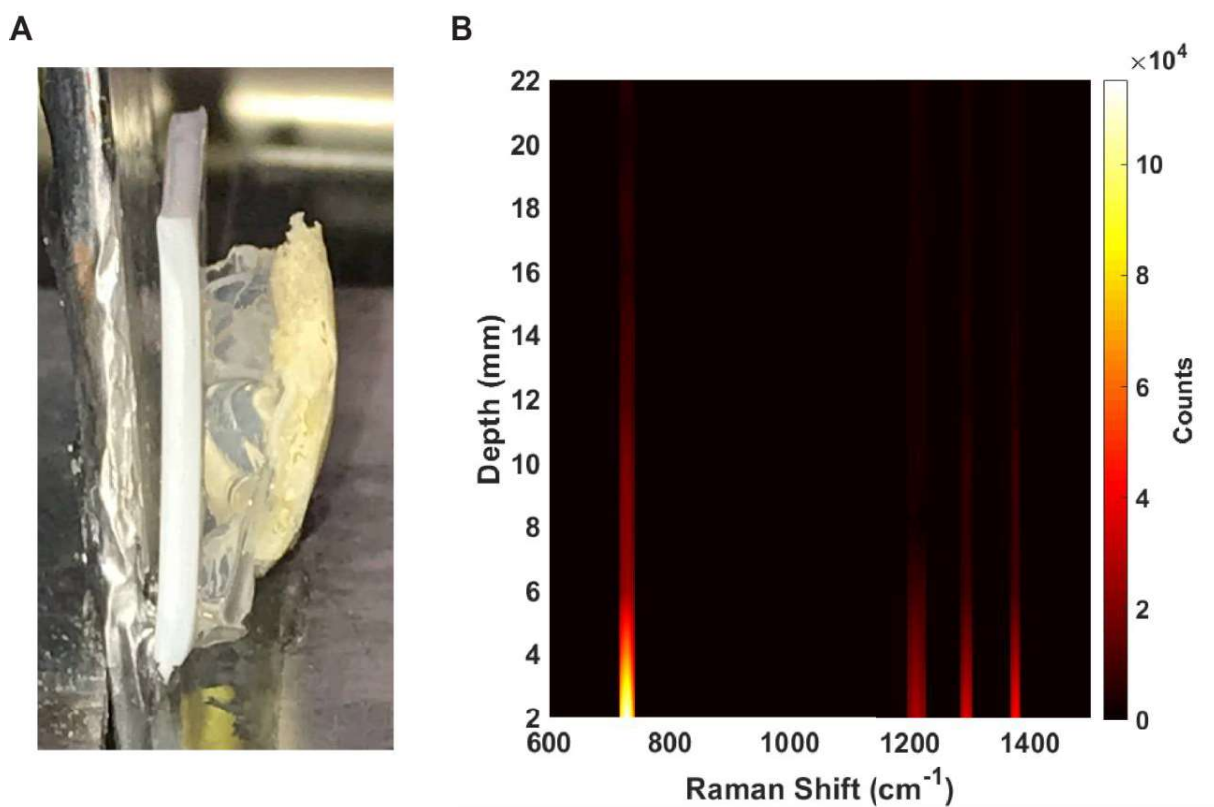
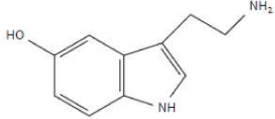
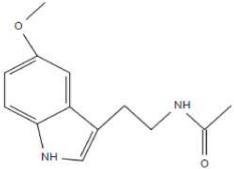


Figure 5.2. Schematic of the geometry of Teflon, agarose gel, and bone used for the depth study (A) and the heat map of the SORS detection depth of Teflon using a spatial offset of 2 mm (B). The false color is characteristic of signal intensity at various depths of agarose gel from highest (white) to lowest (red) intensity. $\lambda_{\text{ex}}=785$ nm, $P=90$ mW, $t=120$ sec.

We have previously determined the SERS limits of detection (LOD) of neurotransmitters in aqueous solutions and artificial body fluids.³⁰ Here we obtain the SESORS spectra of melatonin, serotonin, epinephrine, norepinephrine, and dopamine in the agarose gel behind a rat skull at various concentrations to establish the LODs through the skull. The rat skull ranges in thickness from 0.5 mm to 1.25 mm, and we determined the optimal spatial offset to achieve the highest signal through this thickness of bone was found to be 2 mm. Spectra were acquired at concentrations ranging from 100 μ M to 100 nM. The 0 mm offset spectrum was subtracted from the 2 mm offset SESORS spectra in a scaled subtraction based on the 960 cm^{-1} peak (GRAMS, Thermo-Fisher). Each spectrum was then baseline corrected using GRAMS software. The subtracted and baselined data were imported into MatLab for further processing. The spectra were filtered with a Savitzky-Golay filter, with order (2) and frame length (7) input by the user. The data was zeroed based on the minimum value of each spectrum. The spectra were then barcoded based on the second derivative. If the sign of the second derivative of the spectrum was positive at a given Raman shift, the intensity of the shifted spectrum at that point was set to zero. If the sign of the second derivative was negative at a given Raman shift, the spectral intensity at that point was not modified. The user is then prompted to enter a threshold percentage based on the maximum intensity peak of the original spectrum. Any signal in the spectra below this threshold value is set to zero, essentially filtering out spurious noise in the spectra.

We find that we achieve limits of detection similar to what was found in aqueous solution, generally in the low hundreds of nM concentration range. The neurotransmitters are separated into two classes based on their chemical structures. The first class of neurotransmitters include serotonin and melatonin which have very similar double ring structures including amine groups. Table 5.1 represents peak positions followed by heat maps showing the LODs based on intensities at the specified peak positions for serotonin and melatonin (Figure 5.3).

Table 5.1 Peak positions associated with specified vibrational modes as well as the chemical structures of serotonin and melatonin.

Bands	Serotonin (cm ⁻¹)	Melatonin (cm ⁻¹)
		
b _{CH}	632	648
b _{NH}	820	817
r _{OH}	1221	1224
b _{CNH}		1302
w _{HCH}	1341	1357
st _{CC}	1496	
b _{NH}	1522	
st _{CC}	1593	

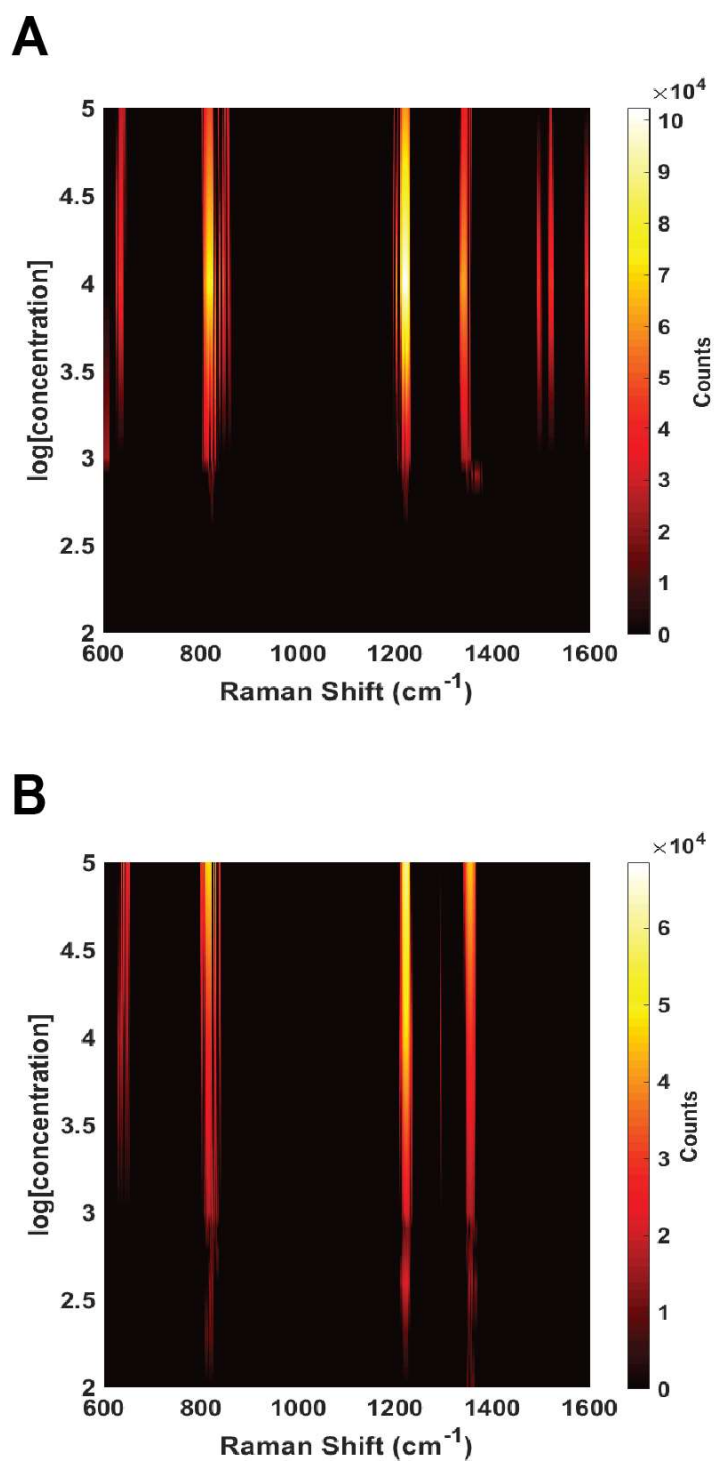


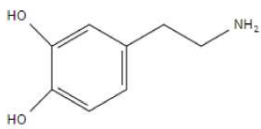
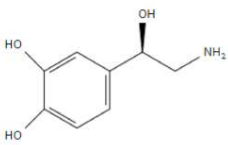
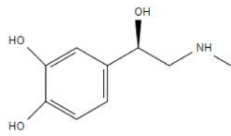
Figure 5.3. Heat map of the SESORS spectra for the LOD of serotonin (A) and melatonin (B) through a rat skull using a spatial offset of 2 mm. The false color is characteristic of signal intensity at various depths of agarose gel from highest (white) to lowest (red) intensity. $\lambda_{\text{ex}}=785$ nm, $P=90$ mW, $t=120$ sec.

The second class of neurotransmitters include dopamine, norepinephrine, and epinephrine. These neurotransmitters have an aromatic ring structure containing two hydroxyl groups, where the carbon side chains differ in the position of the amine groups and the presence of hydroxyl groups. The major peaks associated with each of these neurotransmitters are listed in Table 5.2. The LODs for each neurotransmitter are demonstrated in Figure 5.4 where we see LODs of 1 μ M for dopamine (Figure 5.4A), 400 nM for norepinephrine (Figure 5.4B), and 800 nM for epinephrine (Figure 5.4C).

In the brain, we are unlikely to find isolated neurotransmitters present. Thus, we collected SESORS spectra of mixtures. The mixtures investigated are of neurotransmitters that are most similar in chemical structure, as well as mixtures of neurotransmitters seen in specific areas of the brain including the *substantia nigra*, where Parkinson's disease develops. The neurotransmitters present in the *substantia nigra* are dopamine, GABA, and DOPAC. DOPAC is a metabolite of dopamine which is present in the degradation process of dopamine. Detection of GABA with AuNPs is very difficult as previously demonstrated³⁰, where no signal is obtained with the as synthesized AuNPs. The AuNPs have a strong affinity for indole rings, in aromatic neurotransmitters³¹, however, they do not bind to the carboxyl groups of the amino acid chain neurotransmitters³². Three mixtures are presented, a mixture containing melatonin and serotonin, a mixture containing dopamine, epinephrine, and norepinephrine, and a mixture that contains dopamine, DOPAC, and GABA.

Since Raman spectra are indicative of the vibrational modes of a molecule, mixtures of neurotransmitters with a very similar structure will have spectra that are almost identical, with some peak broadening, which arises due to slight shifts in peak positions from the small structural differences in the molecules. There will also be a few Raman peaks that are unique to a

Table 5.2 Peak positions associated with specified vibrational modes as well as the chemical structures of dopamine, norepinephrine, and epinephrine.

Bands	Dopamine (cm ⁻¹) 	Norepinephrine (cm ⁻¹) 	Epinephrine (cm ⁻¹) 
stCO	1282	1281	1272
bCNH			1314
WHCH	1370	1365	
stCC			1485
stCC	1586	1586	1596

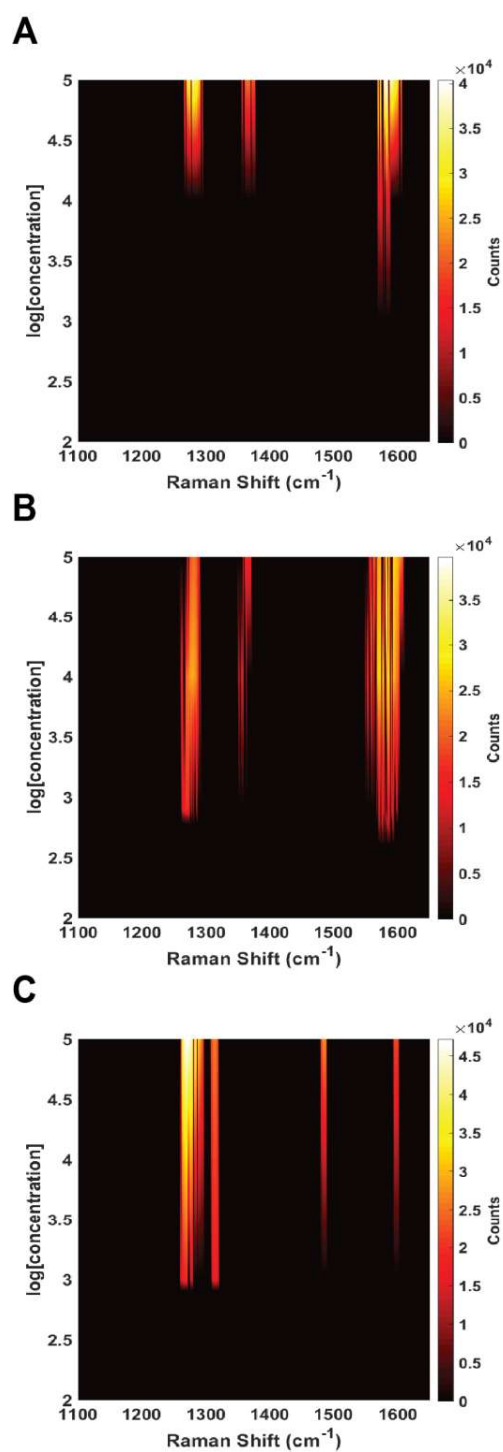
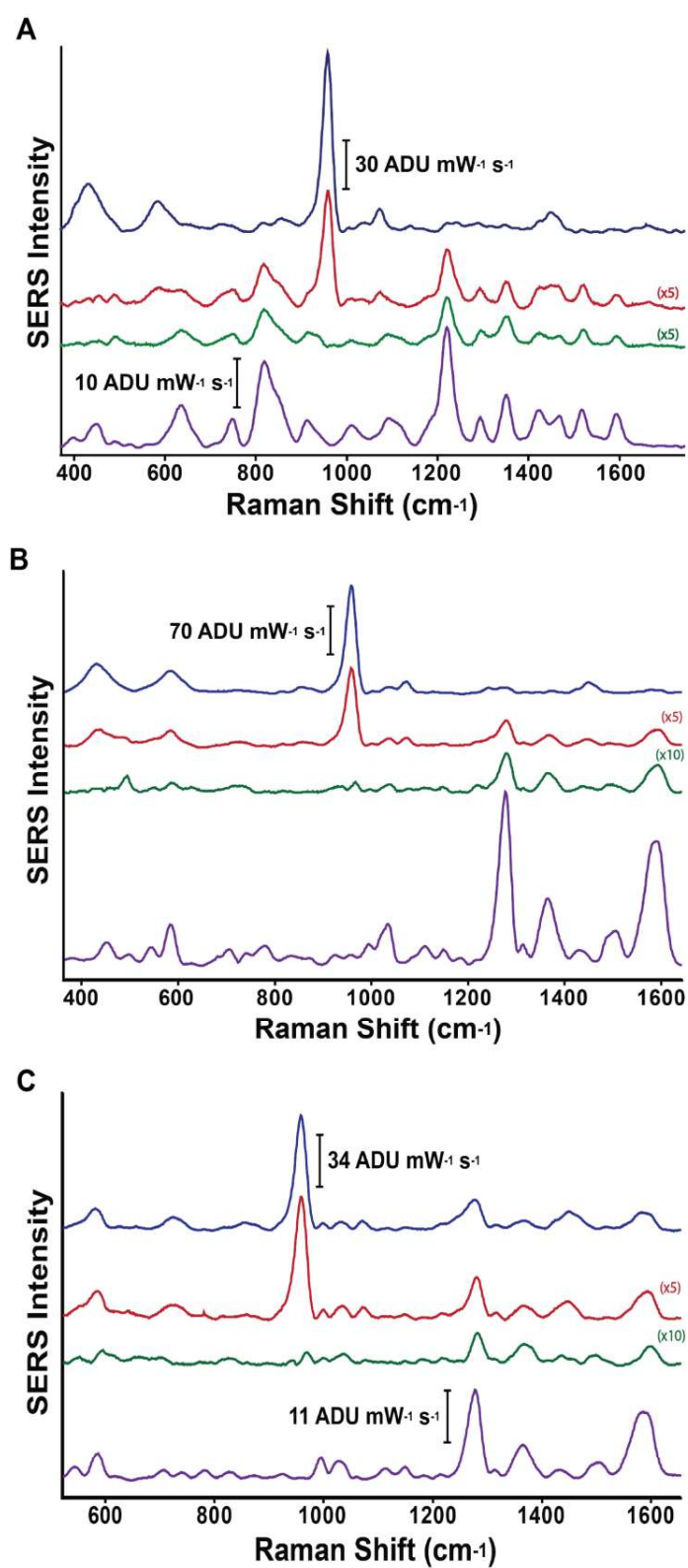


Figure 5.4. Heat map of the SESORS spectra for the LOD of dopamine (A), norepinephrine (B), and epinephrine (C) through a rat skull using a spatial offset of 2 mm. The false color is characteristic of signal intensity at various depths of agarose gel from highest (white) to lowest (red) intensity. $\lambda_{\text{ex}}=785$ nm, $P=90$ mW, $t=120$ sec.

single neurotransmitter which arise from the structural differences. In Figure 5.5A, we show the mixture containing melatonin and serotonin, the peaks at 635 cm^{-1} , 816 cm^{-1} , 1221 cm^{-1} , 1294 cm^{-1} , 1467 cm^{-1} , 1519 cm^{-1} , and 1591 cm^{-1} , are characteristic of both melatonin and serotonin. The peaks at 1004 cm^{-1} , 1088 cm^{-1} , and 1355 cm^{-1} , are characteristic of melatonin and the peaks at 749 cm^{-1} , 918 cm^{-1} , and 1421 cm^{-1} are characteristic of serotonin. The mixture of dopamine, epinephrine, and norepinephrine is shown in Figure 5.5B, with the Raman peaks at 1148 cm^{-1} , 1279 cm^{-1} , 1365 cm^{-1} , 1436 cm^{-1} , 1489 cm^{-1} , and 1587 cm^{-1} are representative of all three neurotransmitters. Dopamine can be represented by a peak at 589 cm^{-1} , characteristic peaks of epinephrine are visible at 730 cm^{-1} , 968 cm^{-1} , and 1039 cm^{-1} , and norepinephrine can be represented by peaks at 1219 cm^{-1} and 1315 cm^{-1} . Finally, in the mixture of dopamine, GABA, and DOPAC (Figure 5.5C), there are no peaks characteristic of GABA as it does not have an indole ring and the AuNPs do not bind with the terminal carboxyl group. The characteristic peaks of dopamine are 594 cm^{-1} , 1367 cm^{-1} , and 1455 cm^{-1} , the characteristic peaks of DOPAC are visible at 616 cm^{-1} , 1436 cm^{-1} , and 1595 cm^{-1} , and the peaks at 1281 cm^{-1} , 1497 cm^{-1} are characteristic of both dopamine and DOPAC. It is demonstrated in Figure 5.4 that it is possible to clearly differentiate between the Raman peaks of different mixtures of neurotransmitters.

To demonstrate the feasibility of SESORS as an *in vivo* method for animal models, we collected SESORS spectra posthumously of exogenously injected serotonin in the brain of a mouse model. The mouse skull is approximately 0.5-0.8 mm thick with a layer of skin covering it at a thickness of 1 mm. We shaved the head of the mouse to remove light scattering effects due to the hair. Prior to injecting the brain with serotonin and nanoparticles, we collected SORS spectra at a 0 mm offset of the brain through the skull (Figure 5.6, blue trace). The spectrum shows the prominent bone phosphate vibration at 960 cm^{-1} . Additionally, we see broadened peaks that arise from degrading brain tissue at 1072 cm^{-1} (ν_{CC}), 1445 cm^{-1} (ν_{CH}), 1661 (ν_{CO} , δ_{NH}).

Figure 5.5. SESORS spectra of mixtures containing melatonin and serotonin (A), dopamine, epinephrine and norepinephrine (B), and dopamine, GABA, and DOPAC (C). The top spectrum for each panel represents the contribution from the bone with no spatial offset (blue). The red spectrum is the SESORS spectrum of the neurotransmitters in the agarose gel behind the rat skull with a 2 mm spatial offset. The green spectrum shows a subtraction of the bone spectrum with no spatial offset from the SESORS spectrum with a 2 mm offset revealing a pure spectrum of the neurotransmitter. A SERS spectrum of the neurotransmitters and AuNPs embedded in the agarose gel is displayed in purple. $\lambda_{\text{ex}}=785$ nm, $P=90$ mW, $t=120$ sec.



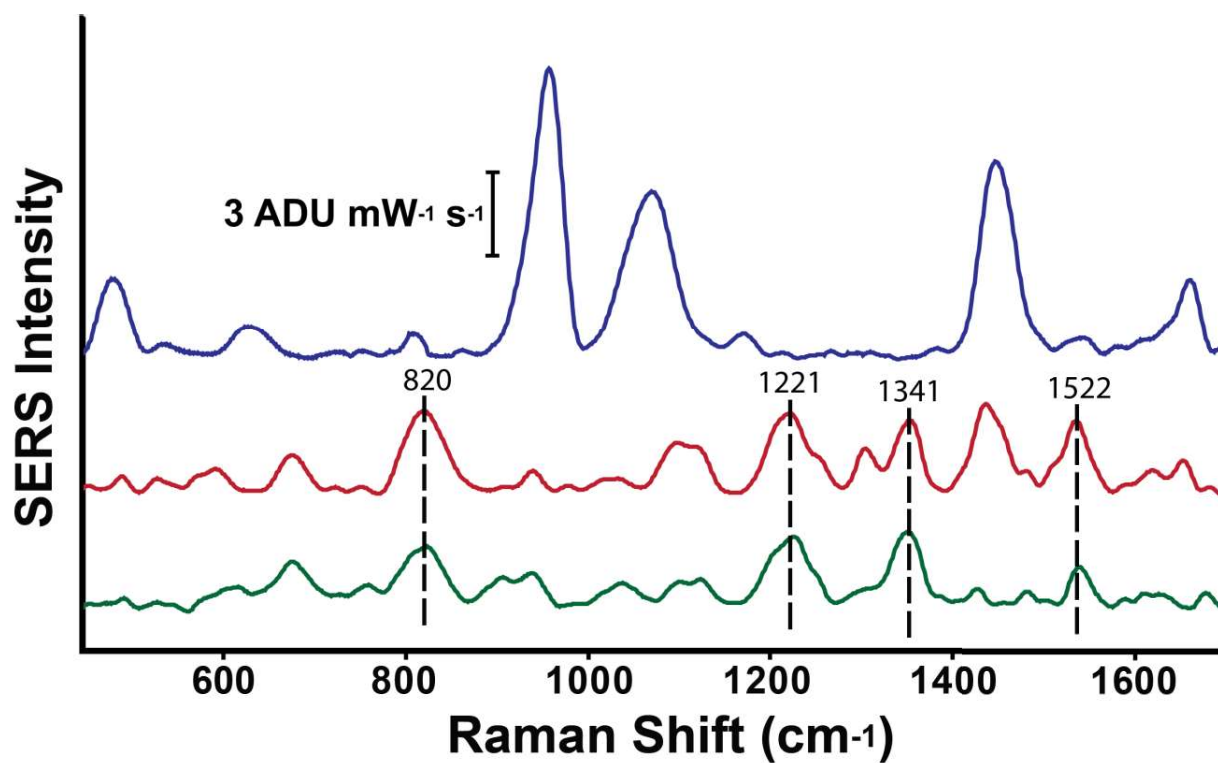


Figure 5.6. SORS spectra of the mouse brain through the skull without the injection of serotonin or AuNPs (top, blue). SESORS spectra of the mouse brain after the injection of 100 μM serotonin at a 0 mm offset (red) and a 1 mm offset (green). $\lambda_{\text{ex}}=785$ nm, $P=90$ mW, $t=120$ sec.

Due to decomposition of the brain tissue, we injected the brain with serotonin and AuNPs. The mouse skull is very thin, which allowed SESORS spectra from the neurotransmitter to be obtained with no spatial offset (Figure 5.6, red trace). The highest Raman intensity, however, is achieved for the characteristic peaks of serotonin at a 1 mm offset demonstrating the ability of this technique for a non-invasive detection of neurotransmitters. Similar to the SESORS spectra of serotonin the modes at 820 cm^{-1} (b_{NH}), 1221 cm^{-1} (r_{OH}), 1341 cm^{-1} (w_{HCH}), and 1522 cm^{-1} (b_{NH}) are detected through the skull (Figure 5.6).

5.5 Conclusion

We have presented the use of SESORS for a minimally invasive detection method for neurotransmitters through the skull. AuNPs are an excellent choice for a potential *in vivo* detection method due to their exceptional stability and low toxicity. Previous studies show the best enhancement for the AuNPs at an excitation wavelength of 785 nm, which also mitigates interferences from fluorescence resulting from the biological material. These AuNPs also mitigate signal from the ‘background’ amino acid chain neurotransmitters such as GABA and allow the detection of the aromatic neurotransmitters commonly known to be associated with neurological diseases. We investigated the dispersion of the AuNPs in a brain mimic where the results show an even distribution of AuNPs throughout the agarose gel. Neurotransmitters and AuNPs were then embedded in agarose gel to find LODs for serotonin, norepinephrine, melatonin, epinephrine, and dopamine through a rat skull. SESORS measurements were also acquired for mixtures of neurotransmitters including neurotransmitters that are very similar in chemical structure and those present in the *substantia nigra* which is associated with Parkinson’s disease. The thickness of the skull in this area of the brain is approximately 3 mm thick, where our technique shows great potential for an *in vivo* method. To further demonstrate the potential of SESORS for an *in vivo* detection method, serotonin was detected in the brain of a mouse

model at a concentration of 100 μM . Detection of individual neurotransmitters is not a trivial task. Future work will include the use of capture molecules including aptamers and antibodies to increase the specificity of the nanoparticle SERS sensor and allow capture of a target analyte.

5.6 Acknowledgements

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Chapter 6

Electrochemical Surface-Enhanced Raman Spectroscopy for the Selective Identification of Dopamine and Metabolites

Chapter 6 is an adaption of a research article submitted to the Journal of the American Chemical Society by Moody, A.S. and Sharma, B. entitled “Electrochemical Surface-Enhanced Raman Spectroscopy for the Selective Identification of Dopamine and Metabolites”. This article describes the combination of two commonly used sensing techniques for neurotransmitters, SERS and electrochemistry to selectively identify dopamine and DOPAC in a mixture, a common limitation of electrochemistry. The paper also describes the SERS detection of dopamine and its metabolites in aqueous solution, artificial urine, and artificial CSF.

6.1 Abstract

Early disease diagnosis is imperative during the development of neurological diseases, such as Parkinson’s disease, where treatment would be most effective. This requires the use of detection techniques that are sensitive and selective to identify the neurochemicals, which are often similar in chemical structure. Surface-enhanced Raman spectroscopy (SERS) allows for this selective detection, and when combined with electrochemistry permits the sensitive and selective localized detection of analytes with similar oxidation potentials. We present here the SERS detection of neurochemicals associated with Parkinson’s disease, including dopamine, 3,4-dihydroxyphenylacetic acid, homovanillic acid, and 3-O-methyldopamine. A carbon screen-printed electrode modified with gold nanoparticles served as the SERS-active substrate during the simultaneous electrochemical detection of dopamine and DOPAC. We demonstrate that the combination of electrochemistry and SERS allows for highly sensitive and selective identification of dopamine and DOPAC in a mixture.

6.2 Introduction

Parkinson’s disease (PD) is the second most common neurodegenerative disorder in the United States, affecting approximately 1 million individuals. The disease is typically characterized by resting tremor, rigidity, bradykinesia or slowness, gait disturbance, and postural

instability.¹⁻² Recently, other symptoms have been associated with PD including sleep disturbances, cognitive dysfunction, autonomic failure, and impaired olfaction, which precede the loss of motor function.³ The motor symptoms of PD are caused by the degeneration of the dopaminergic neurons in the *substantia nigra pars compacta* region of the brain resulting in a deficiency of the neurotransmitter dopamine and the presence of Lewy bodies. Disease diagnosis is based solely on evaluation of symptoms that arise due to the loss of motor functions, however, these symptoms are usually not present until approximately 70% of the dopaminergic neurons have already degenerated.⁴ Due to this delay in diagnosis, there is a high incidence of misdiagnosis and the primary method for disease confirmation is autopsy.¹ There is little known of the mechanism that results in the loss of dopaminergic neurons, thus there are no effective treatment methods that combat the progressive nature of PD. The need continues to grow for a reliable diagnostic biomarker that would allow for an understanding of the pathology of the disease and the ability to monitor the progression of the disease during treatment.

There have been many studies on achieving a sensitive and selective detection of the neurotransmitter dopamine due to its implications in various neurological diseases.⁵⁻²⁶ High performance liquid chromatography (HPLC) is commonly used in the detection of dopamine,²⁷⁻²⁸ but requires long run times and lacks the specificity. Electrochemical detection methods are highly sensitive and selective for electroactive molecules. Recently, efforts have been made to improve the sensitivity, selectivity and biocompatibility of electrochemical sensors.^{15, 29-31} Further, electrochemical detection methods have been paired with HPLC by modifying electrodes with molecules that have a specific affinity for dopamine to improve the sensitivity and selectivity.^{13, 32-33} The combined electrochemical-HPLC is very selective and sensitive, however, it requires considerable sample preparation and the inclusion of electroactive molecules. Ultra-performance liquid chromatography (UPLC) can be combined with mass spectrometry (MS) in

order to easily assess complex biological matter while maintaining the sensitivity and specificity required to analyze neurotransmitters and their metabolites.³⁴

The limitations involved with the aforementioned methods include complex and expensive instrumentation, long run times, laborious sample preparation, and poor sensitivity and selectivity. Surface enhanced Raman spectroscopy (SERS) can overcome these limitations with extreme selectivity and sensitivity to target molecules in complex media with simple instrumentation. SERS detection of dopamine has been previously demonstrated with modified plasmonic nanoparticle substrates.^{5, 35-37} While SERS is a powerful technique, it requires that the target molecule be within 1-2 nanometers of the nanoparticle surface.³⁸⁻³⁹ Some target analyte molecules do not have an affinity for binding to the metallic nanoparticle surface, thereby impeding detection. Electrochemical methods can be applied to bring the target molecule closer to the surface of the gold nanoparticles by taking advantage of the redox properties of electrochemically active molecules. During the oxidation-reduction reaction, the analyte is attracted to the electrode surface, which when modified with SERS nanoparticles, can be used for localized SERS detection.

Electrochemical sensors operate by reacting with the analyte of interest through an oxidation-reduction reaction, producing an electrical signal proportional to the analyte concentration. The technique offers sub-second time resolution, high spatial resolution, and excellent sensitivity; however, the selectivity is only moderate. Electrochemical methods used for detection of dopamine with limits of detection in the low nanomolar range have been reported.⁴⁰ The problem of selectivity still exists, however, where interfering compounds such as dihydroxyphenylacetic acid (DOPAC), ascorbic acid, epinephrine, and uric acid are all oxidized at similar potentials, leading to the overlap of voltammetric responses.⁴¹ To improve upon the selectivity of electrochemical methods and the surface interaction of SERS, SERS and

electrochemical measurements can be performed simultaneously. Applying a potential alters the redox state of the analyte, which results in increased attraction of the analyte to the SERS substrate, giving rise to an increased molecular fingerprint signal. This spectroelectrochemical method has been used previously to study serotonin⁴², 2-amino-5-(4-pyridinyl)-3,4-thiadiazole, pyridine⁴⁴, rhodamine 6G⁴⁵, drugs⁴⁶, anthrax⁴⁷, as well as *para*-aminothiol and melamine⁴⁸.

Here we present the SERS detection of dopamine and its metabolites on gold nanoparticles (AuNPs) as well as the spectroelectrochemical detection of dopamine and DOPAC on a AuNP modified carbon screen printed electrode (CSPE). The results show that SERS enhances the capabilities of electrochemical detection by providing a molecular fingerprint of the analytes in solution with similar oxidation potentials, allowing for a clear identification of species present.

6.3 Experimental Section

6.3.1 Reagents and Materials

Citric Acid (99%), dopamine (hydroxytyramine hydrochloride, 99%), agarose, dihydroxyphenylacetic acid (98%), 3-O-methyldopamine (99+%), L(-)-glutathione, oxidized (98%), and hydrogen tetrachloraurate (III) trihydrate (HAuCl₄) were purchased from ACROS Organics. Homovanillic acid (98+%) and L-glutathione reduced (97%) were purchased from Alfa Aesar. Potassium chloride (99%), ethanol (99.5%), hydrochloric acid, and nitric acid was purchased from Fisher Scientific. Carbon screen printed electrodes were purchased from Pine Research Instrumentation. All glassware was cleaned in aqua regia and rinsed with Millipore water ($\geq 18 \text{ M}\Omega \text{ cm}$) prior to use.

6.3.2 Preparation of AuNPs

Citrate reduced AuNPs were synthesized as previously described by using methods established by Frens⁵⁰. Briefly, a 0.01% by weight solution of HAuCl₄ was brought to a boil

where a 1% solution of citric acid was added and allowed to boil for 5 minutes. The solution was allowed to cool before centrifugation. An aliquot of 4 mL of AuNPs were centrifuged for 15 minutes at 4200 rpm. The supernatant was removed and discarded. The AuNPs were characterized with an Agilent Cary 5000 UV/Vis/NIR spectrophotometer and scanning electron microscopy (SEM) and showed an LSPR max at 525 nm with an average size distribution of 60 nm.

6.3.3 Preparation of SERS-Active Electrodes

Carbon screen-printed electrodes were purchased from Pine Research Instrumentation. The electrodes have carbon working and counter electrodes and a Ag/AgCl reference electrode. The electrodes were fabricated into SERS-active substrates by dropcasting three 5 μ L layers of AuNPs onto the carbon working electrode surface of the screen-printed electrode⁴⁷. The AuNPs were allowed to dry between addition of each layer and the third layer was allowed to dry for 24 hours before use. The compact voltammetry cell kit (Pine Research) was used for the electrochemical cell, which consists of a glass vial, cell grip, cell cap, and a mini-USB style to banana jack cell cable.

6.3.4 SERS Measurements

Initially we collected SERS spectra of each neurochemical. A 4 mL aliquot of AuNPs was centrifuged and the supernatant was removed. The AuNPs were then resuspended in 200 μ L of the neurochemical solution at various concentrations. The pH of the neurotransmitter/AuNP solution was changed through addition of 0.75 μ L of a 3 M solution of HCl. The SERS spectra were acquired immediately after the pH change. The laser (785 nm, Innovative Photonic Solutions) was directed through a Nikon Ti-U inverted microscope equipped with a 20x objective and focused onto the sample. The Raman scattered light was directed into an IsoPlane 320 spectrometer (Princeton Instruments) in a 180° backscattering geometry and subsequently

dispersed onto a PIXIS400 camera (Princeton Instruments). A laser power of 5 mW was used to illuminate the sample for SERS spectra with an acquisition time of 60 s.

6.3.5 Spectroelectrochemical Measurements

The SERS-active carbon screen-printed electrodes were placed into the compact electrochemical cell with 10 mL of the analyte in a 0.1M KCl solution. The cell was then connected to the potentiostat (Pine Research Instrumentation ultra-portable WaveNow Potentiostat). The electrochemical cell was then incorporated into a home-built macro-Raman system to collect spectroelectrochemical measurements. The laser (785 nm, Innovative Photonic Solutions) was directed onto the sample using a dichroic mirror and focused on the SERS-active working electrode. The Raman scattered light was then directed into an Acton LS 785 spectrometer in a 180° backscattering geometry and detected with a CCD camera (PIXIS400, Princeton Instruments). An average laser power of 40 mW was used to illuminate the sample. The spectra were collected using 30 s acquisition times in a cathodic direction at 5 mV/s. All solutions were purged with nitrogen gas prior to measurement.

6.4 Results and Discussion

The life cycle of dopamine begins with the uptake of tyrosine by the dopaminergic neurons of the *substantia nigra* which is subsequently converted into dihydroxyphenylalanine (L-DOPA) through tyrosine hydroxylase. L-DOPA is then either converted into dopamine or quickly metabolized to form 3-O-methyldopamine. The metabolism of dopamine results in a main intermediate metabolite of 3,4-dihydroxyphenylacetic acid and an end product of homovanillic acid (HVA).¹²

We achieved the SERS detection of dopamine, DOPAC, HVA, and 3-O-MD on AuNPs. The AuNPs have an enhancement factor of 10^5 and the extinction spectrum has an LSPR maximum of 525 nm. SEM images show that the AuNPs have an average diameter of 60 nm.

(Figure 6.2) The SERS spectra were acquired at concentrations ranging from 100 μ M to 100 nM in an effort to find a limit of detection (LOD). We obtained the SERS spectra of dopamine and its metabolites in aqueous solution, artificial urine, and artificial cerebrospinal fluid (aCSF) (Figure 6.1). The spectra were baseline corrected, zapped for cosmic rays, and the background contributions from the citrate on the nanoparticles, artificial urine, or aCSF were subtracted.

The most intense modes present for each neurochemical are represented in Table 6.1. We are able to characterize each analyte based on shifts in peak position and the appearance of new peaks.

LODs of the neurochemicals in comparison to the normal cerebral concentrations are listed in table 6.2. We achieve LODs that are within or lower than the normal brain concentrations of dopamine and DOPAC, which ensures detection of normal and deteriorating concentrations of both analytes present during the development of Parkinson's disease.

Although SERS gives excellent chemical specificity, it is a localized detection technique, consequently if the molecule does not have an affinity to the nanoparticle surface, detection is impeded. Electrochemistry is also limited in the ability to identify chemicals with similar oxidation potentials. Dopamine and DOPAC have very similar oxidation potentials which overlap when both are present in solution, making the identification of each analyte difficult. HVA and 3-O-MD are typically oxidized at more positive potentials which allow them to be more easily identified.⁵⁴ In an effort to aid in the diffusion to the nanoparticle surface with SERS and the identification of dopamine and DOPAC during the electrochemical detection, we have combined the two techniques. CSPEs were made SERS active by dropcasting three 5 μ L layers of AuNPs onto the carbon working electrode. SEM images of the Au modified electrodes are shown in Figure 6.2.

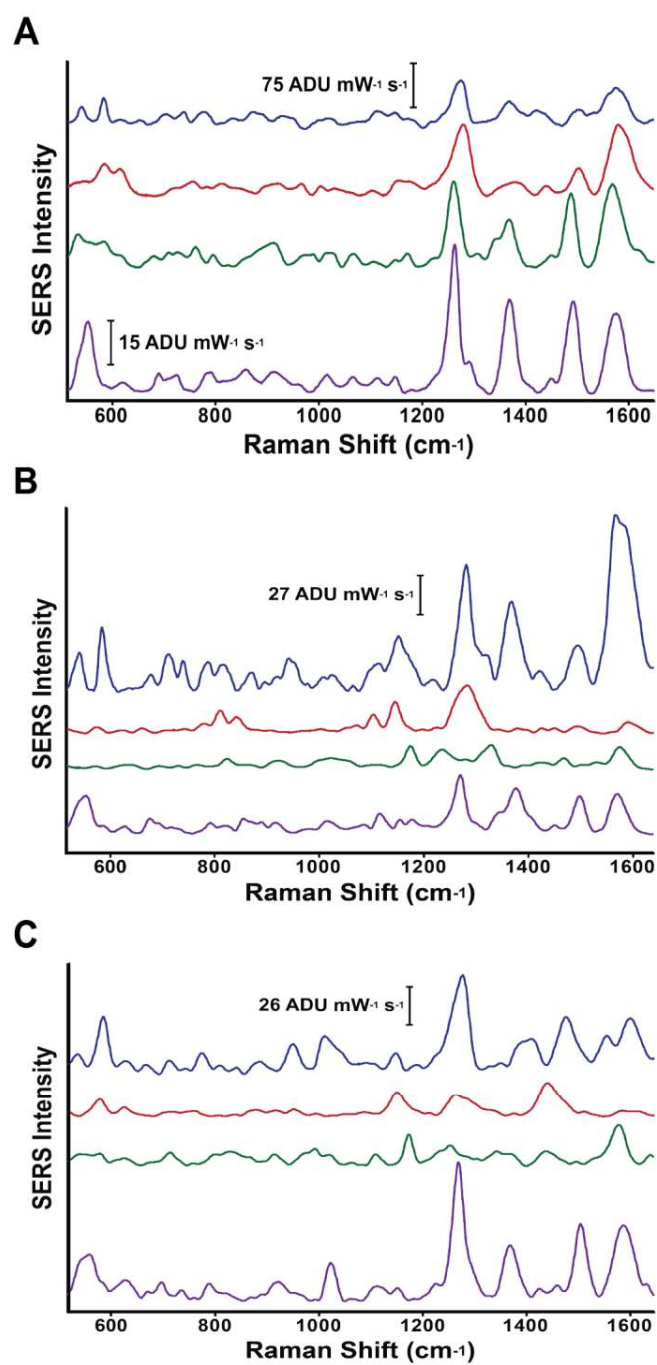


Figure 6.1. SERS spectra of dopamine (blue), DOPAC (red), HVA (green), and 3-O-MD (purple) in H_2O (A), artificial urine (B), and aCSF (C). Representative SERS spectra are shown at a concentration of $100 \mu\text{M}$. $\lambda_{\text{ex}}=785 \text{ nm}$, $P=5 \text{ mW}$, $t=60\text{s}$.

Table 6.1: Band assignments for the SERS shifts of dopamine, DOPAC, HVA, and 3-O-MD as well as chemical structures for each neurotransmitter.

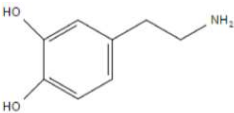
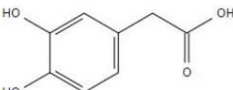
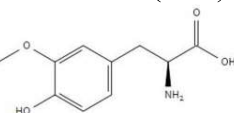
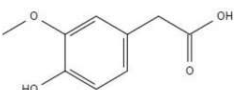
Bands	Dopamine (cm ⁻¹) 	DOPAC (cm ⁻¹) 	3-O-MD (cm ⁻¹) 	HVA (cm ⁻¹) 
ν_{C-N}	585		552	
ν_{C-C-C}	1152	1151	1016	1175
ν_{C-O}	1276	1281	1265	1260
ν_{C-C}	1369		1371	1339
$\nu_{C=O}$		1438	1438	1438
ν_{C-C}	1486		1497	1468
ν_{C-C}	1594		1579	1576

Table 6.2: Normal brain concentrations as well as limits of detection for dopamine, DOPAC, HVA, and 3-O-MD in H₂O, artificial urine, and aCSF.

Neurochemical	[Normal Brain] (μM)	Limits of Detection (μM)		
		H ₂ O	artificial urine	aCSF
Dopamine	1-3 ²⁶	0.4	0.2	0.4
DOPAC	7-18 ⁵⁰	0.4	1	0.6
HVA	0.035-0.250 ⁵¹	10	10	0.8
3-O-MD	0.014-0.020 ⁵²⁻⁵³	0.1	0.2	0.6

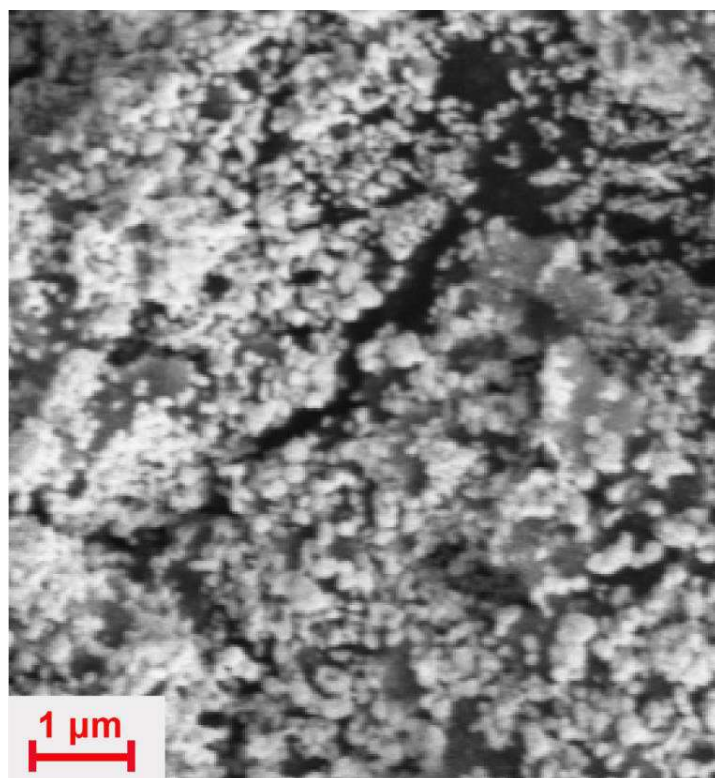


Figure 6.2. SEM images of Au modified electrodes.

The electrochemical activity of the electrodes is not impeded by the deposition of the AuNPs and the AuNPs will permit acquisition of a molecular fingerprint SERS spectrum of each neurochemical. Previously, silver nanoparticles (AgNPs) have been combined with CSPEs for a spectroelectrochemical detection of *para*-aminothiophenol and melamine.⁴⁸ To the best of our knowledge, AuNPs, which are more biocompatible, have not been combined with CSPEs to monitor and identify dopamine and DOPAC during the simultaneous electrochemical reduction or oxidation process.

As a proof-of-concept of the SERS activity and electrochemical performance of the AuNP-coated electrodes, the spectroelectrochemical detection of pyridine was performed. Pyridine is a model system to test SERS activity due to the large Raman cross section and it also exhibits excellent electrochemical behavior. The SERS spectra were collected for 10 s (Figure 6.3) while the potential was swept in a cathodic direction from +1.0 V to -2.2 V at a rate of 10 mV/s. The results show that the maximum SERS intensity is achieved at a voltage of -0.8 V which is consistent with previous studies.²⁶

The combined spectrochemical detection of dopamine and DOPAC were then obtained (Figure 6.4). The spectra were collected with an acquisition time of 30 s during a cathodic potential sweep from +1.0 V to -1.25V. For dopamine, the signal increased during the cathodic potential sweep where a maximum was reached at a potential of +0.4 V. During the sweep from +1.0 V to -1.25 V, dopamine is reduced to DAH after the diffusion of dopamine to the Au modified surface of the electrode occurs. This results in peaks visible at 1162 cm⁻¹ (δ_{CHHC}), 1498 cm⁻¹ (ν_{CC}), 1607 cm⁻¹ (ν_{CN}), and 1657 cm⁻¹ (δ_{OH}). For DOPAC, the potential increases until a maximum is reached at -0.2 V. The reduction of DOPAC to DOPAC-H results in peaks visible at 1036 cm⁻¹ ($\nu_{\text{O-CH}}$), 1268 cm⁻¹ (ν_{OC}), 1310 cm⁻¹ (ν_{CH}), 1364 cm⁻¹ (ν_{CC}), 1423 cm⁻¹ (δ_{CH}), 1667 cm⁻¹ (δ_{OH}).

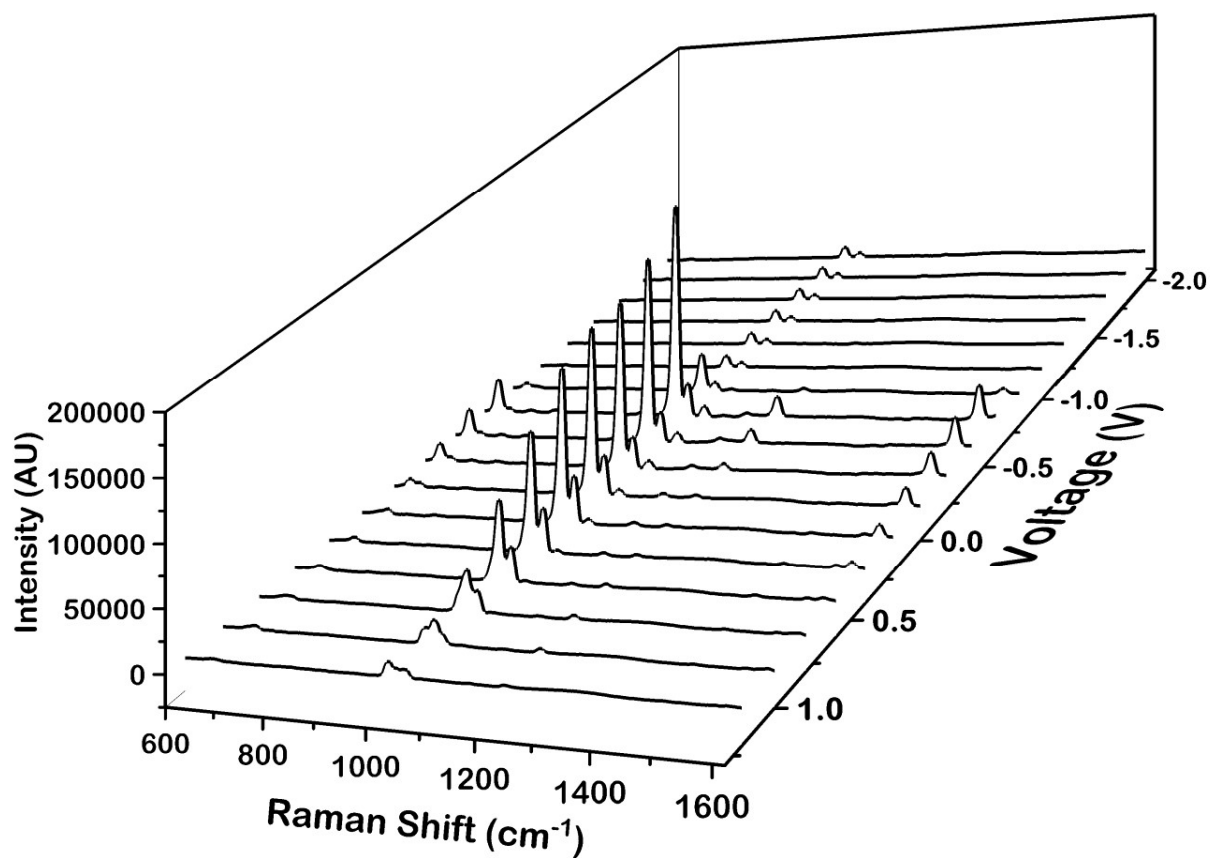


Figure 6.3. SERS spectra of 50mM pyridine on AuNP modified carbon screen printed electrode as the voltage was swept from +1.0 V to -2.0 V. λ_{ex} =785 nm, P=4 mW, t=10s.

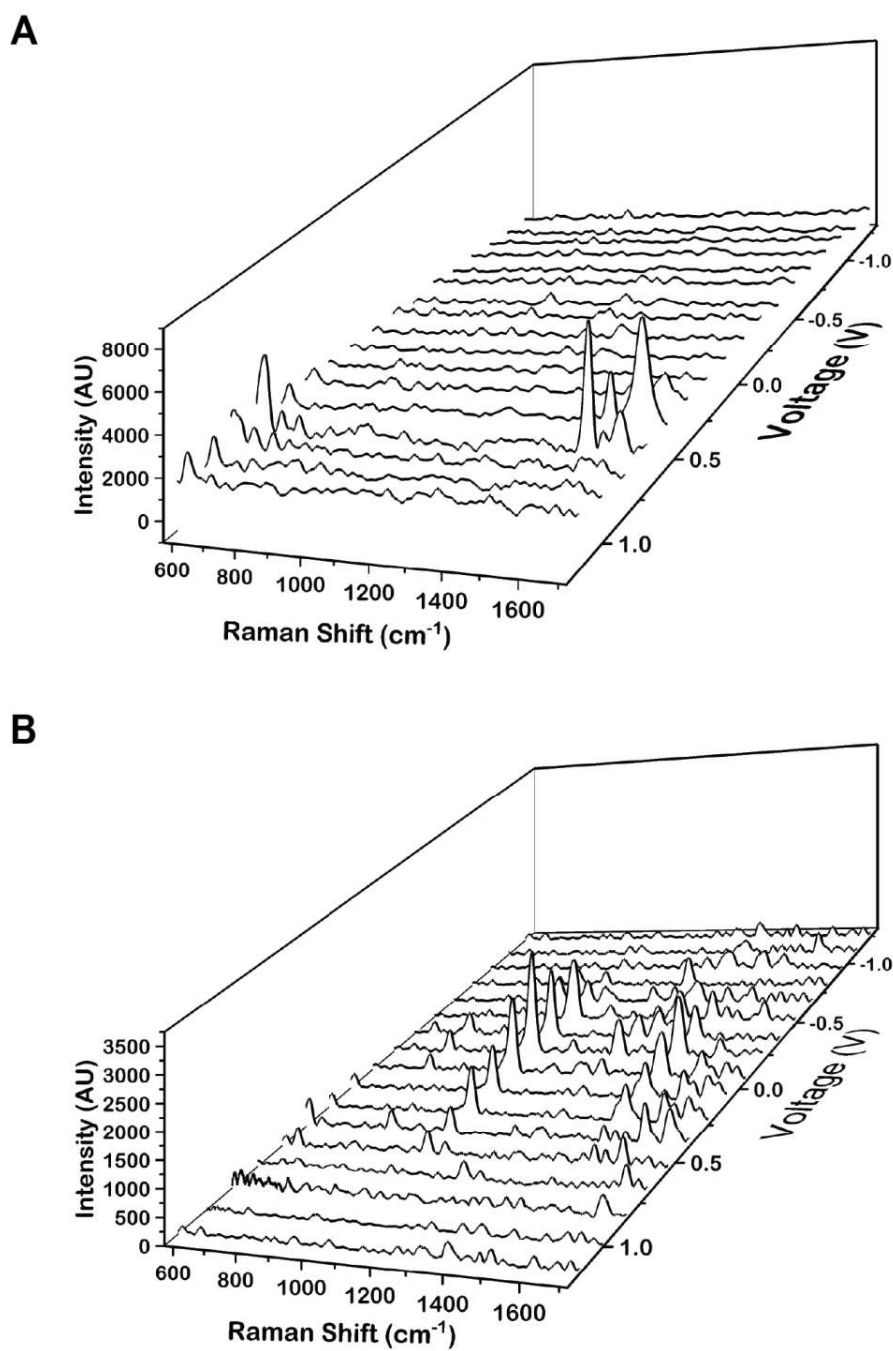


Figure 6.4. SERS spectra of 10 μM dopamine (A) and 10 μM DOPAC (B) on AuNP modified carbon screen printed electrode as the voltage was swept from +1.0 V to -1.0 V. λ_{ex} =785 nm, P=40 mW, t=30s.

In order to determine if dopamine and DOPAC could be differentiated in solution, a spectroelectrochemical detection of a mixture of dopamine and DOPAC at a concentration of 10 μ M was performed which is similar to the concentrations of each analyte present in the brain under normal conditions. SERS spectra were collected as the potential was scanned cathodically from +1.0 V to -1.25 V (Figure 6.5A). Similar to what is seen with the individual analytes, we see a maximum SERS intensity for dopamine at a potential of +0.4 V and -0.2V for DOPAC. The intensity of the dopamine peaks is much higher than the intensity of the DOPAC peaks, so the spectrum containing peaks representative of each analyte at the maximum SERS intensity are extracted in Figure 6.5B. These results show that the analytes can be clearly identified in a mixture.

6.5 Conclusion

Here, SERS spectra of dopamine, DOPAC, HVA, and 3-O-MD are presented in aqueous solution, artificial urine, and artificial CSF. We have also presented for the first time the spectroelectrochemical detection of dopamine and DOPAC using Au modified carbon screen printed electrodes. We demonstrate that the combined spectroelectrochemical technique is able to definitively identify each species in a mixture of dopamine and DOPAC, which mitigates common misidentification of the two with a purely electrochemical detection. Future work includes establishing limits of detection for dopamine and its metabolites using this electrochemical SERS technique. We also intend to investigate the use of capture molecules such as aptamers and antibodies to further increase the selectivity of the sensor.

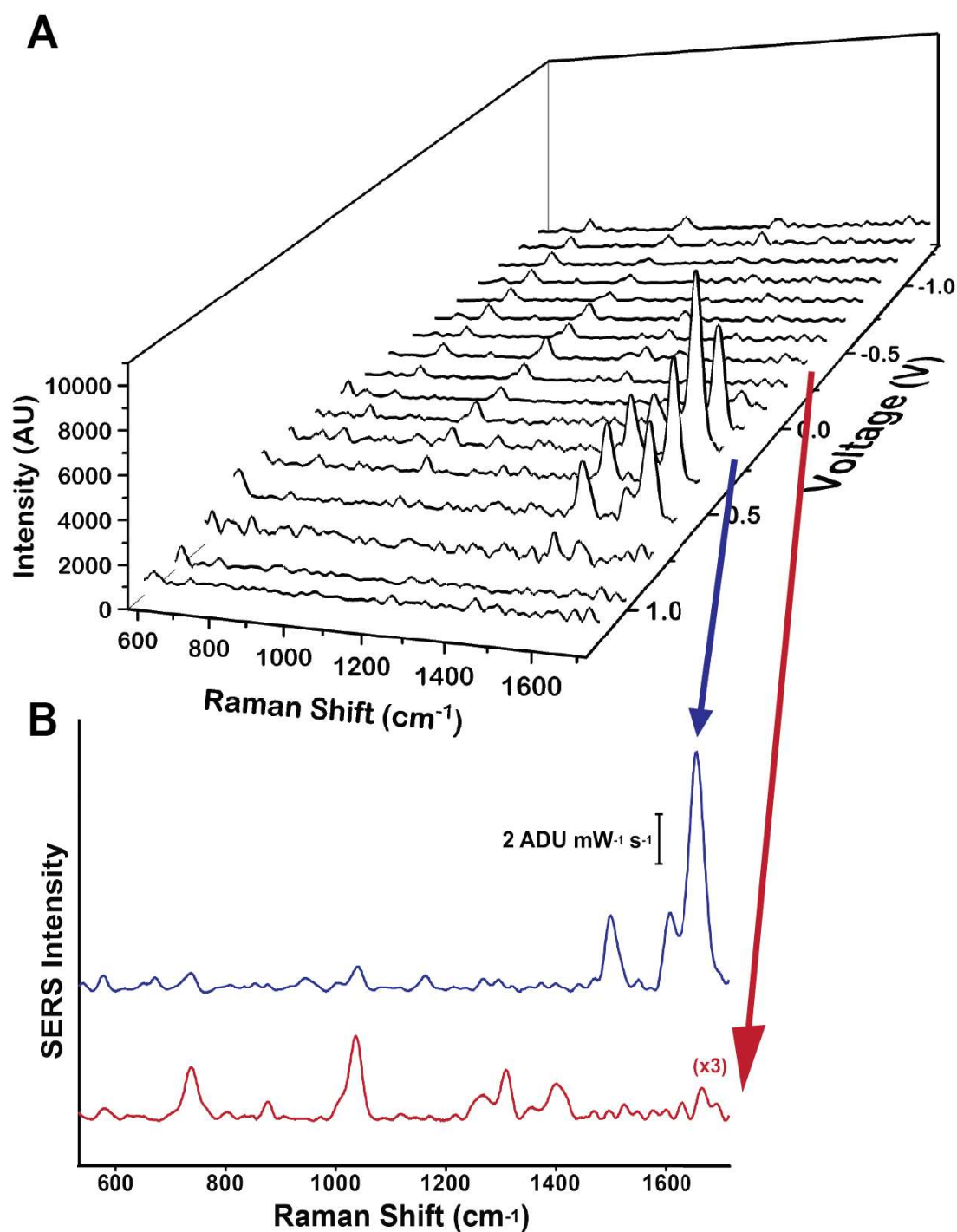


Figure 6.5. SERS spectra of 10 μ M mixture of dopamine and DOPAC on the AuNP modified carbon screen printed electrode (A) as the voltage was swept from +1.0 V to -1.0 V with the maximum intensity SERS spectra for both dopamine (blue) and DOPAC (red) extracted (B). λ_{ex} =785 nm, P=40 mW, t=30s.

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Chapter 7

Conclusion and Future Directions

Raman spectroscopy, specifically SERS and SORS show excellent potential as a detection method for neurochemicals to monitor and diagnose neurological disease. Initial SERS investigations have been conducted which involve the optimization of SERS substrates and excitation wavelength for the detection of seven neurotransmitters. Commonly used SERS substrates for excitation wavelengths in the visible and NIR wavelength range are silver and gold nanoparticles. Results show that for silver nanoparticles, the most intense Raman signal is obtained at an excitation wavelength of 633 nm and an excitation wavelength of 785 nm gives the most intensity for gold nanoparticles. These SERS substrates were also used for the detection of the neurochemicals in biological fluids using gold nanoparticles at the optimal wavelength of 785 nm.

To extend this SERS detection method for a non-invasive detection of neurochemicals through the skull, SESORS was used. Proof of concept experiments show that melatonin, serotonin, and epinephrine could be detected in a brain mimic through a cat skull at concentrations of 50 mM and 100 μ M. Subsequent investigations involved finding LODs for 5 neurotransmitters including melatonin, serotonin, epinephrine, norepinephrine, and dopamine where all LODs were in the μ M-nM concentration range. The AuNPs in the brain mimic were also shown to be evenly dispersed with dark field imaging. A maximum detection depth was also found to be 18 mm through a rat skull with 2 mm thickness. This method was shown to be suitable for an *in vivo* detection method through the detection of 100 μ M serotonin in a mouse model.

Finally, to mitigate the limitations of SERS and electrochemical detection methods, the two techniques were combined. A carbon screen printed electrode was modified by dropcasting AuNPs on the surface of the carbon working electrode. The redox process during an electrochemical detection allows for the diffusion of the analyte to the surface of the modified

working electrode to aid in the localized SERS detection. SERS is then able to identify various analytes, even with very similar oxidation potentials that are difficult to discern in electrochemistry. The spectroelectrochemical detection of dopamine and DOPAC were obtained where the two molecules are easily identified based on the molecular fingerprint obtained with SERS.

Future directions for these projects include optimization of SERS based sensors for a more selective detection. Conjugation of the nanoparticles with capture ligands such as antibodies, aptamers, and boronic acids which selectively capture diols such as dopamine, would allow for a more specific capture of target biomarker molecules for disease. SERS based sensors which are specific to a target analyte would make the SERS detection in more complex mixtures including human samples (CSF, blood, urine, saliva) feasible. SESORS detection should also be optimized to include a more suitable brain mimic such as functioning slices of a mouse brain and move toward the detection of neurochemicals in a mouse model. The SESORS detection has been obtained through a rat and cat skull, both at ~2 mm thickness. The detection should be extended to include thicker skulls that mimic the human skull which has a varying thickness of 3-14 mm. LODs should also be obtained of dopamine and DOPAC, as well as other dopamine metabolites involved in the development of Parkinson's disease.

Vita

Amber Moody was born in Dillon, South Carolina in July of 1991. She attended high school at Latta High School where she found her love for science through her chemistry teacher, Mrs. Kirkland-Cook. In 2009, Amber went on to pursue her bachelor of science in chemistry at Francis Marion University (FMU). During her undergraduate career, she performed undergraduate research under Dr. Allen Clabo investigating the stereoselectivity of the reduction of benzyl using Gaussian 03/09 as well as under Dr. LeRoy Peterson, Jr. synthesizing metal organic frameworks. She graduated from FMU in May 2013 and began attending the University of Tennessee – Knoxville (UTK) in August of 2013 to pursue her Ph.D. in Physical Chemistry.

During her time at UTK, Amber worked under Dr. Bhavya Sharma applying various forms of Raman spectroscopy towards investigation of biomedical problems. Her dissertation work involved use of normal Raman spectroscopy, surface enhanced Raman spectroscopy, and spatially offset Raman spectroscopy to develop a detection method for neurotransmitters with the goal of early diagnosis of neurological disease. Amber used these techniques to achieve a non-invasive detection of neurotransmitters through a rat skull with potential to be used as an *in vivo* detection method.

Amber was secretary of the executive committee on the Association of Chemistry Graduate Students (ACGS) in 2015 and participated on the recruiting committee in 2017 and 2018. She also worked at the UTK Forensics Camp for middle school children in June of 2014-2016 then directed the camp in 2017 and 2018. She was awarded the Chancellor's Fellowship as well as the Gleb Mamantov Graduate Chemistry Scholar Award (the highest graduate level award) in the spring of 2018. She will graduate in August of 2018 and begin a postdoctoral position at the National Center for Toxicological Research at the FDA lab in Jefferson, Arkansas.