

Neurological Disease Detection Using Surface Enhanced Spatially Offset Raman Spectroscopy (SESORS)

Chemistry Honors Thesis
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Abstract

Surface enhanced spatially offset Raman spectroscopy (SESORS) has the potential to serve as a tool for non-invasive, early detection of a variety of disease states, especially neurological disease. In this study, a great deal of preliminary work was completed in order to achieve the SESORS detection of major neurochemicals and their mixtures in a brain tissue mimic through bone. First, seven neurotransmitters were detected at physiological concentrations using surface enhanced Raman spectroscopy (SERS), and their individual limits of detection were established. Next, Teflon signal was collected through bone and brain tissue mimic at depths of 18 mm using spatially offset Raman spectroscopy (SORS). Then, a low concentration of serotonin was sensed in a mouse brain through the skull using SESORS. Finally, methods were developed to selectively capture and detect dopamine molecules using SERS.

Introduction

Neurotransmitters serve as possible biomarkers for neurological diseases, which are difficult to detect in their early stages when treatment would be most beneficial. The basic functions, structures, and physiological concentrations of several important neurotransmitters are shown in Table 1. For example, dopamine is linked to motor control and is involved in Parkinson's disease. The death of dopamine-producing neurons in the substantia nigra of Parkinson's patients results in loss of motor function (1), and the disease is typically not diagnosed until symptoms have caused irreversible damage.

Recently, the need has been recognized for better methods to monitor neurochemicals *in vivo*. Current methods, such as microdialysis (2) and cyclic voltammetry (3), are highly

invasive and often involve removing portions of the skull to surgically implant the required technology. Raman spectroscopy, in particular SERS, has garnered attention as a possible tool for biosensing and disease diagnosis (4), yet in practice, SERS is also an invasive technique, since it requires obtaining bodily fluids or tissue samples for analysis. SESORS is unique in that it can provide highly selective, label free, and minimally invasive detection of low concentrations of chemicals with rapid sample analysis. SESORS could lead to earlier detection of neurological diseases and provide critical knowledge about their progression.

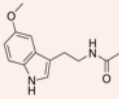
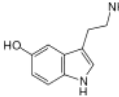
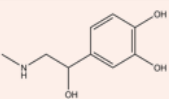
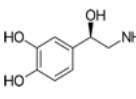
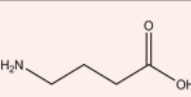
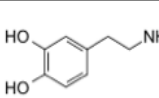
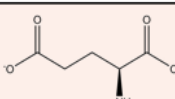
Name	Involved with:	Structure	Physiological Concentration
Melatonin	Regulates the sleep-wake cycle; free-radical scavenger		100 nM
Serotonin	regulation of mood, appetite, and sleep; cognitive functions		1 μ M
Epinephrine (aka adrenaline)	Secreted in conditions of stress		1 μ M
Norepinephrine	Mobilizes brain & body for action (fight or flight); sleep-wake cycle		1 μ M
γ -aminobutyric acid (GABA)	Inhibitory action at receptors		1 mM
Dopamine	motor control, motivation, cognition, and reward		3 μ M
Glutamate *most prominent neurotransmitter	Neural communication, memory formation, learning, regulation		1 mM

Table 1: Neurotransmitter structures, functions, and limits of detection (Adapted from Sharma group).

Background

Raman Spectroscopy

Raman spectroscopy is an inelastic light scattering technique that provides information about the identity of a molecule based on its vibrational modes. For a vibration to be Raman active, the molecule must undergo a change in polarizability when it is excited to a virtual state with a monochromatic light source. When the molecule is excited, an induced dipole is created, according to the equation $\mu = \alpha E$, where α is the polarizability and E is the electric field. Each Raman active molecule has a characteristic spectrum, or a “molecular fingerprint”. Raman signal is highly specific, sensitive, non-destructive, and can be rapidly collected. On the other hand, its major disadvantage is that it is inherently weak. Only one in every 10^7 incident photons is Raman scattered (5), so certain techniques must be used to enhance the strength of Raman signal, one of which is by increasing the electric field surrounding the molecule.

Surface Enhanced Raman Spectroscopy

Surface enhanced Raman spectroscopy (SERS) yields amplified Raman signal by taking advantage of the electric field produced by the excitation of conduction electrons on the surface of noble metal nanoparticles (6). This phenomenon is known as the localized surface plasmon resonance (LSPR). When a molecule encounters this electric field, its Raman signal can be enhanced by up to a factor of 10^8 (7). SERS enhancement allows for the identification and quantification of very low concentrations of molecules.

Spatially Offset Raman Spectroscopy

Spatially offset Raman spectroscopy (SORS) enables the detection of Raman signal from the subsurface layers of diffusely scattering media. Photons enter the deeper layers of a sample, migrate laterally, and then exit the sample. To gather information from these subsurface layers, signal is collected at a point that is offset from the location of the incident light. Inverse SORS differs from conventional SORS in that it involves illuminating the sample in a circle and collecting the scattered light from a point in the center of the circle (8). The radius of the circle equals the degree of spatial offset. A larger offset permits incident photons to penetrate deeper in the sample, and Raman signal can be obtained from further beneath the surface of the sample. The combination of SERS and SORS gives surface enhanced spatially offset Raman spectroscopy, or SESORS.

Experimental Methods

Nanoparticle Synthesis and Characterization

On a regular basis, gold nanoparticles (AuNPs) were synthesized using a citrate reduction method based on the procedure reported by Frens (9). Due to its lack of toxicity, gold is an excellent material for *in vivo* experimentation. Silver nanoparticles (AgNPs) were occasionally synthesized as well using the protocol outlined by Lee and Meisel (10). After each synthesis, the batch of nanoparticles was characterized using ultraviolet-visible extinction spectroscopy to identify its localized surface plasmon (Figure 2), which is an indication of the shape and size distribution of the particles. The LSPR of the gold colloids tended to be around 540 nm, while that of the silver colloids was usually about 420 nm. To test their SERS activity,

100 μ M benzenethiol- a common Raman reporter molecule was adsorbed onto the surface of the colloids for SERS analysis. At such a low concentration, no normal Raman signal could be obtained from the benzenethiol, but if the nanoparticles were SERS active, they enhanced the Raman signal of the molecule to a measurable level (Figure 3). Additionally, the nanoparticles were imaged using scanning electron microscopy (SEM) to better understand their structure (Figure 4).

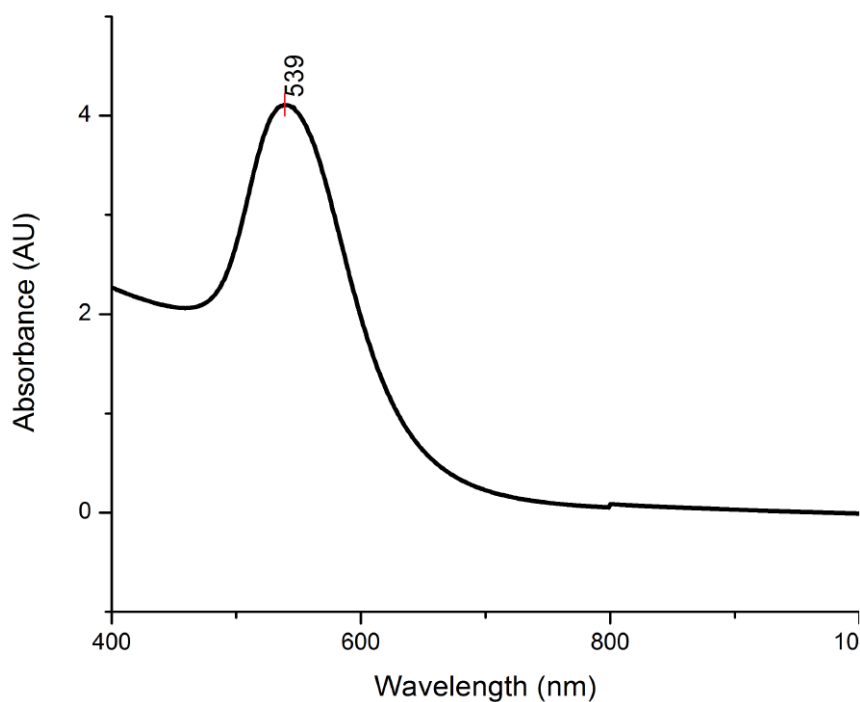


Figure 2: Localized surface plasmon resonance of AuNPs, with the maximum wavelength at 539 nm (Adapted from Sharma group).

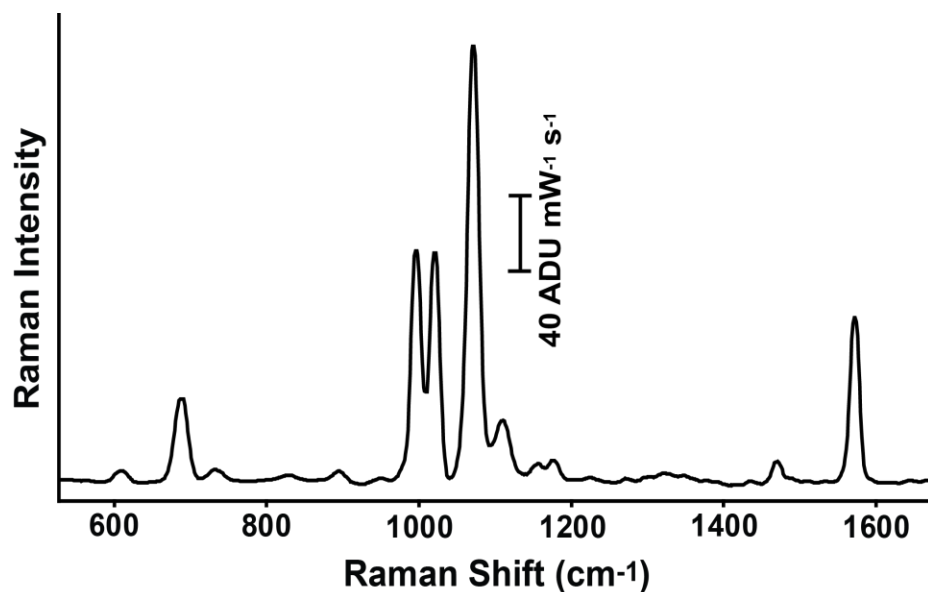


Figure 3: SERS spectrum of benzenethiol on AuNPs. $\lambda_{\text{ex}}=785$ nm, $P=5$ mW, $t=1$ s (Adapted from Sharma group).

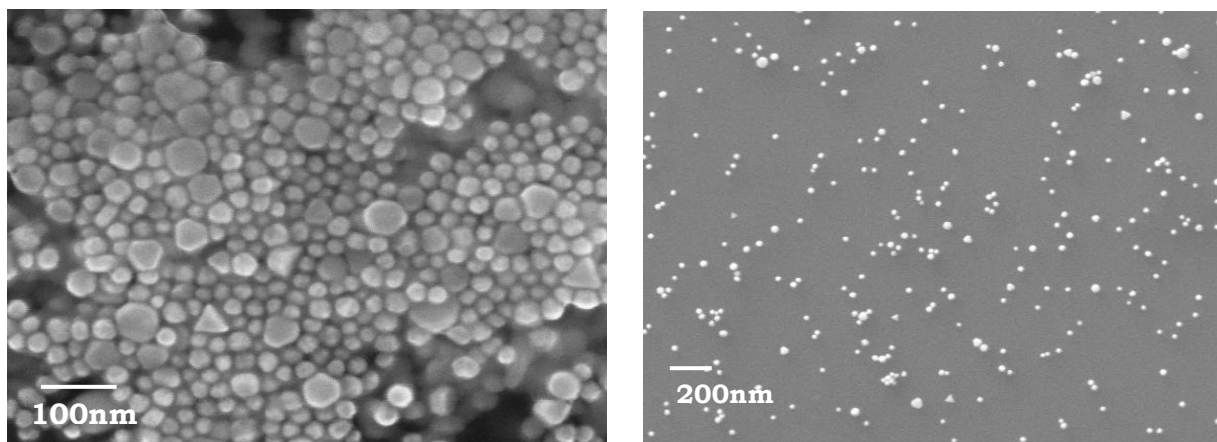


Figure 4: SEM images of AuNPs (Adapted from Sharma group).

Instrumentation

The Sharma lab is equipped with homebuilt macro- and micro- Raman optical systems, which allow the group to perform experiments using normal Raman, SERS, SORS, and SESORS. The macro system (Figure 5) is equipped with a 785 nm continuous wave laser, and signal is

collected in a 180° backscattering geometry. This system has the capability to achieve various spatial offsets using axicon lenses so that inverse SORS signal can be collected from the sample. The micro system (Figure 5) includes four laser sources with wavelengths of 488 nm, 532 nm, 633 nm, and 785 nm on the same beam path, and it incorporates a confocal microscope to focus the laser light onto the sample before it is collected by the spectrometer. Only the 785 nm laser is represented in the figure below.

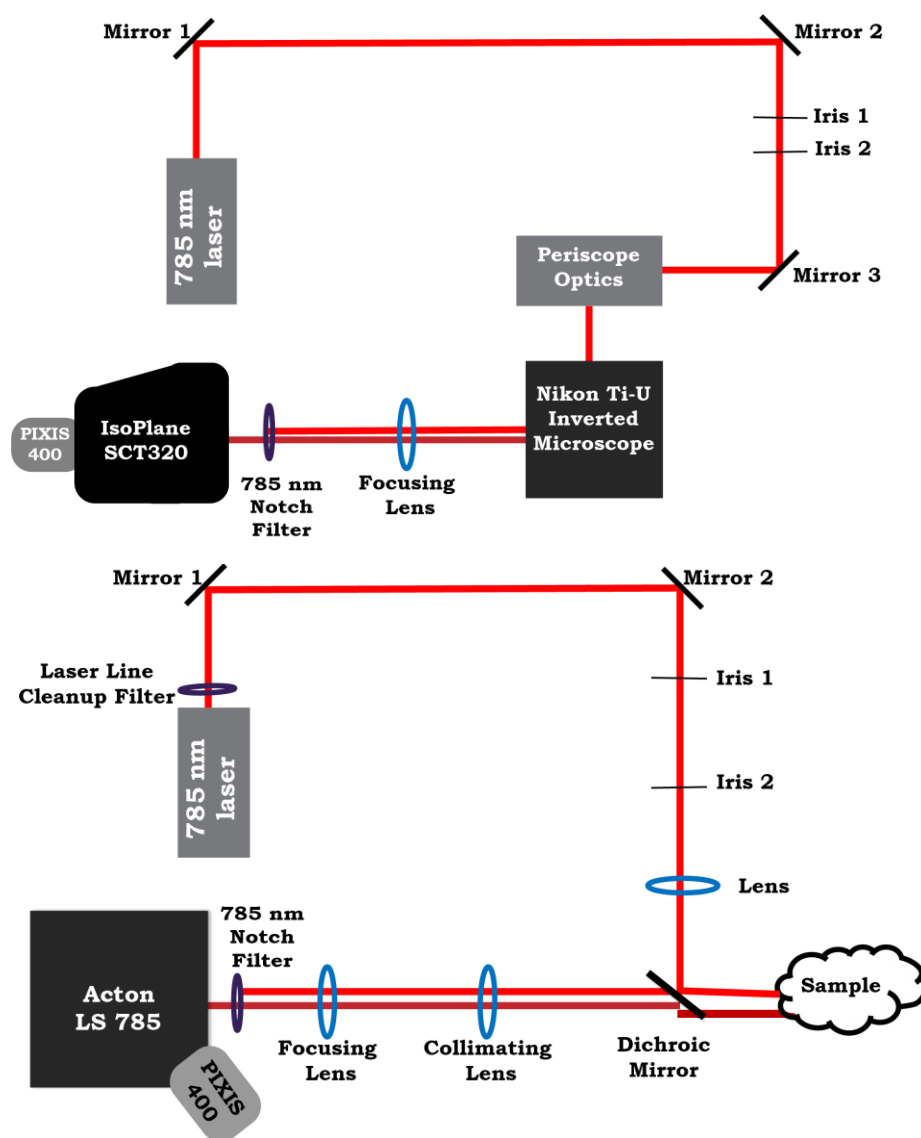


Figure 5: Sharma lab micro setup (*top*) and macro setup (*bottom*) (Adapted from Sharma group).

Normal Raman and SERS Measurements of Seven Neurotransmitters

Dopamine, serotonin, melatonin, glutamate, GABA, epinephrine, and norepinephrine were purchased in their solid forms, and their normal Raman spectra were acquired using excitation wavelengths of 532 nm (Figure 6), 633 nm (Figure 7), and 785 nm (Figure 8). The solid state data provided information about the major vibrations of each molecule. It also showed that although the positions of the vibrations were consistent in each spectrum, certain vibrations were more intense at different wavelengths. Since the intensity of Raman signal increases at a rate inversely proportional to the fourth power of the excitation wavelength ($\frac{1}{\lambda_{ex}^4}$), the strongest signal was observed at 532 nm. The spectra of GABA and glutamate displayed much weaker Raman signal than the other neurotransmitters because they have linear structures and lack rings containing conjugated pi bonds. These spectra were used as a basis for understanding the Raman bands that would be expected from the neurotransmitters when incorporated into solution.

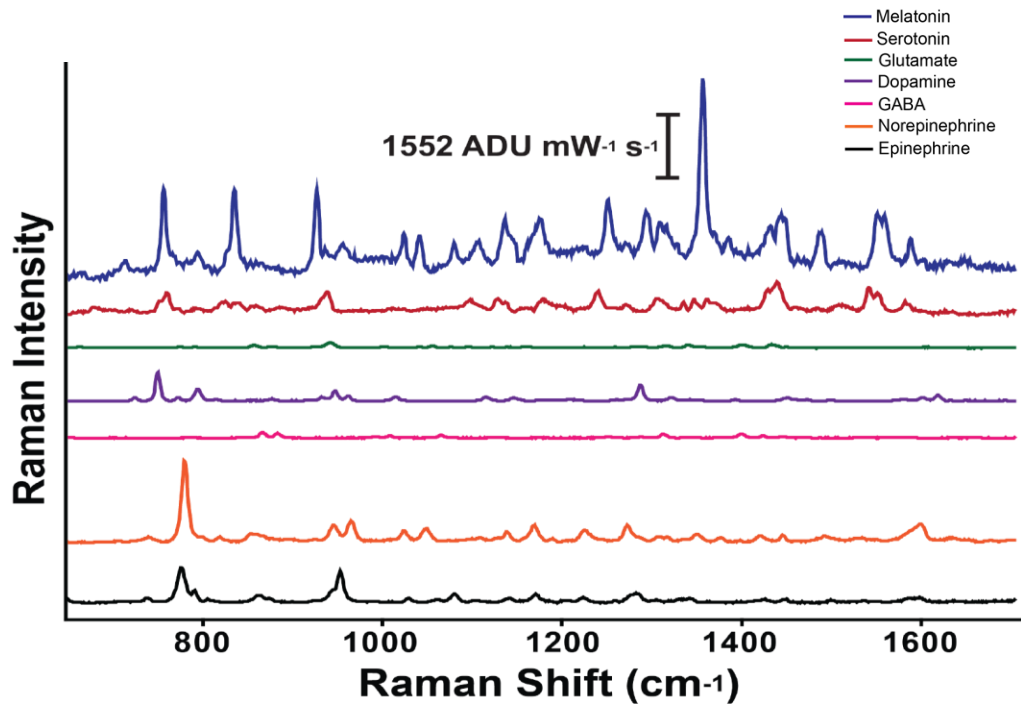


Figure 6: Spectra of solid neurotransmitters at $\lambda_{ex} = 532 \text{ nm}$, $P=10 \text{ mW}$, $t=30 \text{ s}$ (11).

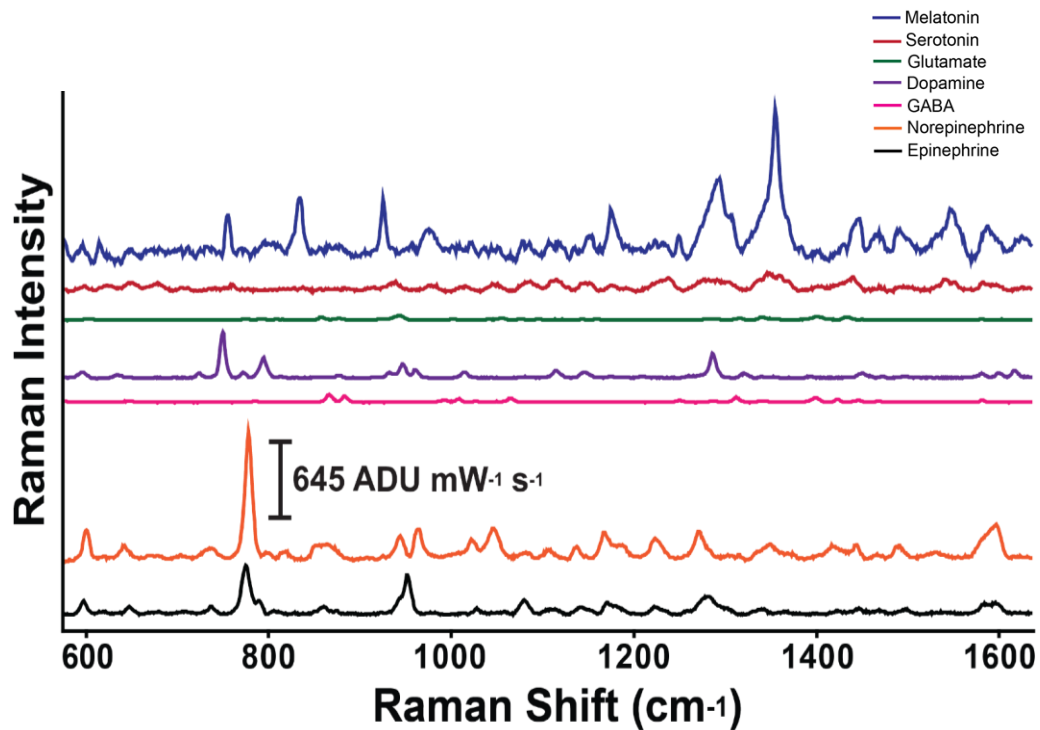


Figure 7: Spectra of solid neurotransmitters at $\lambda_{ex} = 633 \text{ nm}$, $P=10 \text{ mW}$, $t=30 \text{ s}$ (11).

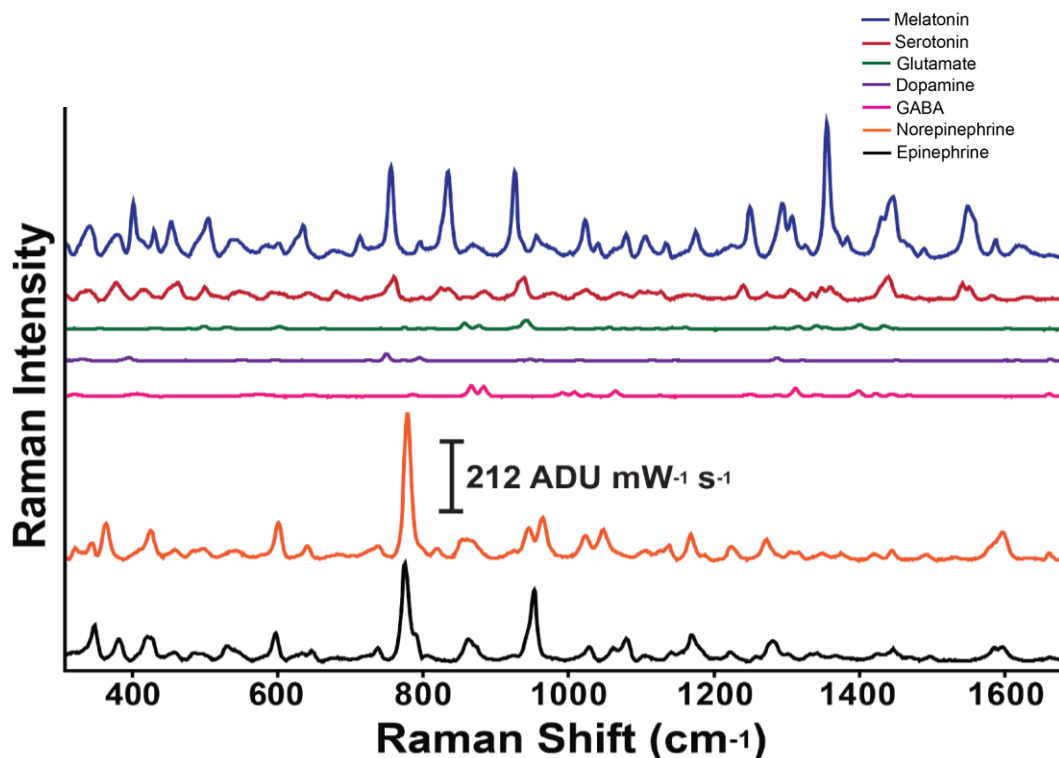


Figure 8: Spectra of solid neurotransmitters at $\lambda_{ex} = 785$ nm, $P=10$ mW, $t=30$ s (11).

Next, a series of SERS spectra were collected for the seven neurochemicals. In order to maximize the intensity of the SERS signal, the optimum experimental conditions were determined. For instance, the centrifuging speeds and durations for both the gold and silver particles were optimized to remove the maximum number of particles from the supernatant. The best centrifuging conditions were 4200 rpm for 15 minutes for gold and 5000 rpm for five minutes, a total of two times, for silver.

Additional optimization included adjusting the amount of time that the analyte was allowed to incubate with the nanoparticles before the SERS measurements were taken, along with changing the pH of the nanoparticle solution. The neurotransmitter solutions gave the strongest SERS signal after an hour of incubation and at a pH of 3. Decreasing the pH of the nanoparticles effectively protonated their negatively charged surfaces, which allowed the neurotransmitters to more favorably adsorb onto the colloids. The negatively charged citrate

molecules remaining on the nanoparticle surface create the repulsive forces that keep the nanoparticles dispersed in solution. At a pH below 3, the nanoparticles become unstable and quickly aggregate and fall out of solution.

Various concentrations (between 1 nM and 500 μ M) of the neurotransmitter of interest were adsorbed onto gold nanoparticles with a pH of 3, and SERS signal was obtained at 633 nm and 785 nm excitation wavelengths. The same concentrations of neurotransmitters were adsorbed onto silver nanoparticles as well, and the solutions were excited at wavelengths of 532 nm, 633 nm, and 785 nm to obtain SERS signal. Figure 9 shows the SERS spectra at 785 nm of four concentrations of melatonin and exemplifies the proportional relationship between analyte concentration and SERS intensity in this range.

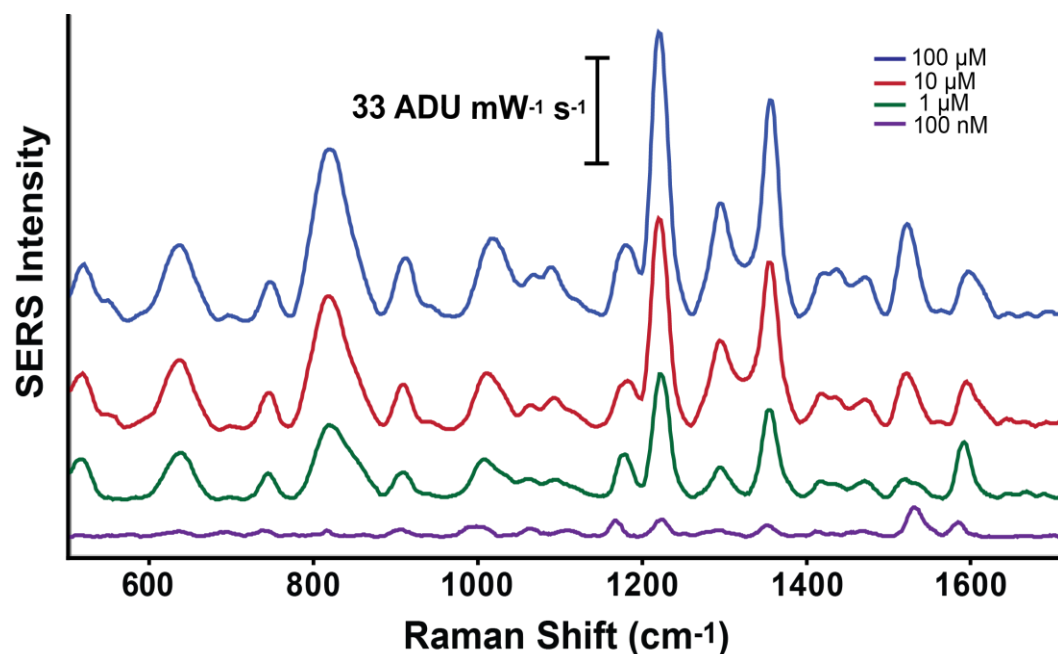


Figure 9: SERS of melatonin on AuNPs. λ_{ex} =785 nm, P=5 mW, t=60 s (12).

Shown below are the spectra at two excitation wavelengths for 50 μM concentrations of each of the seven neurochemicals on gold colloids (Figures 10 and 11). Each molecule exhibits a distinct Raman spectrum, which demonstrates the chemical specificity of the approach.

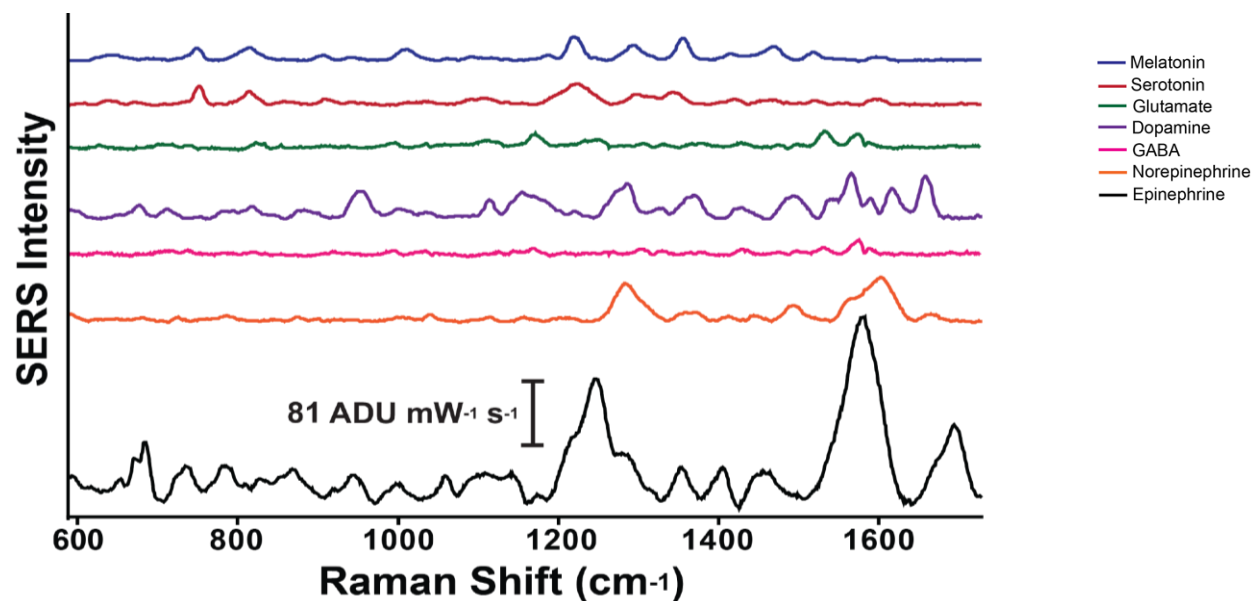


Figure 10: SERS of 50 μM neurotransmitter solutions on AuNPs. $\lambda_{\text{ex}}=633 \text{ nm}$, $P=3 \text{ mW}$, $t=60 \text{ s}$ (11).

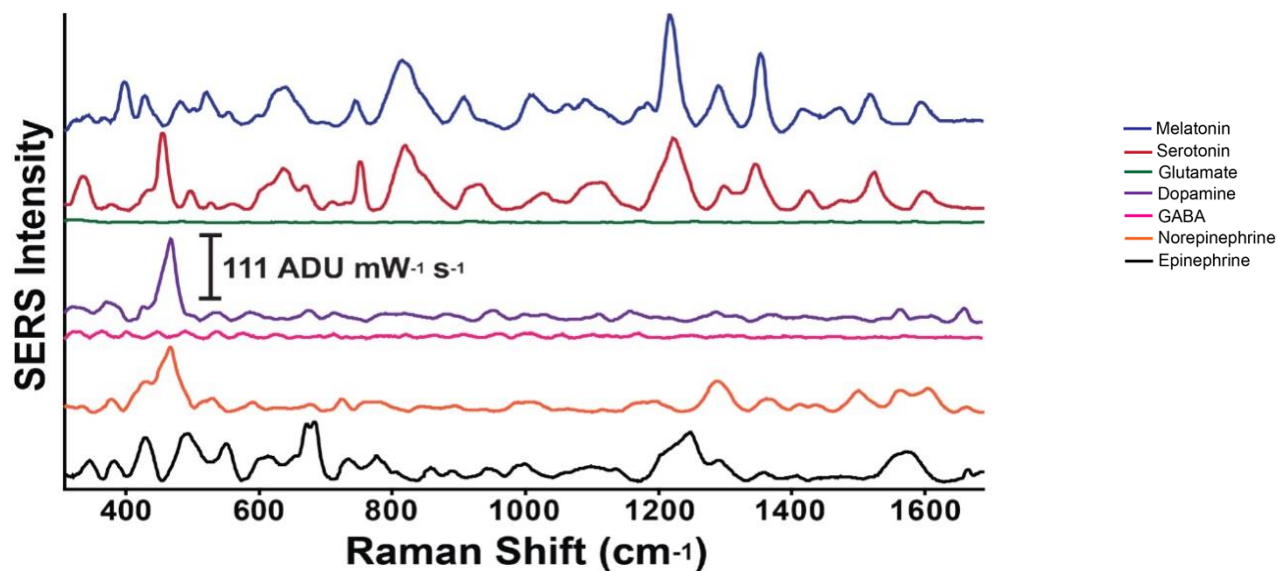


Figure 11: SERS of 50 μM neurotransmitter solutions on AuNPs. $\lambda_{\text{ex}}=785 \text{ nm}$, $P=5 \text{ mW}$, $t=60 \text{ s}$ (11).

Given that the amount of data collected during this study was quite large, spectral differences were difficult to discern by eye. Therefore, principle component analysis (PCA) was used to analyze the spectra, distinguish differences between them, and determine limits of detection. PCA is a multivariate mathematical technique that reduces the number of variables in a data set into principal components, which are represented by loadings that serve as reference spectra to which the software compares each sample. In PCA, the first principal component has the largest variance, which means it represents the source of the largest variability in the data. Each successive principle component represents the next highest source of variance. When the principle components are graphed, samples with similar scores cluster, meaning that they are highly similar. In this experiment, the limit of detection of a molecule was defined as the spectrum of lowest concentration that was discernably different from, or not clustered with, the other spectra. Two representative PCA graphs, along with the loadings for their principle components, are shown here (Figures 12 and 13), and the various clusters of neurotransmitters have been circled. The limits of detection of each neurotransmitter using each type of nanoparticle and each excitation wavelength are compiled into Figure 14.

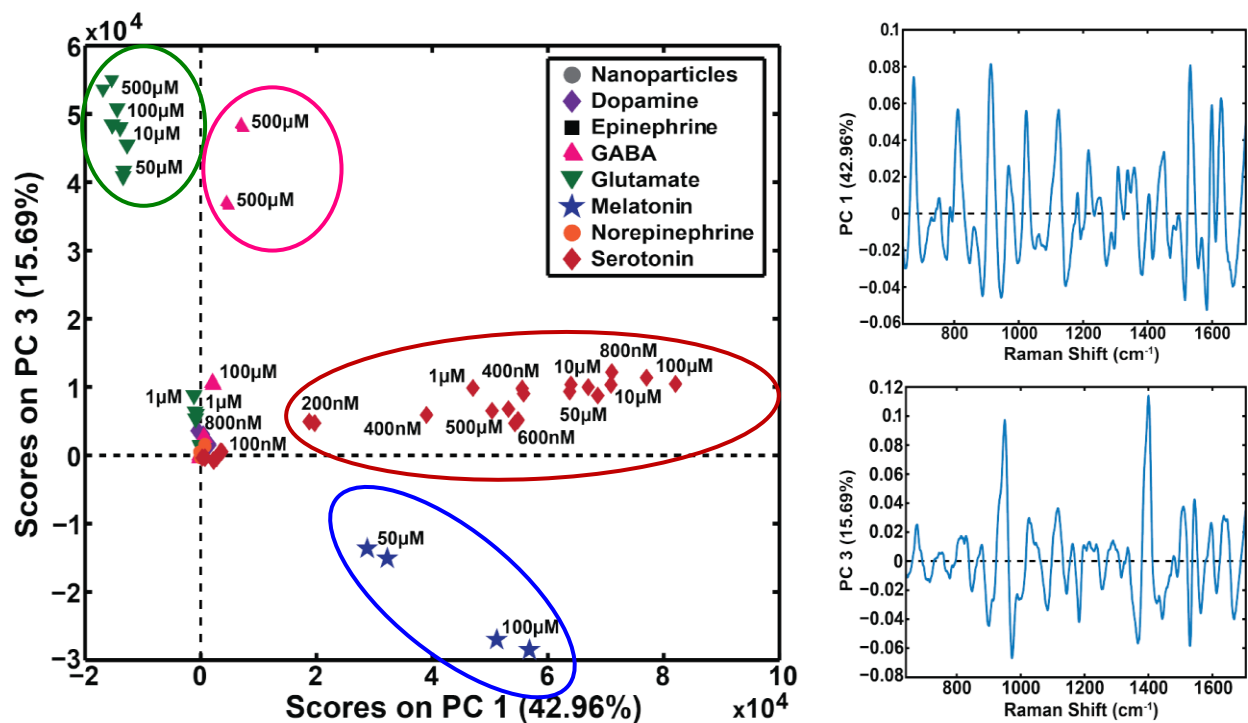


Figure 12: PCA of neurotransmitters (1 nM to 500 μ M) on AgNPs at 532 nm (11).

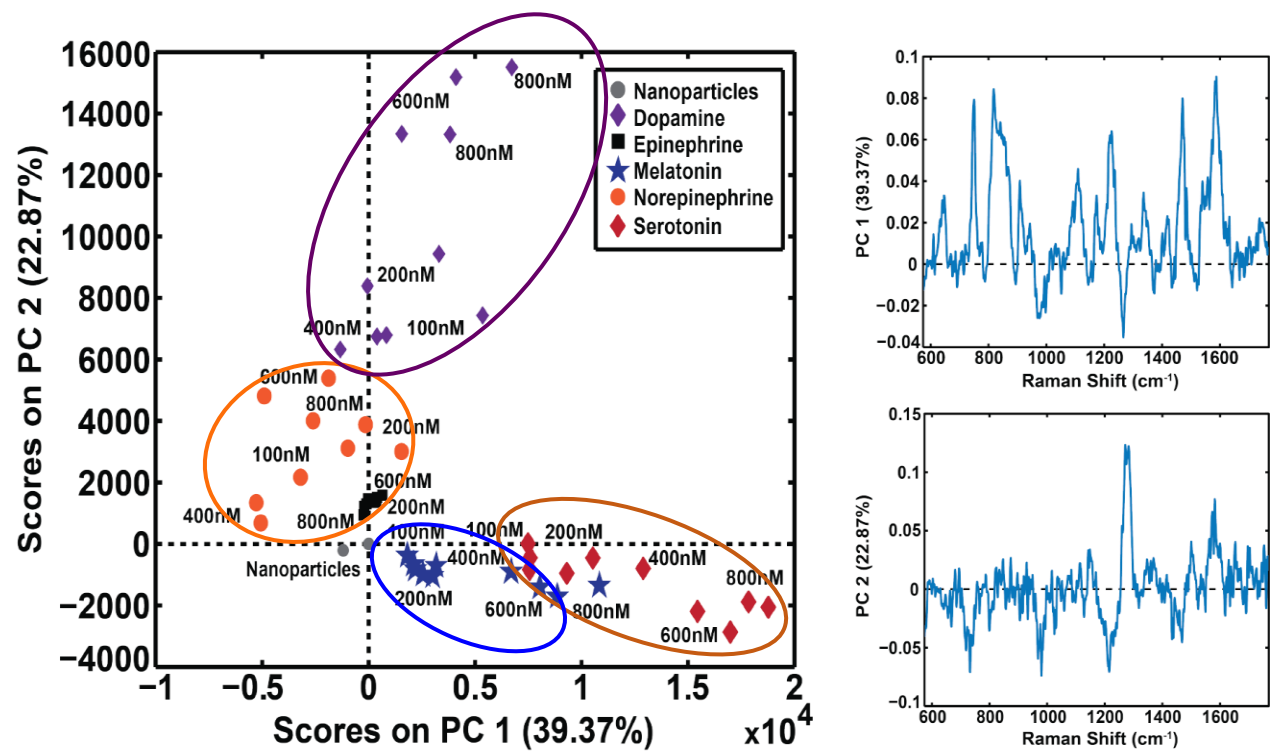


Figure 13: PCA of neurotransmitters (800 nM to 1 nM) on AuNPs at 633 nm (11).

Neurotransmitter	AgNPs @ 532 nm	AgNPs @ 633 nm	AgNPs @ 785 nm	AuNPs @ 633 nm	AuNPs @ 785 nm	Physiological Concentration
Melatonin	50 μ M	50 μ M	200 nM	100 nM	100 nM	100 nM
Serotonin	200 nM	100 nM	200 nM	100 nM	100 nM	1 μ M
Glutamate	50 μ M	600 nM	10 μ M	--	--	1 μ M
Dopamine	10 μ M	10 μ M	10 μ M	100 nM	100 nM	1 μ M
GABA	500 μ M	100 nM	200 nM	--	--	1 mM
Norepinephrine	10 μ M	10 μ M	10 μ M	100 nM	200 nM	3 μ M
Epinephrine	10 μ M	10 μ M	500 μ M	10 μ M	10 μ M	1 mM

Figure 14: Limits of detection of neurotransmitters using SERS (11).

SERS Measurements of Neurotransmitter Mixtures

Since neurochemicals rarely exist naturally in isolation, SERS experiments were also performed in more complex environments using mixtures of neurotransmitters. First, a mixture was prepared using 50 μ M concentrations of all seven neurotransmitters in water. A second aqueous mixture was made using concentrations of the seven neurotransmitters based on the quantities typically found in the human brain. The SERS spectra of these mixtures on gold nanoparticles at 785 nm are shown in Figure 15. Individual characteristic peaks of the majority of the neurotransmitters can be seen and are labeled in this figure. The same peaks appear in both mixtures, although they exhibit different intensities when the proportions of the neurotransmitters in the mixture are changed.

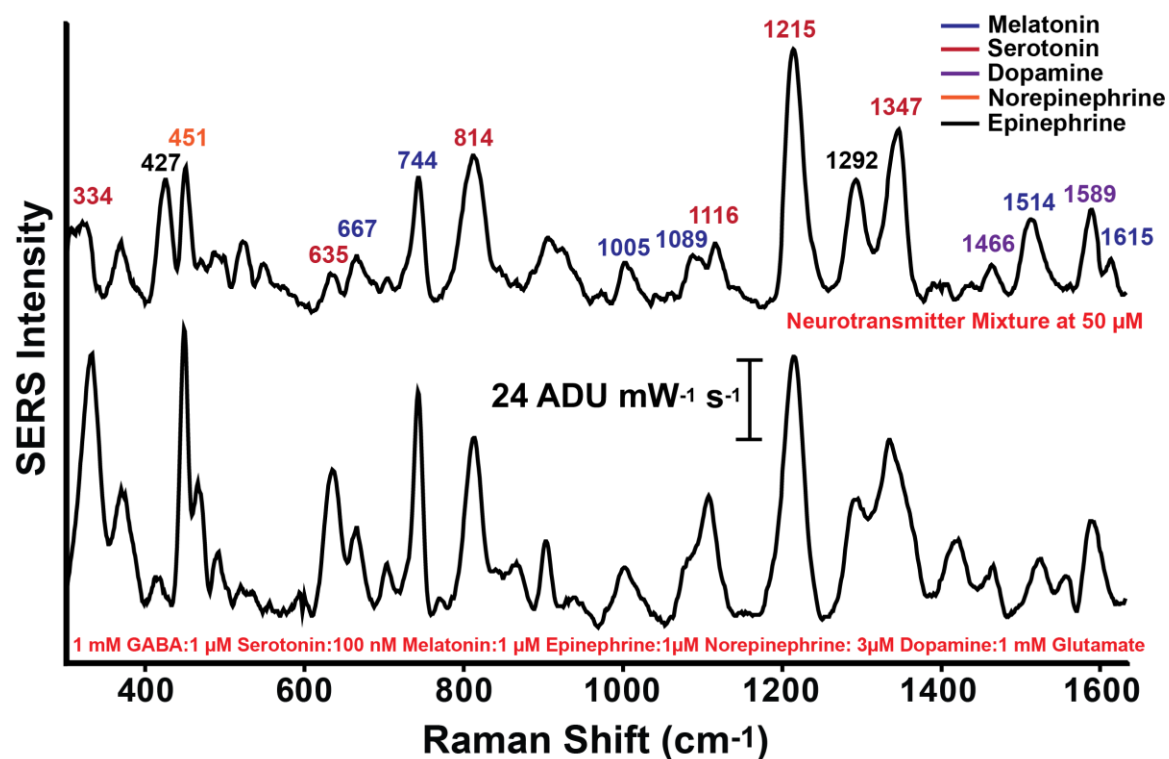


Figure 15: SERS of mixtures of neurotransmitters on AuNPs. $\lambda_{\text{ex}}=785 \text{ nm}$, $P=5 \text{ mW}$, $t=60 \text{ s}$ (Adapted from Sharma group).

SORS Depth Study

Next it was shown that the inverse SORS detection of Teflon- another common Raman reporter- could be achieved through a 1 mm piece of bone and up to 18 mm of agarose gel, which serves as a brain tissue mimic. Square pieces of agarose gel were prepared, each with a thickness of 2 mm. A flat portion of a rat skull was placed in front of a piece of Teflon, and a 785 nm laser was focused onto the bone. A Raman spectrum was collected in this position, termed the 0 mm offset, before an axicon was placed in the laser line to create a 4 mm annular beam on the bone, achieving a 2 mm offset. Slices of the brain tissue mimic were placed in 2 mm increments between the bone and the Teflon, and the SORS measurements were repeated at both offsets until no Teflon signal could be seen using the 2 mm offset. For each depth, a scaled

subtraction was performed. The 0 mm offset spectrum was subtracted from the 2 mm offset spectrum to remove the contribution of the bone and make the Teflon signal more easily visible. As can be seen in Figure 16, the Teflon peak at 734 cm^{-1} persisted until the agarose gel thickness reached 20 mm. This study demonstrated the viability of inverse SORS as a method for obtaining Raman signal from the human brain through the skull, which is only 3 mm thick at the substantia nigra.

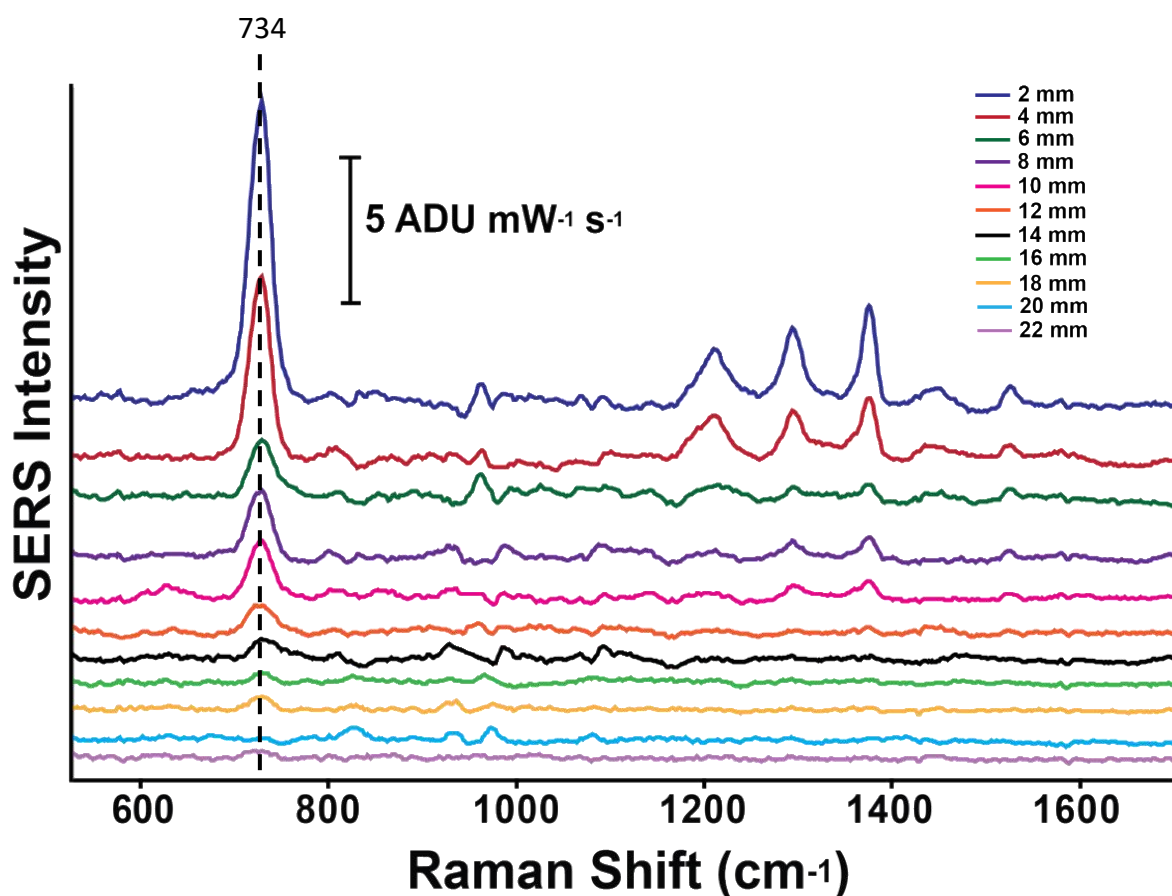


Figure 16: SORS of Teflon through bone and increasingly greater depths of agarose gel. $\lambda_{\text{ex}}=785\text{ nm}$, $P=90\text{ mW}$, $t=60\text{ s}$.

SESORS of Neurotransmitters in a Brain Tissue Mimic through the Skull

Because Raman signal is weak, it would be difficult to obtain neurochemical information using SORS alone; it was important to show that a spatial offset could be combined with surface enhancement to achieve the SESORS detection of low concentrations of neurotransmitters in a brain tissue mimic through bone. Pieces of agarose gel were incubated with 100 μM neurotransmitter solutions for one hour to mimic brain slices with neurochemicals present. Then, gold nanoparticle solutions with a pH of 3 were added to the gels and allowed to incubate for another hour. The samples were placed onto the sample holder of the macro system and the SESORS signal of each was acquired at 785 nm through the piece of rat skull mentioned earlier. Before collecting the final data, experimental parameters such as the time the analyte was allowed to incubate on the gel and the degree of spatial offset were optimized to achieve maximum SESORS signal. A 2 mm offset provided the strongest signal. The procedure was repeated with mixtures of neurotransmitters, such as melatonin mixed with dopamine, serotonin mixed with dopamine, and serotonin mixed with melatonin.

The data was processed by subtracting the 0 mm offset from the 2 mm offset to remove the majority of the bone signal at 960 cm^{-1} . For a few of the samples, figures were compiled showing the 0 mm offset spectrum, the 2 mm offset spectrum, the subtracted spectrum, and the spectrum of the gel alone (Figures 17 – 20). Characteristic peaks from the neurotransmitters were observed in all of the spectra, including the spectra of the mixtures. Overall, by using a 2 mm offset- versus a 0 mm offset- more signal was obtained through bone from the neurotransmitter or neurotransmitters of interest. The ratio of analyte signal to bone

signal increased as the offset increased. In other words, by employing a greater offset, more scattered light was being collected from the brain tissue mimic beneath the bone.

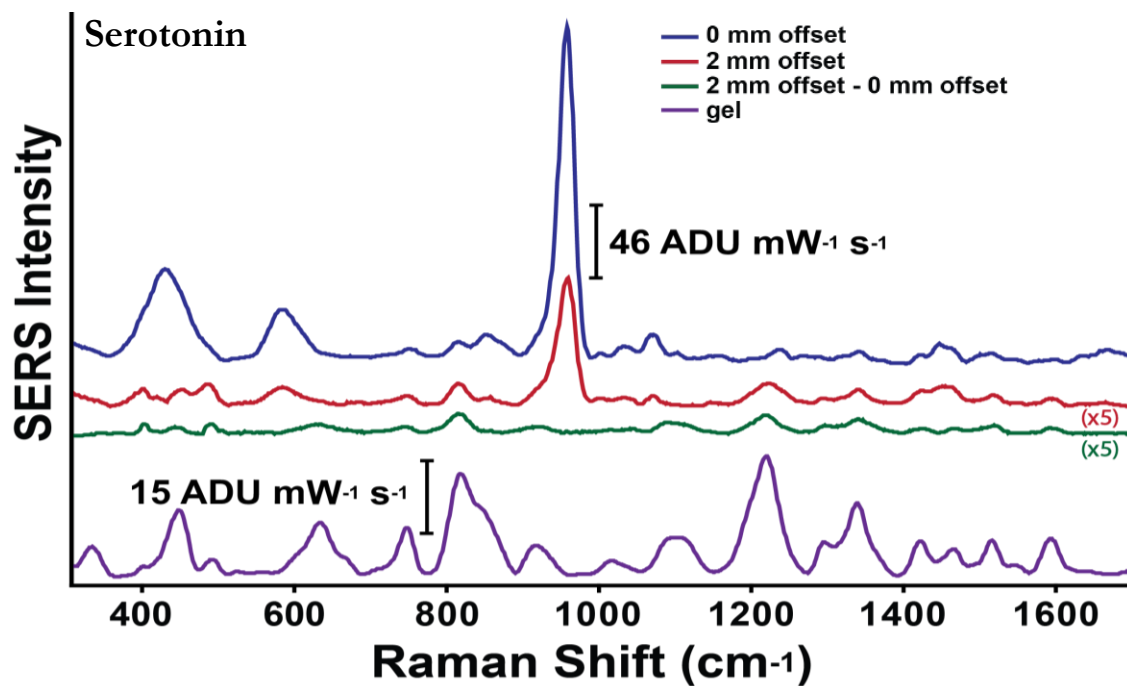


Figure 17: SESORS data for serotonin in agarose gel through bone. $\lambda_{\text{ex}}=785 \text{ nm}$, $P=90 \text{ mW}$, $t=120 \text{ s}$ (12).

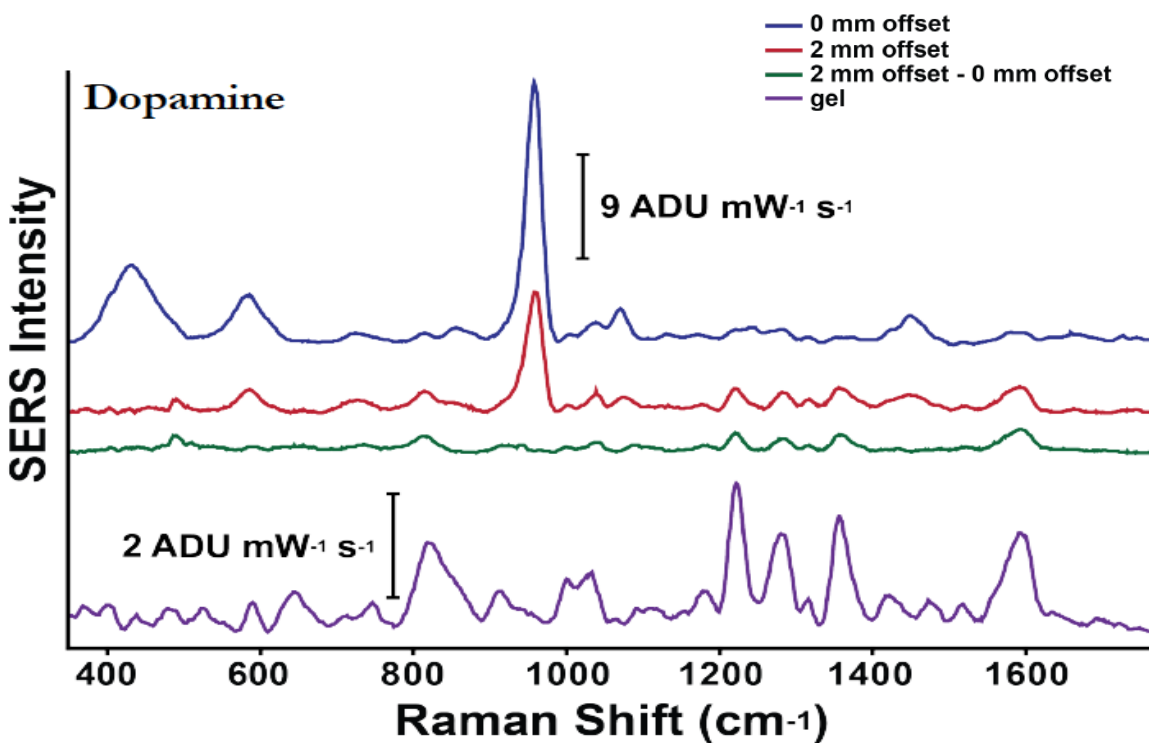


Figure 18: SESORS data for dopamine in agarose gel through bone. $\lambda_{\text{ex}}=785$ nm, $P=90$ mW, $t=120$ s (12).

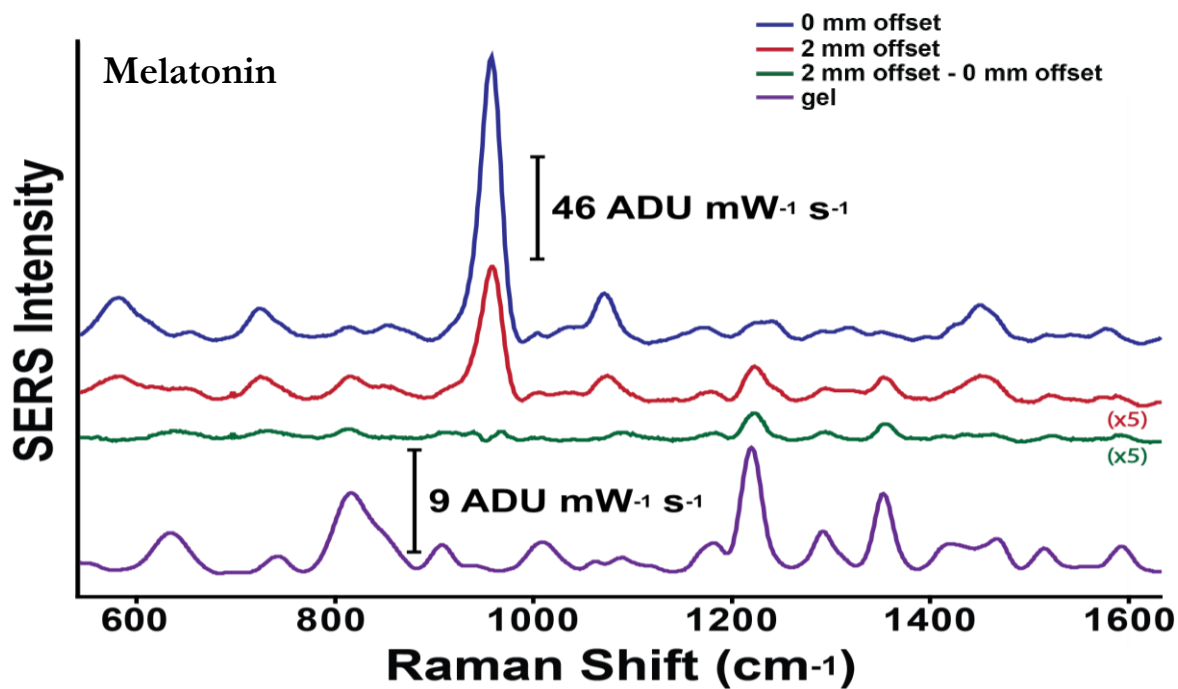


Figure 19: SESORS data for melatonin in agarose gel through bone. $\lambda_{\text{ex}}=785$ nm, $P=90$ mW, $t=120$ s (12).

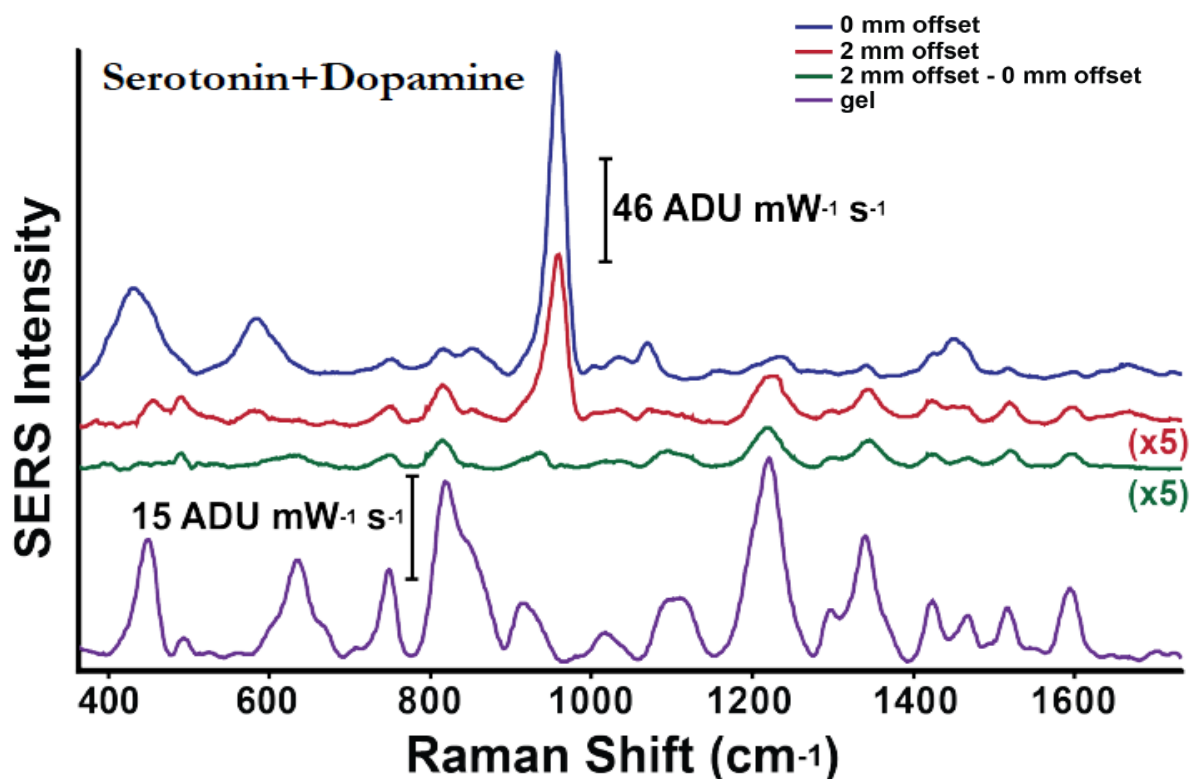


Figure 20: SESORS data for a serotonin and dopamine mixture in agarose gel through bone. $\lambda_{\text{ex}}=785$ nm, $P=90$ mW, $t=120$ s.

SESORS Detection of Serotonin in vivo (Mouse Model)

A sacrificed mouse was obtained from the Sparer group at the University of Tennessee. The head was shaved, and a SORS spectrum was acquired through the skull using a 785 nm laser at a 0 mm offset (Figure 21). Since bone is partially transparent at this wavelength and the mouse skull is very thin, some subsurface information was gathered even at a 0 mm offset. The observed peaks corresponded to bone and decomposing tissue in the brain. The skull was opened, and 100 μM serotonin and gold nanoparticles were injected into the brain. The bone was measured to be about 0.5 mm to 0.8 mm thick, suggesting that a 1 mm offset would allow chemical information to be retrieved from the brain. The skull was replaced, and another spectrum was acquired with no offset (Figure 21). Finally, a SESORS spectrum was collected at a

1 mm offset. The final two spectra exhibit peaks at 820 cm^{-1} , 1221 cm^{-1} , 1341 cm^{-1} , and 1522 cm^{-1} , which are characteristic of serotonin (Figure 21), meaning that this technique has the ability to detect neurotransmitters *in vivo* through the skull.

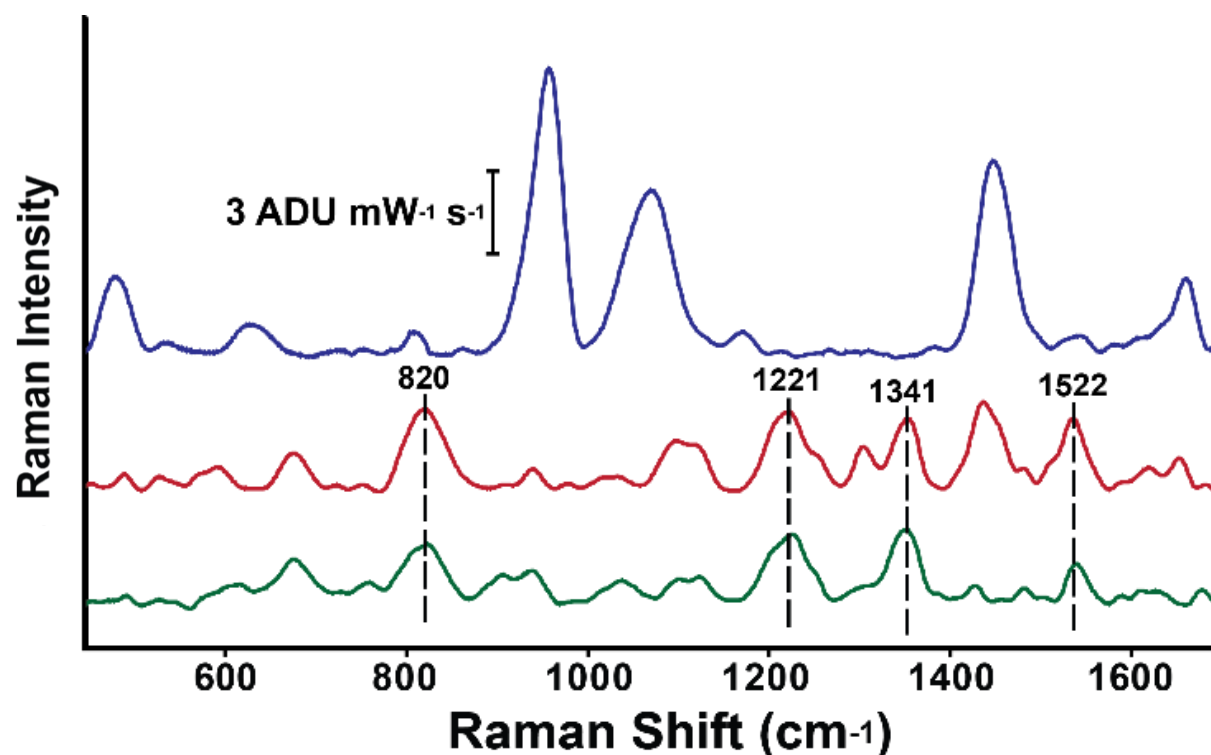


Figure 21: Initial SORS spectrum of the mouse brain through the skull (top, blue). SESORS spectrum (with initial SORS spectrum subtracted) of the mouse brain through the skull after a 100 μM serotonin injection at 0 mm offset (red, middle) and at 1 mm offset (green, bottom). $\lambda_{\text{ex}}=785 \text{ nm}$, $P=90 \text{ mW}$, $t=120 \text{ s}$ (13).

Selective Detection of Dopamine using Boronic Acid

Selectively targeting molecules of interest would allow for better SESORS detection of neurochemicals in the brain. In order to selectively capture and detect dopamine molecules in a brain tissue mimic, boronic acids were employed as capture ligands. A 100 μM solution of 4-mercaptophenylboronic acid (4-MPBA) was prepared in PBS. The sulfur groups of the acid molecules were bound to the surface of gold nanoparticles, leaving the diol groups available to

target the diol groups of dopamine. Various concentrations of dopamine were added to the boronic acid-conjugated gold nanoparticles (BA-AuNPs) and were allowed to incubate for one hour. Then, the solutions were added to pieces of agarose gel, and SERS spectra were collected 24 hours later. The SERS data collected after the full day incubation period is displayed below (Figure 22). The bands at 1006 cm^{-1} , 1258 cm^{-1} , 1344 cm^{-1} , and 1488 cm^{-1} correspond to dopamine, while the other peaks represent the contribution from the remaining boronic acid molecules and gold nanoparticles in the gel. These peaks correlate well with those observed by Yu et al. in their SERS data of dopamine on silver nanoparticle dimers (14). This data suggests that boronic acid-functionalized gold nanoparticles are a viable option for achieving the selective SERS detection of dopamine.

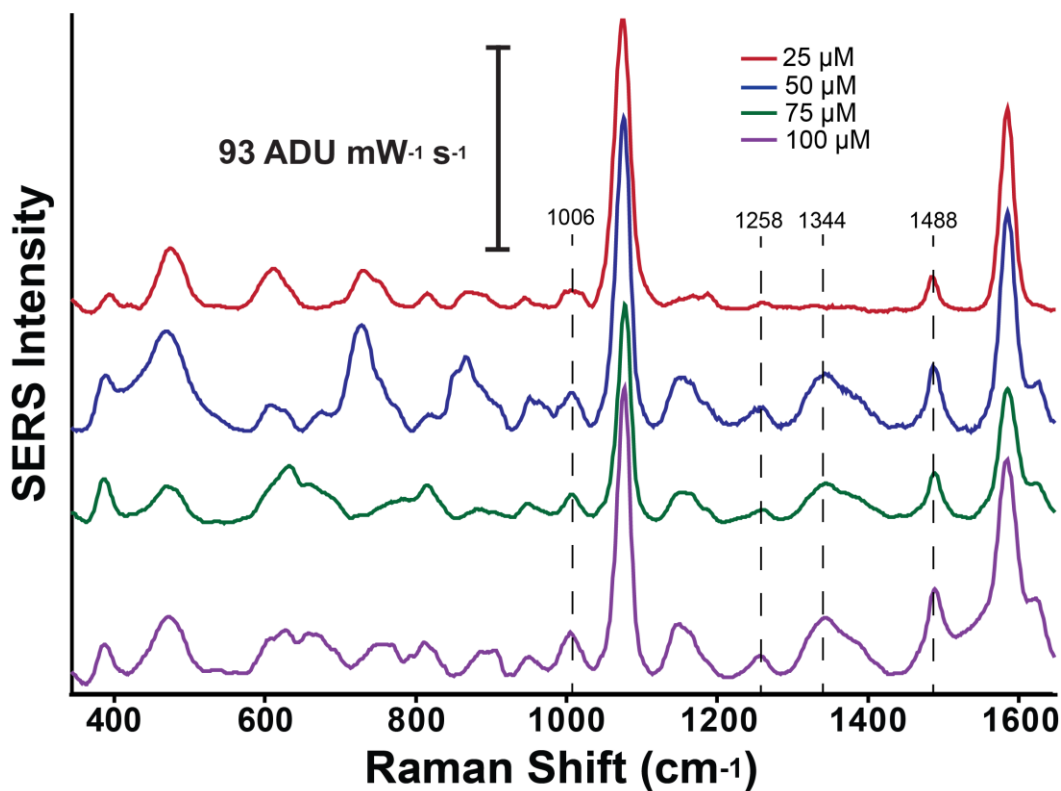


Figure 22: SERS detection of dopamine in agarose gel using BA-AuNPs.

Conclusions and Future Work

The series of experiments outlined in this work represent the groundwork for using SESORS for the *in vivo* diagnosis of neurological disease. First, gold and silver nanoparticles were successfully synthesized and were used to detect low concentration of various neurotransmitters at multiple excitation wavelengths using SERS. With the help of PCA, the limits of detection of our method were established for seven neurotransmitters under various experimental conditions. Individual neurotransmitters were detected within physiologically representative mixtures. Serotonin, dopamine, melatonin, epinephrine, and norepinephrine were identified at concentrations of 100 μ M in a brain tissue mimic through a rat skull using SESORS. Then, mixtures of serotonin, dopamine, and melatonin were detected using the same methodology. After 24 hours of incubation, dopamine was captured using boronic acid-conjugated nanoparticles, and signal was obtained using SERS.

There remains a significant amount of work to be done in the future to develop a SESORS technique to be used in the clinical setting. For instance, the brain contains numerous biomolecules, so steps should be taken to improve the selective detection of the neurotransmitters of interest. The selectivity of the BA-AuNPs described above should be tested on dopamine in the presence of other neurotransmitters and other biological molecules. The BA-AuNPs should be incorporated into SESORS experiments to detect dopamine signal through bone, and they should be used to sense dopamine in the brain of an animal model. Eventually, this technology is likely to be tailored for use in patient care, as SESORS shows great promise as a diagnostic tool. In conclusion, this work represents the first steps in developing a minimally invasive method for the early detection of neurological disease.

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