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Optimization of Aggregating agents and SERS Substrates for SERS detection of Cotinine and trans 3'-hydroxycotinine

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Optimization of Aggregating Agents and SERS Substrates
for SERS Detection of Cotinine and Trans 3’-hydroxycotinine

by

Sungyub Han

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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Dedication

I would like to dedicate this work to my parents. They have always supported and trusted me with endless love. Also, I want to thank my wife to always be with me whenever I have had trouble in our life.
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I would like to express my great appreciate to my advisor, Dr Li, for valuable guidance to complete this research during Ph. D course. She has always encouraged and motivated me to continue many researches whenever my studies had faced trouble. In 2009, she accepted me as one of her group members and allowed me to learn Raman spectroscopy for around half of a year. During the first year since I was involved in her group, although my experimental data had many problems, she had provided me with valuable advice. Her support drives me to finish the Ph. D program now.

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Abstract

This dissertation mainly focuses on applications of Surface Enhance Raman Scattering (SERS) to detect tobacco-related biomarkers with optimized experimental conditions (pH and aggregating agents) and SERS substrates (silica core and silver shell nanoparticles). Cotinine (COT) and trans 3-hydroxycotinine (3HC), metabolized from nicotine as one of main chemicals of tobacco, have been used as tobacco biomarkers because their half-life are longer than that of nicotine, which enable to monitor the tobacco exposure. The effects of aggregating agents and pH on SERS detection of COT and 3HC were investigated. Aggregating agents play an important role in SERS detection of target molecules since the strong SERS enhancements are observed from junctions of nanoparticles which can be induced by aggregating agents, and so called “hot spot”. That is, the more hot spots are created among the nanoparticles by aggregating agents, the higher the SERS enhancement is. Five cationic (K⁺, Na⁺, Mg²⁺, Li⁺, Ca²⁺) and three anionic (Cl⁻, Br⁻, I⁻) aggregating agents were tested. Interestingly but not surprisingly, optimal concentrations of 11 kinds of aggregating agents for COT and 3HC detections vary dramatically within two orders of magnitude. In addition, the effect of pH conditions on SERS intensity of COT and 3HC was investigated since the protonated or deprotonated molecules induced by various ranges of pH values produces change in SERS intensity of the molecule. The highest
SERS enhancement is obtained using 1.5 mM MgCl$_2$ for COT at pH 7 and 50 mM NaBr for 3HC at pH 3. Both cations and anions strongly influence the SERS enhancement.

SERS enhancement depends also significantly on the type of metallic substrates. This indicates the choice of metallic substrate is critically important to achieve strong SERS enhancement. While Ag is the most commonly used materials for SERS substrates and has been demonstrated to exhibit high enhancement. It has the disadvantage of limited selection of excited wavelengths, which prevents to apply Ag SERS substrates to biological field. Dielectric core and metallic shell structure has been theoretically studied and it has been proposed that silica core and silver shell (SiO$_2$@Ag) nanoparticles produces higher plasmon resonance than that of silver nanoparticles and their surface plasmon are tunable by controlling shell thickness. Here, SiO$_2$@Ag nanoparticles were successfully fabricated and their activity as substrates for surface-enhanced Raman scattering (SERS) were examined. Both the core and the shell thickness exhibit strong effect on the SERS activity. Using Rhodamin 6 G (R6G) as a probe molecule, it was observed that SERS intensities of R6G were susceptible to change in Ag shell thickness and the size of core-shell nanoparticles. The 76 nm SiO$_2$@ 23 nm Ag shell nanoparticles shows highest SERS intensity of R6G. Moreover, 76nm SiO$_2$@ 23 nm Ag nanoparticles have higher SERS enhancements of R6G, 4-aminothiophenol (4-ATP), and cotinine (COT) than that of both silver nanoparticles and SiO$_2$@Ag nanoparticles of previous studies. Also, the tuneability of surface plasmon of core-shell structure is flexible by changing in the size of either core or shell.

In addition, three Raman spectroscopy application in material science fields were studied: MP-11 encapsulated inside of Tb-mesoMOFs, poly(methyl methacrylate)
composites of copper-4,4’-trimethylene dipyridine, and surfactant-free TiO₂ surface hydroxyl groups. For the first study, the interaction between the ligands of Tb-meso MOFs and MP-11 was examined. Individual Raman bands of MP-11 and the ligands of Tb-mesoMOFs were distinguished and some of bands were shifted from the complex of MP-11@Tb-mesoMOFs. It is turned out that the interactions is involved through π⋯π interactions between the heme and the conjugated triazine and benzene rings of TATB ligand. Next, Raman was used to study the interaction between poly methyl methacrylate (PMMAP) composites and copper-4,4’-trimethylene dipyridine (CU-TMDP). Copper contained in polymer materials has shown improvement performance (thermal and mechanical stability). The Raman results reveal a red-shift of vibrational peaks associated with pyridine ring of CU-TMDP when CU-TMDP is dispersed into PMMA. This interaction, a dipole-dipole interaction or London dispersion force, may produce the stability improvement of metal-containing polymer. The last application is about the effect of pH levels on the phase of TiO₂ crystalline. TiO₂ crystal has attractive advantage of self-cleaning property. The efficiency of self-cleaning of TiO₂ is dependent on the phases (anatase, rutile, and brookite) of TiO₂. Raman study revealed that the formation of the anatase phase of TiO₂ is interrupted as the pH level increases.
CHAPTER ONE: INTRODUCTION

1.1 History of Raman and Surface-enhanced Raman Scattering (SERS)

Raman scattering was discovered by Chandrasekhara Venkata Raman and K. S. Krishnan in 1928. Raman was born in Trichinopoly, India on 7th November 1888. The Raman scattering is the inelastic scattering of the light that produces a difference in photon energy between the incident radiation and the scattered one from atoms or molecules. Although, in 1923, the light scattering was firstly proposed by Austrian physicist A. Smekal who predicted the possibility of the phenomenon related to Raman effect, Raman was the first researcher to experimentally prove the phenomenon. In 1921, when Raman was travelling to Europe, he watched a beautiful blue light of glacier reflected from the surface of the Mediterranean Sea. This beautiful event of nature motivated him to unveil with scientific approach. After the traveling, he came back to India and started to study this phenomenon. He filtered sunlight into a monochromatic light then irradiate the sample to obtain scattered light by applying another filter to block the incident monochromatic light. This work was published in 1928, titled A New type of Secondary Radiation and won him a Nobel Prize in Physics in 1930. The fact that it took only two years after the publication of his work to win the prize shows the significance of his work. When light is interacting with molecules, the light may be absorbed, scattered or penetrate through the molecule without any interactions. In absorption the photon
energy of light matches the excitation energy of a molecule from a ground state to an excited state. Scattering, however, does not need to satisfy such same energy criteria. There are two types of scatterings: elastic and inelastic scatterings. Elastic scattering is called Rayleigh scattering, where the energy of the scattered light is the same as that of the initial light. Rayleigh scattering is a more predominant event than inelastic scatterings. Inelastic scattering is called Raman scattering. When a molecule absorbs the energy of a photon, it is excited from the ground state to a virtual state. Then, the molecule reemits the energy in the form of a photon and relaxes back to the ground vibrational state which has a higher energy than the initial ground state. In this case, the scattered radiation will have a lower energy (frequency) than the energy of incident radiation, which is called Stock scattering. Likely, molecules already in an excited state can absorb the incident radiation. When the molecules are excited and returned to the ground state, the scattered light has higher energy than the incident one, which is called anti-stock scattering as shown in Figure 1.1. In Raman scattering, stock scattering is more preferable because most of molecules at room temperature are present in the lowest vibrational state. Thus, the stock scattering is more frequently used in Raman spectrum due to its higher intensity. Anti-stock scattering, on the other hand, can be more useful if a sample has fluorescence interference.

Not all molecules are Raman active. Raman signals can only be observed from the molecules whose polarizability changes during the vibration. In the case of Raman scattering, when the electric field of the radiation is interacting with molecules, momentary distortion of the electron cloud in the bonds occurs, followed by the reemission of the radiation from the molecules as it returns to a ground state. Ordinarily,
Figure 0-1 Sketch of Raman scattering: Rayleigh, Stokes, and Anti-Stokes

symmetric vibration modes of molecules can produce the largest change in their polarizability and generate strong Raman scattering. For example, carbon dioxide (CO$_2$) molecules have three vibrational modes: symmetrical stretch, deformation, and asymmetric stretch. In a Raman spectrum, the strongest Raman peak is assigned to its symmetric stretch mode. In contrast to Raman scattering, Infrared absorption can display the most intense peak from asymmetric stretch mode of CO$_2$ due to the larger change in the dipole moment of CO$_2$.

Raman spectroscopy has several advantages: simple or no sample preparation, rapid analysis, and little interference by water, allowing the Raman technique to be applied in the biologic field.$^{3-7}$ Solid samples can be directly analyzed on the stage without any sample treatment, but keep it in mind that sample degradation is possible by the use of laser. This problem can be resolved by employing a higher wavelength of excitation. Likewise, the use of spinning disc or water as solvent can also help reduce the sample decomposition by distributing the localized energy of the laser. Although
these minor problems can occur during Raman analysis, there are many attractive benefits that prove the technique to be beneficial for researchers in the study of molecular structures. One of the major benefits of Raman spectroscopy is small sample requirements and nondestructive analysis. In the case of ancient artworks, gathering a large sample to test would cause damage and loss in the value. Raman analysis only needs micrograms of a sample to run a spectrum. Even when one cannot collect micrograms of a sample the confocal technique can be implemented. The confocal technique is collecting a sample from layers under artwork, which can then be used to take Raman spectra of the sample without damaging the art work. This technique enables the Raman technique to distinguish original work from restoration and forgery. For the study of biological molecules, the Raman spectroscopy can be used in situ in aqueous systems. Biomolecules having thiol, cyanide, disulfate, alkene and aromatic rings provide strong characteristic peaks in a Raman spectrum. Other groups such as carbonates and phosphates are also detectable in Raman bands. However, carbonyl, amine, and amides show relatively weak Raman intensities. Fortunately, this can be improved by the introduction of a metallic colloid to the biomolecule sample, which is called Surface-enhanced Raman spectroscopy (SERS).

SERS is a technique that uses special substrates from a coinage metal (Ag, Au, Cu) to improve the Raman scattering. When target molecules are adsorbed to metal surfaces under electromagnetic waves of incident light, the target molecules are influenced by a strong surface plasmon generated from the metal surface. The surface plasmon is composed of conductive oscillating metal electrons which enhances both the incident light and the Raman scattering of the target molecules and results in high
Raman intensities. This phenomenon was first observed by Fleischmann in 1974.\textsuperscript{10} He used a rough silver electrode in aqueous solution as the SERS substrate to generate strong Raman signal of pyridine when it adsorbed on the electrode. The strong intensity of pyridine in his experimental conditions was achieved by the roughened surface of the electrode because it allows more molecules to be held on the surface and provides oscillation of electrons perpendicular to the surface resulting in more scattering. This discovery has inspired many researchers to study and develop the SERS technique.

1.2 Theories of SERS

Since the discovery of SERS, many explanations of the strong enhancement mechanism have been proposed. Among those, two mechanisms of SERS are generally accepted so far: Electromagnetic enhancement (EM)\textsuperscript{11-13} and Chemical enhancement (CM)\textsuperscript{14,15}. Electromagnetic enhancement mechanism theory is related to the surface plasmon on the metal surface. When the incident laser irradiates on the metal surface, it excites the surface plasmon on the metal. These excited surface plasmon can generate a strong electromagnetic field on the metal surface that magnifies both the incident laser and the Raman scattering. This EM is a predominant process for the SERS enhancement compared to CM and the enhancement can be normally achieved up to $10^6$. The chemical mechanism involves strong interaction such as charge transfer between the analytes and metal surface through their bonds. There are two types of charge transfer, one electron is transferred from the highest occupied molecular orbital (HOMO) to the Fermi Level of the metal and the other from the Fermi level of the metal to lowest unoccupied molecular orbital (LUMO). These charge transfers cause a change in the polarizability of the molecule. The SERS enhancement
by CM is up to a factor of $10^2$. A simple explanation of electromagnetic SERS is based on a single metal sphere smaller than the wavelength of exciting light. Although strong SERS enhancement has been experimentally observed from the aggregation of metallic nanoparticles (NPs), it is useful to use a single sphere model to understand the fundamental background of SERS enhancement. When the exciting laser is resonant with the surface plasmon of NPs, the NP can radiate light. In this case, molecules absorbed on the NP will be excited by both incident light and the surface plasmon on the surface of a NP. This magnitude of electric field that excites the molecule, $E_s$, is expressed by the equation (1).\(^{16}\)

$$E_s = gE_0 \quad (1)$$

Where $g$ is the field enhancement averaged over the surface of the particle and $E_0$ is the magnitude of the incident field. Since Raman scattered light produced by molecule (E$_R$) can be proportional to $E_s$, it is expressed again as followed in equation (2).

$$E_R \propto \alpha_R E_s \quad (2)$$

Where $\alpha_R$ is a Raman tensor. Equations (1) and (2) indicate that Raman scattering of molecules absorbed on the metal surface is dependent on the electromagnetic waves of the incident laser light and electric field on the metal surface. Moskovits defined “SERS enhancement”, $G$, as the ratio of the Raman-scattered intensity in the metal particle to that in the absence of the metal particle.\(^{16}\)

$$G = |\alpha_R/\alpha_{Ro}||g g'|^2 \quad (3)$$

Where $\alpha_{Ro}$ is Raman polarizability of the isolated molecule and $g'$ is a factor enhancing light at the Raman shifted wavelength that the metal particle scatters. As equation (3) shows, the major contribution to SERS is more likely from scattering by the metal.
sphere than by the molecule, which means the magnitude of surface plasmon on the metal surface plays a pivotal role in SERS intensity.

Since the SERS enhancement is achieved at the metal surface, the distance between the analyte and metal surface is one of the important factors for good enhancement. As the distance between them increases, SERS enhancement will dramatically decrease. The strong SERS enhancement is expected within 1 nm. The other factors affecting SERS enhancement are the radius of the sphere and dielectric constant of both medium around the metal sphere and metal itself. The relationship between the total electric field at distance \( r \) from metal surface (\( E_r \)) and these factors is described by\(^{17}\)

\[
E_r = E_0 \cos \theta + g \left( \frac{a^3}{r^3} \right) E_0 \cos \theta \quad (4)
\]

Where \( a \) is the radius of the metal sphere, \( \theta \) is the angle relative to the direction of the electric field, and \( g \) is a dielectric constant. Even though this equation shows \( E_r \) is proportional to the radius of the sphere, the radius is not allowed to go to infinity. As the size of NP increases, EM enhancement can increase, but as the particle size increases past the point where the strongest EM enhancement is produced, the metal sphere absorbs less light and scatters more through inelastic scattering.\(^{18}\) Fundamentally, the condition for radius of the metal sphere must be confined smaller than the wavelength of the exciting laser light. Since the surface plasmon is produced at the interface of the metal surface and dielectric environment (water, air, organic solvents etc), as shown in the above equation, the electric field created by the surface plasmon is sensitive to the dielectric constants, which can be further classified and expressed by equation (5)

\[
g = \frac{\varepsilon_1(V_L) - \varepsilon_0}{\varepsilon_1(V_L) + 2\varepsilon_0} \quad (5)
\]
Where $\varepsilon_0$ and $\varepsilon_1$ are the dielectric constants of the medium surrounding the metal sphere and of the metal sphere, respectively, $V_L$ is the frequency of the incident radiation. In order to obtain higher electric field at the metal surface, the denominator should be small. Since $\varepsilon_0$ is usually 1 in vacuum and approximately 1 in air, the $g$ becomes the maximum value when $\varepsilon_1$ is -2. However $\varepsilon_1$ will have different values where the environment is different around the metal sphere.

Although the two mechanisms of SERS enhancement allow us to theoretically understand the background and concepts about SERS, the SERS enhancements can also be experimentally demonstrated to better understand how much SERS enhancement can be practically observed in the experimental setup. In general, there are three points of view to consider when looking at the SERS enhancement: the single molecule, the SERS substrate, and the analytical chemistry. A single molecule’s enhancement depends on the Raman tensor of the probe and the orientation of metallic SERS substrate for the local field, which is dependent upon the orientation and position of probe on the metal surface. Thus, this is suitable for the theoretical estimation of SERS enhancement. The equation about single molecule enhancement factor (SMEF) is defined by equation (6).

$$SMEF = \frac{I_{SERS}^{SM}}{\langle I_{RM}^{SM} \rangle} \quad (6)$$

$I_{SERS}^{SM}$ is SERS intensity of single molecule under the consideration, and $\langle I_{RM}^{SM} \rangle$ is the average Raman intensity per molecule.
The SERS substrate enhancement factor (SSEF) has been commonly used in many studies because the average SERS enhancement measured can be compared to that used by different SERS substrates, which is expressed by equation (7)\textsuperscript{20-23}

$$SSEF = \frac{I_{SERS}/N_{Surf}}{I_{RS}/N_{Vol}}$$  \hspace{1cm} (7)

Where $I_{SERS}$ and $I_{RS}$ are the intensities of SERS and normal Raman of the probe, respectively. $N_{Surf}$ is the average number of molecules that adsorb on the surface in the scattering volume of SERS, and $N_{Vol}$ is the average number of molecules in the scattering volume of normal Raman. The analytical enhancement factor (AEF) is limited to when comparing to the SERS enhancement factor in the use of different SERS substrates because it ignores the number of molecules adsorbed on the metal surface. Thus, AEF will be effective way to calculate the enhancement factor at the condition where the number of molecules adsorbed on the metal surface is below that of a uniform monolayer of the molecules. AEF is shown below.\textsuperscript{19}

$$AEF = \frac{I_{SERS}/C_{SERS}}{I_{RS}/C_{RS}}$$  \hspace{1cm} (8)

Where $C_{SERS}$ and $C_{RS}$ are the concentration of the analyte used in SERS measurement and in normal Raman measurement respectively.

1.3 Metallic nanoparticles as SERS substrates

The choice and synthesis of the SERS substrate are critical to perform SERS experiment because SERS intensity is dependent on surface plasmon resonance generated by the metallic substrates. Strong enhancement can be achieved by controlling the shape, size, and composition of the SERS substrate. There are many
types of SERS substrates: electrode, thin film, and metal colloid. Most commonly used nanostructures for SERS is metal colloids because wet chemistry provides an inexpensive and easy approach to metal NP synthesis. The use of the colloid solution is to reduce the burning of the sample and being able to obtain an average spectrum. Among many elements of metal, coinage metals, such as silver and gold, have been widely used because the plasmon frequencies of them are observed in the visible light region where commercial lasers are available.

The silver nanoparticles (Ag NP) has much higher SERS signal achieved than gold, generally about 100 times higher. This is due to its d-s band gap in the UV region, damping less plasmon mode than gold. For Ag NP fabrication, AgNO₃ or Ag₂SO₄ can be reduced by sodium citrate or sodium borohydride. The reduction of Ag⁺ in a boiling solution by addition of citrate is the common method for the synthesis of Ag NPs as SERS substrate, called the Lee and Meisel method. Briefly, the process is adding 2 ml of 1 % sodium citrate solution to 100 ml of 1 mM AgNO₃ and boiling the mixture for 1 hour. Once the addition of citrate in a temperature range between 95 ~ 100 °C in made, the solution quickly changes to a gray color. The absorption band on UV spectroscopy usually observed at 406 nm. This citrate reduced Ag NP solution is stable for a month or longer and is durable against a change in pH, extending from pH 2 to 12. The durable stability of Ag NP allows its applicability to the biological field. Another way to fabricate Ag NPs is to use sodium borohydride as a reducing agent. The process is simple. 10 ml of 1 X 10⁻³ M AgNO₃ is added into 30 ml an ice-cold solution of 2 X 10⁻³ M sodium borohydride with stirring. The color of the mixture is turned to yellow whose absorption is observed at 391 nm. The borohydrated reduced Ag NP is less stable than
the citrate reduced Ag NP because of a lower electrostatic barrier of borohydrate ions existing on the surface of Ag NPs that make Ag NPs aggregated. The slight difference in UV absorption bands between two Ag NP solutions is explained by the difference in their average sizes produced by the experimental conditions compared to that of the citrate reduction method. Ag NPs are good SERS substrates due to it being inexpensive, its simple fabrication process and stability, and its high SERS enhancement. The toxicity of Ag NP, however, can frequently prevent the use of Ag NPs in biological field. Its toxicity is related to the high surface to volume ratio of Ag NPs which upsurges an interaction with serum, saliva, mucus or lung lining fluid components and interacts in new unpredicted ways with biological systems.

The other excellent SERS substrate is Au NPs. A simple wet chemical method has been used to synthesize Au NPs as well. The reduction of chloroauric acid (HAuCl₄) is similar to that with silver: 2 ml of 1 % sodium citrate solution is added to 10 ml of 1 X 10⁻³ M HAuCl₄ when the mixture starts boiling. The color of the mixture is turns to red wine after about 10 minutes after adding the citrate. The resulted Au NPs show a surface plasmon absorption at around 520 nm. In another synthesis, one can use NaBH₂ to reduce Au⁺: 100 ml of 5 X 10⁻³ M HAuCl₄ solution is added to 300 ml of ice-cold 2 X 10⁻³ M NaBH₄ solution with vigorous stirring, followed by the addition of 50 ml of 1 % poly vinyl alcohol during the reaction. The mixture is boiled for 1 hour to decompose the excess NaBH₄ and the final volume of the mixture is adjusted to 500 ml. The Au colloid solution shows red-violet color with absorption band at 535 nm. As mentioned before, although Au NPs do not create higher SERS enhancement then that
of Ag NPs, it is a robust SERS substrate due to its high stability, easy to control NP size, and biocompatibility.

The optical properties of Ag and Au NPs strongly depend on the size and shape of the particles, resulting in varied magnitudes of SERS enhancement.\textsuperscript{31,32} The size of both Ag and Au NPs ranging from 20 to 100 nm is effective to generate SERS enhancement and the strongest SERS enhancement can be achieved with \~{}50 nm of both Ag and Au NPs.\textsuperscript{32,33} This result in the optimal size of the NPs is expected from the competition between EM field increase and higher scattering efficiency as the diameter of NP increases. Similarly, the shape of NPs can also affect SERS enhancement. So far, various shapes of NPs such as nanorods, cube, star, and flower have been fabricated and applied as SERS substrates.\textsuperscript{34-36} For example, in case of the nanorod, the plasmon excitation produces maximum electric field on the tips of rods, which dominates the SERS signal. Similarly, cube shape NPs have strong electric fields concentrated at coroners and edges.\textsuperscript{37} This strong electric field observed at the tip or edge is caused by the charges being concentrated and the field intensity becomes much higher than in a rounded area.

Hybrid structures such as core and shell and sandwich structures have been used as SERS substrates due to strong and adjustable surface plasmons created by the property of the structure.\textsuperscript{25,38} For solid metallic NPs such as Au and Ag nanospheres, the optical plasmon resonance is basically a fixed frequency resonance. In order to improve and adjust plasmon frequency, it is necessary to change the shape of metallic substrates. However, the process of creating different shapes of metallic NPs is complicated. One of the convenient ways is through the use of core-shell
nanostructures. Their optical property is dependent on the size and thickness of metallic shell.\textsuperscript{39} This nanostructure, by controlling the relative radii of the inner and outer shell, can have surface plasmons extended to near Infrared region. The interaction between two fixed plasmon frequencies produced from the inner and outer shell generates a strong electric field, resulting in higher SERS enhancements than that of spherical structure.\textsuperscript{40} For example, silica core and silver NP have absorption peaks in the UV-vis spectrum that are susceptible to change in either of the size of silica core or the diameter of silver shell. Moreover, it is available to extend absorption bands from visible to near Infrared region, which can apply the silica core and silver shell NP in biological field because of the possibility to use longer wavelength excitation lasers that do not damage on cells. According to Tanabe’s study, the field enhancement of the silica core and silver shell nanostructure can be much higher than that of silver monosphere NP when the core to shell diameter ratio becomes about 0.9.\textsuperscript{40}

SERS metallic substrates mentioned so far have shown strong electromagnetic field on their surface. The most intense field, however, is observed at junctions of aggregated NPs, which is called a “hot spot”. In the case of single molecule-single NP, the maximum enhancement factor is estimated as $\sim10^6$.\textsuperscript{41} On the other hand, the hot spots created by aggregations of NPs can provide the enhancement factor with as high as $10^{13}$.\textsuperscript{42} This large enhancement leads the SERS technique to single molecule detection. The addition of salts to a colloid solution makes NPs aggregated because they break the stable balance of colloidal NPs between negatively charged ions surrounding nanoparticles that are used in the reduction of Ag$^+$. Thanks to the excess in anions, such as citrate, each of the NPs repulse one another to prevent their
aggregation. It is believed that halide anions play a critical role in the aggregation of NPs. However, our previous study proved their cations coupled with halide ions also can influence SERS enhancements. From the trial of various salts, it was found that the types and concentrations of aggregating agents (salts) were the other factors that affect the enhancement and the best aggregating agent for the maximum SERS enhancement is depended on the target molecules.\textsuperscript{43} Another way to produce the aggregation of NP is to use the Langmuir-Blodgett technique. This is one of the simpler methods: colloidal NP solution was sprayed over water surface and the formed NP layer was transferred to silicon or quartz substrate. The monolayer NP substrate is naturally aggregated and creates hot spots, resulted in the broad localized surface plasmon resonance that enhances both incident exciting photons and Raman scattered photons.\textsuperscript{36} The other way to create hot spots on NPs is to use the self-assembled monolayer method. First, clean glass plates are functionalized with 3-aminopropyltrimethoxysilane (APTMS) through the interaction between the surface silanols of the glass and Si-OH groups of APTMS. The APTMS functionalized glass is immersed in NPs solution for 6 hours to form close-packed three-dimensional NP arrays.\textsuperscript{44} This method is cheap and simple when compared to the Langmuir-Blodgett technique. But, the problem is controlling the arrangements of NPs on the slide, which produces poor reproducible data. Although the assembled NP layers bring a reproducibility problem to SERS measurement, the use of spinning disc is one of the solutions to overcome it.

1.4 Applications of SERS

The powerful ability of SERS is to identify chemical species and structural information of analytes due to the high enhancement and sensitivity compared to
Raman spectroscopy. One benefit of SERS technique is the possibility to detect biological samples. Au NPs have been widely selected as a SERS substrate in biological field because of the biocompatibility and the coupling with the lower power excitation laser that reduces the invasiveness of the laser on the cells. In addition, since most of biological environments are composed of water, and Raman and SERS bands of water hardly interfere with the spectra of organic compounds, many researchers are encouraged to apply SERS technique in biological fields.

One of attractive advantages of SERS technique is a high selectivity and sensitivity for use in detection of biomolecules. SERS biosensors have been used in discovery of many diseases including cancers, Alzheimer’s disease, and Parkinson’s disease. Cancer is one of the most mortal diseases for human. It caused 8.2 million deaths, which is 15% of all human deaths. Surgery is one of the most effective ways to remove cancers, but the major problem is that the doctors may fail to bring the all of the cancer cells out of human body, leaving behind tumor-positive margins to regenerate and become bigger as time goes by. Mohrs’ research invented a hand-held spectroscopic pen device for intraoperative detection of malignant tumors, based on wavelength-resolved measurements of fluorescence and SERS signals. This device is designed to detect tumor cells and help operators completely remove the tumors during surgery. In this research, the use of Au NP gave rise to sharp signals that are distinct from the broad background of fat. That allowed accurate measurement even at a greater tissue depth. According to Eric’s study, the antibody conjugated Au NP was employed to selectively determine tumor cells and destroy them by using photothermal property of Au NP. When the conjugated Au nanoparticles are attached to
the tumor cells, exposure of invasive near-Infrared laser on it for 20 minutes results in upward of 20 temperature increases at the tumors, resulting in killing the tumor cells with minimal damage to surrounding normal tissues.\textsuperscript{51} This plasmonic photothermal therapy is a minimally-invasive oncological treatment strategy in which photon energy is selectively applied and converted into heat sufficient to induce cellular hyperthermia.

Gas phase detection is important for toxic molecules such as chemical warfare agents and toxic industrial chemicals. To detect toxic gas molecules with SERS, the main issue is to overcome the lack of interaction between the toxic gas molecule and SERS substrate. Brian’s research overcame this problem by using a pressure-driven liquid microchannel flow including Ag NP to trap gas-phase 4-aminobenzenethiol (4-ABT) from the surrounding environment.\textsuperscript{52} Since 4-ABT tends to aggregate NPs to create hot spots, there was no a need to use aggregating agents, which indicates this sensor may need to use an aggregating agent to obtain high SERS signal when trying to detect other toxic gas molecules. However, this system’s microfluidics-based sensor is sensitive for real-time, continuous monitoring of water-soluble gas-phase or airborne agents.

The SERS technique has also has been used in identification of the analytes presented in art object.\textsuperscript{53} To perform a SERS analysis, it needs a sample as small as a single grain of pigment (sub-μg to pg range) with aggregated Ag NPs. Once the pigments are analyzed with SERS, the identification of the art work is completed by comparing the experimental spectrum to a SERS spectral database. There are many ways to use the SERS technique to characterize art works: tracing the origin dye to figure out the history of the art work, authenticating the work by comparison to other
works from the same artist, and reproduction of the original color used in ancient art works.\textsuperscript{54-56}

The invention of portable Raman spectrometers has extended a laboratory based-work beyond the laboratory. Especially, stand-off detection by SERS equipped with optical fiber probes makes it possible to perform \textit{in vivo} measurements or biomedical applications.\textsuperscript{57} The ongoing progress of SERS application bears impressive out comes in many fields and it will continue to intrigue researchers.
CHAPTER TWO: EXPERIMENTAL PROCEDURES

2.1 Glassware Treatment for the Experiments

2.1.1 Sulfuric Acid and Nitric Acid Wash

All the glassware used for the experiments must be free from any contaminations. Acid wash is an effective and inexpensive way to remove chemicals from the glassware. Two Acid baths, sulfuric acid and nitric acid, were designed. The sulfuric acid bath has around a 10 M concentration diluted from around 18 M stock solution purchased from Fisher Scientific Company (A300C-212). Once the used glassware is washed with detergents, they are moved in sulfuric acid for 4 hours with full immersion into the bath. After over 4 hours, the glassware is rinsed with distilled water and dried. Then, they are transferred in the nitric acid bath.

The nitric bath contains a nitric acid solution with 8 M concentration. The concentration was adjusted by diluting an 18 M of stock solution (Fisher Scientific, A200C-212) with distilled water. The glassware needs to stay in the bath for over 2 hours before its removal. During the removal step, one needs to prepare boiling Milipore water because hot water shows effective removal of nitric acid from the glassware. After washing the glassware with hot water and rinsing again with distilled water, the glassware is then dried in air. For safety, all acid baths were placed on a container filled with sodium bicarbonate to neutralize the acid when it is spilt. In addition, before
damping the washing solution for acids, they were also neutralized with solution dissolved with sodium bicarbonate.

### 2.1.2 Nitro-hydrochloric acid (Aqua Regia) Wash

The mixture of nitric acid and hydrochloric acid, optimally in a volume of 1:3 ratios, is an effective and simple way to wash noble metals out of the cuvettes for SERS measurement. All of the cuvettes used were immersed into a bath of aqua regia solution for over two hours, and followed by rinsing of them with hot water several times and dried in air. This mixture solution has a distinctive color. When it is mixed, it turns to yellow immediately which involves a chemical reaction producing volatile products of nitrosyl chloride and chlorine. As time goes by, it quickly becomes an orange color. The orange color of aqua regia solution stands for a while to be capable of the effective removal of organic compounds and metal particles. But, as the volatile products escape from the bath, the color of the solution fades away and returns to the yellow color. This change is caused from the acid solution losing its potency.

### 2.2 Silver Nanoparticles Synthesis

The synthesis of the Ag NPs is described by the Lee and Miesel’s method. Briefly, 0.017 g of AgNO₃ (Fisher Scientific, S486-100) is added to a 125 ml of Erlenmeyer flask filled with 100 ml of Milipore water with a clean stirring bar. The flask is placed on a hot plate (Corning, ANYT-0175) with thermometer inside of the flask. The bottom tip of the thermometer is located at a space between a stirring bar and water surface, but make sure the thermometer is fully immersed into AgNO₃ solution. Heat up the hot plate with the control dial for heating, placing between 4 and 5 and turn the other
dial for stirring speed to 5. When the temperature rises up 90 degrees Celsius (°C), it is
time to add 2 ml of 1 % sodium citrate (Fisher Scientific, S279-500) solution into the
AgNO₃ solution drop by drop using a micropipette. To make 1 % sodium citrate solution,
1 g of sodium citrate is dissolved in 10 ml of Milipore water. After the addition of sodium
citrate solution, one can observe the color change of the solution to yellow a couple of
times, and then becomes gray. The reaction stands for 1 hour keeping 100 °C of
temperature during the process. Once the reaction is done, take the reaction flask off of
the hot plate, and let it cool down in the air where there is no light to avoid
photodecomposition.

2.3 Silica Nanoparticles synthesis

2.3.1 Synthesis of 34 nm Silica Nanoparticles

To synthesize the average size of 34 nm SiO₂ NPs, 3.15 ml of Tetraethyl
orthosilicate (TEOS, Sigma-Aldrich, 131903) is added to a 125 ml of Erlenmeyer flask
filled with 45 ml of ethanol (Fisher Scientific, A405F). To set a water bath, fill a 1 L
beaker with around 200 ml of water and set the beaker on the hot plate. Now, put the
Erlenmeyer flask into the water bath and thermometer is placed inside the water bath. It
should make sure the water level of the water bath is higher than the ethanol and the
bottom tip of the thermometer. The solution is vigorously stirred at 65 °C. Then, 3 ml of
ammonium hydroxide (Sigma-Aldrich, 221228) is immediately added to the flask. The
reaction goes on for 10 minutes after the addition of ammonium hydroxide. As the
reaction continues, the solution becomes cloudy during the formation of silica particle.
When the reaction is complete, put the flask out of the water bath, and cool it at room
temperature. Transfer SiO$_2$ NP solutions into 4 centrifuge tubes (VWR North America, 89039-668), the 4 tubes are placed inside of the centrifuge after making sure that all of tubes have a same weight. Then, one separates the SiO$_2$ NPs from the solvents using Centrifuge (LW Scientific, LWS-Combo-V24 Centrifuge) until a layer of cloudy SiO$_2$ NPs is completely collected at the bottom of the tubes. Using a disposable pipette, only remove the ethanolic solvent from the layer of SiO$_2$ NPs. Fresh ethanol is refilled in the tubes the dispersion of the layer of SiO$_2$ NPs into the ethanol solvent is achieved using an ultrasonic cleaner (VWR North America, P1500-DTH). In order to avoid contamination of SiO$_2$ NPs from the reactant solvent, this process is repeated two more times, and then the combined SiO$_2$ NP solution from all of 4 tubes is added into a 50 ml beaker. Now, one puts the beaker on the hot plate and applies gentle heating below 60 °C to evaporate the ethanol. The beaker stands for a while until all ethanol is evaporated, then one collects white SiO$_2$ NP powders using spatula transferring it into a glass vial. Finally, cover the top of the glass vial with its cap.

2.3.2 Synthesis of 52 nm Silica Nanoparticles

The procedure of the average size of 52 nm SiO$_2$ NPs is almost same steps as those of synthesizing 34 nm SiO$_2$ NPs. 3.12 ml of TESO solution is transferred to an Erlenmeyer flask filled with 45 ml of ethanol. Place the flask inside the water bath which is set up on the hot plate, and apply heating to the water bath with stirring. The speed of stirring is kept the same as that of synthesis of 34 nm SiO$_2$ NPs. But, using the dial for heating, control the temperature to rise up to 60 °C by watching a thermometer placed in inside of the water bath and then add 3 ml of ammonium hydroxide solution to the reaction flask. After 8 hours reaction, take the flask out of the water bath and cool it
down to room temperature. The procedures from separation to evaporation of SiO$_2$ NPs from ethanol are followed the same as previous.

2.3.3 Synthesis of 76 nm Silica Nanoparticles

Deliver 3.12 ml of TEOS to an Erlenmeyer flask containing 45 ml ethanol and put the flask on the water bath. Start stirring the mixture with applying heating to rise temperature up to 55 °C. The stirring speed is the same as previous experiments. While the mixture is mixing, add 3 ml of ammonium hydroxide to the flask and let the reaction go on for 8 hours. During the reaction, frequently monitor the temperature to keep it constant. When the reaction is done, proceed with the separation and evaporation process to obtain SiO$_2$ NP powder.

2.3.4 Synthesis of 113 nm Silica Nanoparticles

To synthesize 113 nm SiO$_2$ NPs, transfer 3.12 ml of TEOS to 45 ml of ethanol in an Erlenmeyer flask and put the flask to the hot plat. In this case, stir the mixture with the same speed as before, and add 3 ml of ammonium hydroxide to the flask. The reaction continues at room temperature for 8 hours. Finally it obtains white and cloudy color of SiO$_2$ NP solution. Take the same steps of separation and evaporation.

2.4 Experimental Procedures

2.4.1 Normal Raman measurements

The Raman spectroscopy invented by Horiba Jovin Yvon is equipped with two different types of lasers. One is an Argon and Krypton laser (Coherent, Innova 70C series) to generate 514 nm and 647 nm of excitation wavelengths. The other is a diode laser (Coherent, Diode Cube) to generate 785 nm of wavelength. There are two
spectrum gratings of 600 and 1200. The 600 of spectrum grating was chosen for all Raman measurements. All experiments were performed on Confocal Raman Microscopy (Olymous, IX71) attached to the Raman spectroscopy. 20 X magnification of microscopic objective lens was used among 40 X and 100 X.

As mentioned before, one of advantages of Raman spectroscopic measurement is simple sample preparation. The measurement is accomplished on the microscope stage where a sample is placed. It is possible to detect all types of samples such as solid, liquid and gas with Raman spectroscopy. In case of solid sample, it can directly be measured on the microscope stage. If the sample is powder and needs to be homogeneous to obtain constant peak intensities of Raman bands, one must grind it into fine powders, and press it in a dese pallet on a glad slide. For the solid sample measurement, first place the glass slide on the microscope stage, and use dials for X, Y, and Z to get a clear image of the sample surface under the incident of white light source. Turn on the switch of the camera view to the laser and irradiate the sample with the laser. It observes a bright white spot on the center of the screen if the focus is corrected. Finally collect Raman spectrum in a wavenumber range from 0 to 3600 cm\(^{-1}\) where most of chemical vibrational mode can be observed. Depending on the condition of the sample, it is required to adjust slight movement of Z axis, exposure time or/and accumulation time to obtain better spectra.

In case of a liquid sample, once the Raman spectroscopy is calibrated with the use of a silica wafer, place 1 ml of quart cuvette including liquid sample on the microscope stage and collect Raman spectrum of the sample with a wavenumber range from 0 to 3600 cm\(^{-1}\). It also needs to get laser focused on the sample by controlling Z
axis dial. In order to get the focus, Raman spectra were collected at every 50 µm unit from 0 to 1000 µm. The optimal height of Z axis was found at 500 nm in where the highest intensity of Raman band is observed. To achieve better spectra, the adjustment of exposure time or/and accumulation time is also required based on the signal/noise ratio.

2.4.2 Surface Enhanced Raman Spectroscopy

SERS is Raman spectroscopy with the use of a metallic colloid. First, transfer a certain amount of Millipore water to the cuvette and add optimal volume of metallic solution followed by the addition of sample. Mix the solution using a micropipette several times. As aggregation of nanoparticles can improve high intensity of the band, one may add aggregating agent to solution if need be. The type and volume of an aggregating agent is dependent on a sample. After the addition of an aggregating agent, the color of the mixture will turn darker, indicating the nanoparticles is aggregated. Finally place the cuvette on the microscope stage where the optimal distance of X axis is already adjusted, and then collect SERS spectra at a range of wavenumber from 0 to 3600 cm\(^{-1}\). The exposure time and accumulation time is adjusted to obtain better spectra.

2.4.3 UV-Vis spectroscopy

UV-Vis spectroscopy (Beckman Coulter DU 640 spectrometer) is employed to characterize metallic nanoparticle colloids. A metallic NP itself is concentrated. Thus, it must be diluted with Millipore water. For preparing the samples for UV-Vis measurement, add 50 µl of metallic colloid to the quartz cuvette filled with 950 µl of
Millipore water, and mix the solution using a micropipette. If the maximum absorption is produced higher than 1, it needs to be diluted again to obtain better results. The quart cuvette has two clear sides where the UV light will pass through. Since the solution is mixed well, wipe the two clear side walls with tissue wipers (VWR, 82003-820) to make sure that there is no interference between the light and any contamination on the walls of the cuvette. To calibrate UV-Vis spectroscopy, set the zero line by inserting a black block into the sample holder and black ground is set by placing a cuvette filled with Millipore water as solvent into the sample holder. Before it is calibrated, make sure the two clear walls of the cuvette are parallel to the pathway of the sample holder where the light will pass through. After the background is set, insert the sample into the sample holder and click the scan button to collect the spectra. The cuvette is rinsed with Millipore water several times for the next measurement. UV-Vis spectras are normalized using the program, thus the concentrations of the solution is not the same as the initial one. The scan range is limited from 200 to 800 nm, which is enough to cover our sample.

**2.4.4 Scanning Electron Microscope (SEM)**

SEM (Hitachi Microscope) located in Nanotechnology Research and Education Center (NREC) on University of South Florida Tampa is employed to evaluate the size and shape of metallic NPs. Before loading the sample on a silicon wafer purchased from NREC, the surface of the silicon wafer should be rinse with ethanol or methanol. If residues of sample remain on the surface, use the tissue wiper soaked with ethanol or methanol to rub the surface several time and rinse it with ethanol. Once the clean silicon wafer is ready, 1 µl of NP solution is loaded on the surface of the silicon wafer using a
micropipette. If there are multiple samples, drop a sample on each of edges of the silicon wafer and then mark each of sample’s location on a note. In order to dry NP solution on the silicon wafer in room temperature, put it on petri dish and cover the petri dish with its cap. Finally place it in a drawer where there is no light. After the sample is dry with showing up their distinct marks on the silicon wafer, load the silicon wafer into the chamber of SEM and start the process of SEM analysis.
CHAPTER THREE: Effects of cations and anions as aggregating agents on SERS detection of cotinine (COT) and trans-3’-hydroxycotinine (3HC)

Note to the reader
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3.1 Abstract
The sensitivity of surface-enhanced Raman spectroscopy (SERS) highly depends on experimental factors including aggregating agents and pH. Using silver nanoparticles as the substrate, the effect of five cationic (K⁺, Na⁺, Mg²⁺, Li⁺, Ca²⁺) and three anionic (Cl⁻, Br⁻, I⁻) aggregating agents was examined on the SERS detection of tobacco-related biomarkers, namely cotinine (COT) and trans-3’-hydroxycotinine (3HC). The optimal concentrations of the aggregating agents with respect to highest SERS
intensity varied widely (from 1.5 mM for MgCl$_2$ to 150 mM for LiCl). Both cations and anions strongly influenced the SERS enhancement. When Cl$^-$ was used as the anion, Mg$^{2+}$ and Na$^+$ exhibited the highest SERS intensities for COT and 3HC, respectively. When Mg$^{2+}$ was used as the cation, Cl$^-$ and Br$^-$ generated the highest SERS enhancement for COT and 3HC, respectively. Clearly, SERS enhancement also depended on the target molecule. Among the 11 aggregating agent combinations tested, the highest SERS enhancement is obtained using 1.5 mM MgCl$_2$ for COT at pH 7 and 50 mM NaBr for 3HC at pH 3.

3.2 Introduction

Raman spectroscopy is a powerful analytical technique for studying chemical structures and detection target molecules, though the weak cross section of most molecular Raman scattering has limited its application in the past. Fortunately, when the analyte is either close to or adsorbed on rough metal surfaces, the Raman signal can be dramatically increased by as much as $-10^{14}$. This phenomenon is known as surface-enhanced Raman scattering/spectroscopy (SERS) and arises from localized plasmon resonance on the metal surface along with direct chemical interactions between the metal and the analyte. Since the advent of SERS in the 1970s, there has been extensive research aimed at understanding the phenomena of SERS enhancement. It is believed that the tremendous enhancement derives from both an electromagnetic mechanism (EM) and a chemical mechanism (CM), and it has allowed SERS to detect a single (or few) molecule(s) in biological samples. SERS offers many advantages including simple preparation, fast analysis, and detection at low concentrations, thus it has been widely applied in many fields.
However, it is difficult to achieve reproducible SERS signals due to the nature of the metal surface or nanoparticles. A number of studies have focused on exploring the effects of three primary experimental parameters on SERS activity; they are the selection and morphology of the metal, the operating pH, and the inclusion of aggregating agents. Firstly, it is well recognized that both the size and shape of metallic nanoparticles strongly influence the SERS enhancement at different plasmon resonances.\textsuperscript{73-75} Secondly, there have been SERS studies that revealed band shift and intensity variation due to pH-induced geometric orientations of the analyte on the metal surface.\textsuperscript{76-78} Lastly, agents added into the metallic colloid can cause the nanoparticles to aggregate and create “hot spots” that generate high surface plasmon resonance.\textsuperscript{79,80} Currently, most researchers have been investigating the anion effect on SERS enhancement.\textsuperscript{81-86} They demonstrated that the addition of anions as aggregating agents can significantly increase the SERS enhancement due to the electrostatic force between nanoparticles. For example, a 100 fold increase of the SERS intensity with the addition of chloride was reported when rhodamine 6G was adsorbed on a silver surface.\textsuperscript{87} They also suggested that the cations of aggregating agents do not influence the SERS intensity and that their contribution to SERS intensity is negligible.\textsuperscript{87,88} However, in 2009, Nicola pointed out that in order to achieve high SERS enhancement, both cations and anions need to be considered.\textsuperscript{89} To clarify the mystery, further understand their roles, and improve SERS enhancement, a systematic investigation of the effect of both anions and cations is needed.

In this study, we explored the effect of various aggregating agents, including LiCl, LiBr, NaCl, NaBr, NaI, KCl, KBr, MgCl\textsubscript{2}, MgBr\textsubscript{2}, CaCl\textsubscript{2}, and CaBr\textsubscript{2}, on the detection of cotinine
(COT) and trans-3'-hydroxycotinine (3HC). COT and 3HC are the primary metabolites from nicotine and have been widely used as tobacco-related biomarkers. Their chemical structures only differ by the existence of a hydroxyl group on the pyrrolidine of 3HC. Interestingly, despite their similar chemical structure, COT exhibits a much higher SERS intensity than 3HC. The effect of other commonly used anions like sulfate and nitrate were not tested due to their interference in the detection of our target molecules.

3.3 Experimental Methods

3.3.1 Materials

Silver nitrate (AgNO₃, 99 %) and sodium citrate (Na₃C₆H₅O₇·2H₂O, 100 %) were purchased from Sigma-Aldrich and Fisher Scientific, respectively. Aggregating agents: lithium chloride (LiCl, 99.99 %), lithium bromide (LiBr, 99.998 %), sodium chloride (NaCl, certified ACS), sodium bromide (NaBr, 99 %), sodium iodide (NaI, 98 %), potassium chloride (KCl, 99 %), potassium bromide (KI, 99.998 %), magnesium chloride (MgCl₂, 98 %), magnesium bromide (MgBr₂, 99.999%), calcium chloride (CaCl₂, 99 %), and calcium bromide (CaBr₂, 99.98 %) were all purchased from Sigma-Aldrich. Cotinine and trans-3'-hydroxycotinine were purchased from Sigma-Aldrich and Toronto Research Chemicals Inc., respectively. All the chemicals were used without further purification. All glassware was acid washed using nitric acid and sulfuric acid, and all the solution were prepared using deionized water (DI water, 18.2 mΩ) from a Cascada BIO-water Purification System (Pall Corporation).
3.3.2 Synthesis of Silver Nanoparticles

Ag colloids were prepared according to the method by Lee and Meisel.\textsuperscript{26} Once the 100 mL solution of 1 mM AgNO\textsubscript{3} reached 95 °C with vigorous stirring, 2 mL of 1 % trisodium citrate solution was immediately added to reduce Ag\textsuperscript{+}. The mixture was heated at 100 °C until the dark grey colloid was obtained (1 h), and then cooled down with vigorous stirring to room temperature. The Ag colloid was stored in a dark place for future use.

3.3.3 UV-Vis, SEM, and SERS Measurements

UV-Vis absorption spectra of the silver colloidal solution were obtained with a Beckman Coulter DU 640 spectrometer. The silver colloid was diluted to ¼ with DI water and the solution was placed into a quartz cuvette. The solution was scanned from 200 to 800 nm for 0.5 s.

Scanning electron microscopy (SEM) images of Ag nanoparticles were obtained using a Hitachi S-800 microscope located at the Nanotechnology Research and Education Center (NREC) on the University of South Florida’s Tampa campus. In order to obtain the SEM measurements, about 2 µL of the silver colloidal solutions were dropped onto the top of the silicon wafer and air dried. The wafers were kept away from light due to silver’s photosensitivity. Samples were prepared by adding 250 µL each of the Ag colloidal solution, an aggregating agent, and either COT or 3HC into a glass cuvette, and then pipetting the mixture several times. To control sample pH, diluted HCl or NaOH solution was added into the mixture.
All the SERS experiments were carried out using Confocal Raman Microscope purchased from Horiba Jovin Yvon, Inc., equipped with an Argon and Krypton laser (Coherent, Innova 70C series). For all experiments, a 514 nm wavelength laser at 42 mW of power, 5 s of exposure time, and 3 accumulations were used. The spectrum grating was 600 grooves/mm and a 20X objective was used throughout the experiments. All the results that were reported have been repeated in triplicate for reproducibility.

3.4 Results

3.4.1. Ag Nanoparticles Characterization.

Figure 3.1.(a) shows a typical UV-Vis absorption spectrum of Ag colloidal solution with and without 50 mM NaCl. The maximum absorption for Ag colloidal solution was observed at 410 nm, which directly indicates the average size of the spherical Ag nanoparticles is ~67 nm.37 The addition of NaCl into the Ag colloidal solution leads to a decrease of absorption in the plasma resonance region. This phenomenon was also observed when other aggregating agents were added and can be attributed to the aggregation of the nanoparticles.28 Figure 3.1.(b) shows a typical SEM image of the nearly spherical Ag nanoparticles. Statistical analysis was performed on manually measured nanoparticles from sampled SEM images, and the mean size of 67 ± 19 nm corroborates the approximation from the absorption spectra.
3.4.2. Normal Raman and SERS Spectra of COT and 3HC.

Figure 0-1 UV-vis absorption spectra and SEM of Ag colloid solution
(a) UV-vis absorption spectra of 250 µM Ag colloidal solution (solid line) and 250 µM Ag colloid solution with 45 mM NaCl (dash line). (b) A typical SEM image of silver nanoparticles.

Figure 0-2 Normal Raman and SERS spectra of (a) COT and (b) 3HC
Normal Raman and SERS spectra of (a) COT and (b) 3HC. (a) SERS spectrum of 1 µM COT with 100 mM NaCl (top) and normal Raman spectrum of 0.1 M COT (bottom) at pH 7. (b) SERS spectrum of 50 µM 3HC with 45 mM NaCl (top) and normal Raman spectrum of 0.1 M 3HC (bottom) at pH 7.0

Figure 3.2 shows the normal Raman and SERS spectra of COT and 3HC. Two intense peaks were observed at 1031 and 1050 cm\(^{-1}\) in the normal Raman spectrum of COT of which only the 1031 cm\(^{-1}\) peak exhibited a strong enhancement. Using the same aggregating agent (NaCl), similar peak behaviors were observed for 3HC, which can be attributed to the similarities in their chemical structures.
No Raman peak assignments for COT and 3HC have been reported to date. Fortunately, Barber et al. used SERS for nicotine determination. Since nicotine also shares structural similarities with COT and 3HC, it can serve as an effective guide for COT and 3HC peak assignments. Thus, the peaks at 1031 and 1050 cm\(^{-1}\) are assigned to the symmetric ring breathing mode and the trigonal ring breathing mode of the pyridine ring, respectively. The strong enhancement of the symmetric ring breathing mode peak observed in the SERS spectra indicates that both COT and 3HC tend to approach the surface of silver nanoparticles through the pyridine ring rather than the pyrrolidine ring. The same behavior was also observed when nicotine is adsorbed on Ag surface, which was attributed to the steric hindrance of the methyl group on the pyrrolidine ring.

At the same concentration and under the same experimental conditions, COT Raman peaks are notably greater than those of 3HC by at least a factor of ten. This phenomenon indicates that 3HC has a much smaller cross section than that of COT due to the presence of the hydroxyl group on the pyrrolidine ring. The relationship between the 1031 cm\(^{-1}\) peak intensities of COT and 3HC translated to the SERS spectra even when the 3HC concentration was 50 times greater than that of COT. The exorbitant difference in SERS intensities can only be partially attributed to their cross sections; therefore, the adsorption of these molecules onto the Ag surface may be playing a significant role in the enhancement. The electromagnetic field generated from the surface plasmon decreases exponentially away from the surface; therefore, the closer the molecules are to the surface, the stronger their SERS signal intensities will be. Thus, we can infer from the remarkable difference in SERS intensity that COT adsorbs
more strongly onto the Ag surface than 3HC, which is likely due to the hydroxyl group hindering adsorption.

For its high intensity and good reproducibility, the peak intensity at 1031 cm\(^{-1}\) was used to indicate the SERS activity and further calculate the SERS enhancement factors of COT and 3HC.\(^{92}\) The SERS substrate enhancement factors (SSEF) of COT and 3HC, when each analyte’s optimal aggregating agents were used, were estimated by eq 1, where \(I_{SERS}\) and \(I_{NR}\) are the respective intensities of the vibrational peak at 1031 cm\(^{-1}\) in SERS and normal Raman (NR) measurements, and \(N_{surf}\) and \(N_{NR}\) are the respective average number of adsorbed molecules in the scattering volume for the normal Raman and SERS measurements.\(^ {19,20,93}\)

\[
SSEF = \frac{I_{SERS}/N_{surf}}{I_{NR}/N_{NR}} = \frac{I_{SERS}C_{NR}N_{A}A_{m}}{I_{NR}d_{NP}4\pi r^{2}\sigma}
\]  \hspace{1cm} (1)

Taking consideration of the surface coverage of the analyte on the nanoparticles, a more rigorous definition of the \(N_{surf}\) and \(N_{NR}\) is also expressed in eq 1. \(C_{NR}\) is the concentration of COT or 3HC for the NR measurement, \(N_{A}\) is the Avogadro constant, \(A_{m}\) refers to the surface area occupied by an adsorbed COT or 3HC molecule, \(d_{NP}\) is the number of metallic nanoparticles in the experimental solution of \(4.92 \times 10^{9}\) mL\(^{-1}\), \(4\pi r^{2}\) is the surface area of a metallic nanoparticle, and \(\sigma\) is the surface coverage of the analyte on metallic nanoparticles. Assuming that both COT and 3HC molecules orient parallel to the metal surface, the value of \(A_{m}\), including pyridine and pyrrolidine rings for both COT and 3HC, was estimated to be around \(9.099 \times 10^{4}\) pm\(^{2}\). The values of SSEF for both COT and 3HC are presented in Section 3.4.5.
3.4.3. Concentration Effect of Aggregating Agents on SERS Intensity.

The addition of aggregating agents serves to achieve higher SERS enhancement. But using excessive amounts results in the instability of the colloidal solution and the eventual precipitation of nanoparticles. Therefore, it is necessary to determine the optimal aggregating agent concentrations. To evaluate the effects of multiple cationic and anionic agents and obtain the best SERS enhancement for COT and 3HC, the effects of eleven inorganic aggregating agents, including LiCl, LiBr, NaCl, NaBr, NaI, KCl, KBr, MgCl$_2$, MgBr$_2$, CaCl$_2$, and CaBr$_2$, were examined: the results are listed in Table 1. Figure 3.3 shows the concentration effect of five aggregating agents on the SERS intensity for COT and 3HC (only five are shown for clarity). All COT and 3HC measurements were performed in solutions at pH 7 and pH 3, respectively, for reasons explained in Section 3.4.4. Generally, as the concentration of the aggregating agents increase, the SERS intensities for both COT and 3HC increase and reach their eventual maxima before experiencing a decline. This behavior occurs because the Ag nanoparticles begin to aggregate and form strong interactions among themselves, which leads to large surface plasmon and high SERS enhancement. However, as more aggregating agents are added, the precipitation of Ag nanoparticles ultimately results in overall decreased enhancement. The concentration of the aggregating agent where maximum SERS EF was achieved will be labeled as the optimal concentration in the latter text and is also shown in Table 1.

As expected, we observed unique behavioral patterns for each of the aggregating agents. The first major theme concerning the effects of the various aggregating agents is that their optimal concentrations are dramatically different from
each other. For example, though 140 mM NaCl generates the highest SERS intensity for COT, the optimal concentration of MgCl\(_2\) is only 1.5 mM. In the case of 3HC, 100 mM LiBr provides the highest SERS EF, but 1 mM is the best concentration for MgBr\(_2\). In both cases, the optimal concentration of Mg\(^{2+}\) is more than ten times smaller than that of the rest of the cations, including Li\(^{+}\), Na\(^{+}\), K\(^{+}\) and Ca\(^{2+}\). This might be explained by the smaller size and strong electrostatic force of the doubly charged Mg\(^{2+}\). Clearly, the electrostatic interactions between the cations and anions play an important role during the SERS measurements. Additionally, the optimal concentration of the aggregating agent also depended on the target molecule. In the case of NaCl, the maximum SERS intensity of COT was observed with a 100 mM NaCl, but the optimal concentration was 45 mM NaCl for 3HC signal enhancement.

The second major theme is that there are notable differences in the relative dependence of SERS intensity on the concentrations of each aggregating agent. As
shown in Figure 3.3.(a), the SERS intensity remains high when MgCl$_2$ concentration is in the range of 1.5 – 10 mM and decreases sharply once the concentration is out of this range. To the contrary, the SERS intensity is at its maximum when MgBr$_2$ has a concentration from 30 to 50 mM and it declines slowly when the concentration is out of this range. In Figure 3.3.(b), the SERS intensity of 3HC is more susceptible to the change in the concentrations of CaBr$_2$ and NaBr than of KBr, LiBr, and MgBr$_2$.

Overall, the maximum achievable SERS intensity critically depends on the combination of an aggregating agent and its optimal concentration and the selected analyte. As shown in Table 1 and Figure 3.3, the maximum SERS intensity achieved for COT is $5.4 \times 10^4$ with 1.5 mM MgCl$_2$, and for 3HC, it is $6.9 \times 10^3$ with 50 mM NaBr. The relevance of these values is discussed in detail in Section 3.4.5.

### 3.4.4 pH Effect on the SERS Intensity.

As mentioned in Section 3.4.3, the solution plays an important factor in SERS enhancement. The protonated and deprotonated forms of the target molecules in the colloid environment can approach the Ag surfaces with differing orientations, thus generating different SERS behaviors.

Figure 3.4.(a, b) shows the SERS spectra of COT and 3HC at pH 7 and 3, respectively. Though the SERS intensity of the peak for COT is much higher at pH 7 than at pH 3, the relative intensity of those vibrational bands is nearly the same. On the other hand, the SERS intensity of the peak at 1028 cm$^{-1}$ for 3HC is much higher at pH 3 than at pH 7. The wavenumber shift from $1031$ cm$^{-1}$ to $1028$ cm$^{-1}$ is due to the protonation of 3HC.
Additionally, the enhancement of the peak at 1050 cm$^{-1}$ for 3HC is so immense, it exhibits almost the same intensity as the peak at 1028 cm$^{-1}$. These observations strongly suggest that 3HC molecules adsorb onto the silver surface by different adsorption geometry in the acidic environment when compared to the neutral environment.

The protonated 3HC might allow the pyridine ring to absorb with closer proximity to the Ag nanoparticle surfaces. In contrast, the absence of the hydroxyl group on COT results in a less pronounced pH effect on its adsorption geometry. We also observed that the low pH condition of 3HC gave rise to the intensity of the peaks at 1188, 1609,
and 1635 cm\(^{-1}\), as shown in Figure 3.4.(d). At low pH, the pyridine ring of 3HC remains in the form of a pyridinium ion; the three aforementioned peaks of 3HC at pH 3 confirm this and are assigned to CH deformation of pyridinium ion (1188 cm\(^{-1}\)) and pyridinium ring vibrations (1609 and 1635 cm\(^{-1}\)).\(^9\) In an acidic solution, the pyridinium ions maintain positive charges on the nitrogen of the pyridine ring, and the halide ions surrounding the pyridinium ion may form an ionic bond with the positively charged nitrogen of the pyridinium ring that further promotes an intermolecular interaction with the silver nanoparticle surface.

Figure 3.4.(c, d) also shows the effects of pH values ranging from 1 to 11 on the SERS intensity of COT and 3HC. As pH increases, the SERS intensity of peak at 1031 cm\(^{-1}\) for COT initially increases until reaching its maximum at ca. pH 7, after which it decreases. As was pointed out earlier, the SERS intensity of 3HC is more sensitive to pH changes than COT. 3HC experiences its SERS peak intensity maximums at pH 3, but then decreases abruptly and levels off at nearly zero above pH 5. All results clearly suggest that pH 7 and pH 3 are the best operating conditions for achieving maximum SERS intensity for COT and 3HC, respectively. The same conclusion can be drawn from the results listed in Table 1. Therefore, in the remaining SERS experiments to be discussed, pH 7 and 3 were applied to investigate the effects of various aggregating agents on SERS activity for the detection of COT and 3HC, respectively.

### 3.4.5 Effects of Different Aggregating Agents on the SERS EF.

Figure 3.5 shows the SERS spectra of 1 µM COT and 5 µM 3HC with various aggregating agents at their optimal concentrations. In both cases, the effects of three kinds of anions (I\(^-\), Cl\(^-\), Br\(^-\)) and five kinds of cations (K\(^+\), Na\(^+\), Mg\(^{2+}\), Li\(^+\), Ca\(^{2+}\)) were
examined. Investigation of the effects of F⁻ and I⁻ (alternative combinations) ion as an aggregating agent was also performed; however, with the exception of NaI, the reproducibility for such SERS experiments was unexpectedly low and the SERS spectra of the blank solution without the target molecules were full of anomalous peaks and were therefore excluded from further study (not reported here). Significant differences were observed in terms of the absolute intensity of the peaks for both COT and 3HC, but little change was observed for the relative intensity of the vibrational peaks for COT with the addition of most aggregating agents (Figure 3.5). When KCl and NaI were used as the aggregating agents, the vibrational bands at 1031 cm⁻¹ and 1028 cm⁻¹ dramatically decreased in both COT and 3HC. Even when the concentration of 3HC was increased by ten times in the presence of KCl, these two peaks still could not be observed in SERS measurements. Such phenomena clearly indicate that the presence of aggregating agents not only affects the aggregating status of the Ag nanoparticles, but also the manner in which COT and 3HC molecules adsorb on the Ag surface. As an exception for those measurements, the peak with the highest intensity is used to calculate the SERS EF.

To better understand the effects of the various aggregating agents on the SERS detection of COT and 3HC, we would like to remind the readers that Table 1 serves as a suitable accompaniment to this portion of the discussion. For the measurements of COT at pH 7, MgCl₂ produces the highest SERS intensity, while LiCl, NaCl, and CaCl₂ generate lower SERS intensity than that of MgCl₂. For the measurements of 3HC at pH 3, NaBr clearly provides the highest SERS intensity. For obvious reasons (explained
previously), the SERS intensity of COT at pH 7 is always greater than at pH 3 for all the aggregating agents tested.

![SERS spectra of COT and 3HC with various aggregating agents](image)

The observation agrees well with the conclusion drawn in Section 3.4.5 that pH 7 is the best condition for the detection of COT. Interestingly, the SERS EF for COT detection at pH 7 without an aggregating agent is close to 1, but that of COT and 3HC at pH 3 is 6.9
and 36. This is due to the chloride anions of the added HCl that was used to adjust the pH.

As shown in both Figure 3.5.(a) and Table 1, when chloride or bromide anions were coupled with different cations and used as the aggregating agents, four out of the five cations, K\(^+\), Na\(^+\), Mg\(^{2+}\), Li\(^+\), and Ca\(^{2+}\), resulted in significant SERS enhancement with a signal intensity above \(2 \times 10^4\) for COT and above \(4 \times 10^2\) for 3HC. However, the SERS signal intensity with iodide was significantly lower for both COT and 3HC than the other halides. Thus, chloride ion is desirable for the detection of COT while bromide is preferable in case of 3HC. The poor performance of iodide can be further examined through literature since much research has already focused on the role of halides and their adsorption onto Ag nanoparticles (Cl\(^-\), Br\(^-\), I\(^-\) and F\(^-\)).\(^{82-88}\) It was suggested that the halide anions do not directly cause silver nanoparticles to aggregate, but rather repulse the other anions present in the solution, such as citrate and nitrate (introduced during Ag\(^+\) reduction), as they compete for metallic surface adsorption with stronger affinities.\(^ {83}\) This halide behavior changes the chemical properties of silver nanoparticles, hence they give rise to the SERS enhancement. Since the electronegativity of I\(^-\) is much smaller than that of Cl\(^-\) and Br\(^-\), it is adsorbed on the silver surface more weakly; therefore, it results in reduced SERS enhancement.

Also interesting to note, when either Li\(^+\), Na\(^+\), Mg\(^{2+}\) or Ca\(^{2+}\) was used as the cationic complement of the aggregating agents, higher SERS intensity was achieved with Cl\(^-\) than with Br\(^-\) for the detection of COT. On the contrary, for all cations, using Br\(^-\) produced higher SERS intensity than Cl\(^-\) during the detection of 3HC. According to the adatom model proposed by Otto et al.,\(^ {95,96}\) molecules adsorb on the specific active sites
of the metal surface and form surface complexes with the halide anion and a single metal atom, which is called the Ag$^+$-molecule-halide ion complex. The complex will have different magnitudes of electrostatic force depending on the chemical properties of both the anions and absorbates. Since 3HC molecules are more likely be protonated at pH 3, they tend to increasingly interact with Ag$^+$ that maintains a relatively lower partial positive charge induced by Br$^-$ compared to that more positive Ag$^+$ charge induced by the more electronegative Cl$^-$. On the other hand, protonation does not occur as abundantly with COT since it lacks the hydroxyl group, and such repulsion between positively charged adsorbate and Ag atom does not apply at either pH 3 or 7. Therefore, Cl$^-$ exhibits a stronger influence on the SERS intensity of COT on Ag nanoparticles than Br$^-$. In addition, both COT and 3HC tend to interact with the Ag surface through the pyridine ring while their methylpyrrolidone rings are positioned away from the surface. The pyrrolidine ring of 3HC is more electronegative than that of COT due to the hydroxyl moiety. Thus, 3HC responds more favorably in the presence of halides of lower electronegativity than Cl$^-$. These factors should encompass why there is a seemingly pH-independent synergy between particular anions and target analytes.

Previous reports suggested that the cationic complements (H$^+$, K$^+$ and Na$^+$) of aggregating agents do not significantly influence the SERS enhancement of Rhodamine 6G (R6G) signals using silver nanoparticles.$^{87,88}$ However, we have observed that there are notable effects, for both the spectra in Figure 5 and the data in Table 1 demonstrate that the cations yield varying SERS signal intensity for both COT and 3HC. When Cl$^-$ was coupled with the five cations, Mg$^{2+}$ produced the highest SERS intensity of $5.4 \times 10^4$ for COT and K$^+$ produced the lowest SERS intensity of $7.3 \times 10^2$ (a difference by a
factor of 74 times). In the case of 3HC detection, when Br\(^-\) was coupled with the five cations, Na\(^+\) produced the highest SERS intensity of 6.9 × 10\(^3\), which is almost 5 times higher than that of Li\(^+\). It was suggested that the cations do not directly adsorb onto the metal surface, but rather remain in the diffuse double layer, which only slightly affect the surface properties of colloidal silver.\(^{97}\) Such differences can be attributed to the fact that the chemical property of the surface complex (Ag\(^+\)-molecule-halide anion) depends not only on the analyte, but also on the existence of cations in the diffuse double layer. It is possible that the cations sufficiently influence the overall electronic interaction of the surface complex (Ag\(^+\)-molecule-halide anion). In the case of R6G, the cationic influence is almost negligible because of the imperviousness of the surface complex to cations caused by its positive charge and large size. When neutral COT forms the surface complex, it has little effect on the electronic interaction between the surface complex and the cations in the surrounding diffusion layers. Therefore, K\(^+\), which generates the lowest electrostatic force, exhibits the smallest SERS intensity among all the five cations tested here. When protonated 3HC molecules are adsorbed onto the surface at pH 3, their positive charge does not favor the strong interaction from the surrounding cations. This explains the smaller effect observed when K\(^+\) is used.

To better compare the SERS EF with different substrates under various conditions, the SERS substrate enhancement factors (SSEF) of both COT with MgCl\(_2\) and 3HC with NaBr were estimated by eq 1 to be 3.2 × 10\(^9\) for COT and 1.3 × 10\(^9\) for 3HC. The surface coverage of COT and 3HC on silver nanoparticles was determined experimentally to be 52 and 50 %, respectively. In order to optimize the SERS intensity, the effect of various aggregating agents and their concentration should be considered.
Figure 0-6 Calibration curves of (a) COT with 1.5 mM MgCl$_2$ at pH 7 and (b) 3HC with 50 mM NaBr at pH 3.

3.4.6 SERS Detection of COT and 3HC.

With the help of MgCl$_2$ and NaBr as the best aggregating agents, we were able to obtain the calibration curves of COT and 3HC shown in Figure 3.6. SERS yielded good linearity in the calibration curves for COT and 3HC. The detection limits are 0.20 nM for COT and 0.12 µM for 3HC. When considering the concentration range of COT (0.4 ~ 38 µM) and 3HC (2 ~ 166 µM) of active smokers in their urine, SERS spectroscopy is capable of detecting those biomarkers in smokers.

3.5 Conclusion

The effect of five kinds of cations (K$^+$, Na$^+$, Mg$^{2+}$, Li$^+$, and Ca$^{2+}$) and three kinds of anions (Cl$^-$, Br$^-$, and I$^-$) as aggregating agents were studied to explore their influence on signal enhancement during the SERS detection of COT and 3HC using Ag nanoparticles. The effects of pH were also investigated and it was found that pH 3 was optimal for 3HC and a neutral environment was optimal for COT detections. We found that the concentration of aggregating agents heavily impacted the SERS intensities for
both target molecules, and the optimal concentrations for maximum SERS intensities were determined. These optimal concentrations differed dramatically for the various aggregating agents, which extends almost two orders of magnitude and depends on the pairing between aggregating agent and target molecule. In the case of COT, the optimal concentration for LiBr is 140 mM while that for MgCl$_2$ is only 1.5 mM. For 3HC, the optimal concentration for LiCl is 150 mM while that for MgBr$_2$ is only 1 mM. Not all of the cations and anions were found to be suitable as aggregating agents since some salts exhibited complicated backgrounds or poor reproducibility. For the suitable anionic aggregating agents, Cl$^-$ gave the most preferable enhancement during COT detection and Br$^-$ was best for 3HC detection. We found that contrary to previous reports, the cations did give noticeably different SERS enhancement for our target molecules, especially in the case of K$^+$ where it gave the lowest enhancement while paired with Cl$^-$ in both COT and 3HC measurements. However, the anions in our research had the most pronounced effect on SERS intensities while using COT and 3HC as target molecules, which can be explained by their different roles on the interaction between the adsorbate and Ag surface. Lastly, the best aggregating agent to achieve the highest SERS intensity is critically dependent on the target molecules, where MgCl$_2$ was found to be the best aggregating agent for COT and NaBr best suited 3HC. In general, both the aggregating agents and their concentrations need to be examined in order to achieve the highest possible SERS EF.
Table 0.1: Effect of aggregating agents on SERS intensities of peak 1031 cm⁻¹ for COT and 3HC

<table>
<thead>
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<th>Aggregating agent</th>
<th>Concentrations (mM)*</th>
<th>SERS Intensity (pH=7)</th>
<th>SERS Intensity (pH=3)</th>
<th>Aggregating agents</th>
<th>Concentration (mM)*</th>
<th>SERS Intensity (pH=3)</th>
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<td>560</td>
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<tr>
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<td>7297</td>
<td>NaBr</td>
<td>50</td>
<td>6938</td>
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</tr>
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</tr>
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<td>700</td>
</tr>
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<td>32904</td>
<td>5868</td>
<td>CaBr₂</td>
<td>10</td>
<td>3591</td>
</tr>
</tbody>
</table>

* The concentrations shown are the ones with maximum SERS intensity.
** No A. A: SERS detection with Ag nanoparticles without any aggregating agent.
CHAPTER FOUR: Optimal SiO$_2$ core Ag shell nanoparticle as SERS substrates

4.1 Abstract

Silica core and silver shell nanoparticles were successfully fabricated and their activity as substrates for surface-enhanced Raman scattering (SERS) were examined. Both the size of the core and the thickness of the Ag shell exhibit strong effect on the SERS activity. We observed that SERS intensities of Rhodamin 6 G (R6G) as a probe molecule are susceptible to change in shell thickness and the size of core-shell nanoparticle. Also, the tuneability of surface plasmon of core-shell structure is flexible by changing in the size of either core or shell. It shows that the optimized size of silica core-silver shell nanoparticles have higher SERS enhancements of R6G, 4-aminothiophenol (4-ATP), and cotinine (COT) than that of silver nanoparticle.

4.2. Introduction

Surface-enhanced Raman scattering (SERS) has attracted much attention in research because of the amplification to Raman signals that results from surface plasmon resonance. Under certain conditions, SERS can even detect a single target molecule, where the SERS enhancement factor can range between $10^{13}$–$10^{15}$. Such major enhancements occur when the target molecules are directly absorbed onto or in close proximity to the surface of SERS substrates, which would typically be silver, gold, or copper. The surface plasmon resonance on the metal surface is induced by incident
light and produces strong local electric fields that lead to intense electronic transitions in target molecules; this is the electromagnetic mechanism of SERS enhancement, and it is significantly affected by the properties of metal substrates.

The surface plasmon frequency of noble metal nanoparticles, such as Ag and Au, can be controlled by their size, shape, and dielectric environment. In general, their plasmon resonances fall within the visible region. In order to extend the application of SERS technique to biological fields, the surface plasmon frequencies of SERS metal substrates need to be tuned to the near-infrared region. A relatively convenient method to accomplish this involves adjusting the relative diameters of a core-shell nanostructure that consists of a dielectric core (silica) and metallic shell. The core-shell structure can also produce greater field enhancement because of the surface plasmon coupling by inner and outer shell. Since Ag and Au shells show a broad tunable scale of extinction peaks, they have been widely used on silica-supported nanostructures.

It has been previously reported that Ag produces a higher field enhancement on a silica core than Au when the core-to-shell diameter ratio is about 0.87. Additionally, the plasmonic tunability of a silver nanoshell is about 10 % greater than that of a gold nanoshell. So far, many studies of core-shell structure for use as SERS substrates have been performed using silica core sizes that ranged from 200 to 300 nm. These studies have demonstrated that core-shell structures are SERS-active substrates. However, there has been no detailed study on the optimal silver shell thickness when using silica cores (to the best of our knowledge). Furthermore, it has been reported that the optimal size for Ag nanoparticles to achieve the highest enhancement is ca. 60 nm. This size is way smaller than the size of SiO$_2$@Ag
nanoaprticles reported so far. Here, we intend to optimize the size of silica core and the Ag shell thickness and explore their effect on the activity of nanoparticles as SERS substrates. The Stöber method\textsuperscript{109} was modified to synthesize silica cores of different sizes (34, 42, 81, and 113 nm). There have been many methods to synthesize the Ag shell including electroless plating method, ultrasound-assisted synthesis, layer-by-layer synthesis, tin-mediated electroless plating method, and surface modifications.\textsuperscript{110-114} The seed-mediated method proposed by Kim et al. was used here because this method is simple and produces high surface coverage of silver on silica surfaces.\textsuperscript{108} The silica core and silver shell nanoparticle examined optical properties and SERS enhancements, which yields the most active SERS substrates.

4.3. Materials and methods

4.3.1 Materials

Sodium citrate (Na\textsubscript{3}C\textsubscript{6}H\textsubscript{5}O\textsubscript{7}•2H\textsubscript{2}O, 100 %) and ethanol (C\textsubscript{2}H\textsubscript{6}O) were purchased from Fisher Scientific. Tetraethyl orthosilicate (TEOS, C\textsubscript{8}H\textsubscript{20}O\textsubscript{4}Si; 98 %), butylamine (C\textsubscript{4}H\textsubscript{11}N, 99.5 %), ammonium hydroxide (28–30 %), Rhodamine 6G (C\textsubscript{28}H\textsubscript{31}ClN\textsubscript{2}O\textsubscript{3}, 99 %), 4-aminothiophenol (C\textsubscript{6}H\textsubscript{7}NS, 97 %), cotinine (COT, C\textsubscript{10}H\textsubscript{12}N\textsubscript{2}O; 98 %), sodium bromide (99 %), magnesium chloride (98 %), and silver nitrate (99 %) were all purchased from Sigma-Aldrich. All chemicals were used without further purification. All glassware was acid washed using sulfuric and nitric acids, and all the solution were prepared using deionized water (DI water, 18.2 M\textOmega\cdot\text{cm}) from a Cascada BIO-water Purification System (Pall Corporation).
4.3.2 Silica nanoparticle

Silica sphere nanoparticles were prepared using the Stöber method, which is detailed elsewhere. In order to obtain the different sizes of silica spheres, we varied the reaction times and temperatures of the nanoparticle solutions. Briefly, 3.12 mL of TEOS and 3 mL of ammonium hydroxide were added into 45 mL of ethanol. The mixture was stirred vigorously, and different reaction times and temperatures were applied in accordance with the desired sizes. The 34, 52, 81, and 113 nm sized silica spheres were synthesized at 55 °C for 10 min, at 60 °C for 8 h, at 55 °C for 8 h, and at room temperature for 8 h, respectively. The resulting silica nanoparticles were centrifuged and dispersed into ethanol several times to purify the silica nanoparticles. The solid powder of silica nanoparticles was obtained after evaporating the ethanol by heat.

4.3.3 Formation of silver shell on silica surface

The silica nanoparticles were initially coated with silver seeds by modifying a method proposed by Kim. Specifically, 0.1 g of silica powder was dissolved into 20 mL of ethanol, then Ag NO₃ was added to the mixture to reach a final concentration of 5 mM. Butylamine was injected into the mixture with vigorous stirring to reduce Ag⁺. The temperature of the reaction vessel was kept at 65 °C for 2 h. The formations of Ag layers on the SiO₂ surfaces were indicated by the solution achieving a gray tint. After the reaction reached an endpoint, the Ag-coated SiO₂ nanoparticles were centrifuged to remove ethanol, then dispersed into the initial volume of distilled water for the second reduction of Ag.
Secondary reduction of Ag⁺ was conducted using various concentrations of AgNO₃ to control the final thicknesses of Ag shells on SiO₂ nanoparticles. An aliquot of 10 mL of Ag seed-coated SiO₂ solution was added to 10 mL of deionized water. First, the desired amount of AgNO₃ solution was introduced to the diluted Ag seed-coated SiO₂ solution under continuous stirring. Subsequently, sodium citrate solution with a concentration 1.5 times higher than that of AgNO₃ was added when the temperature of the mixture exceeded 80 °C. The reaction was permitted to occur for 1 h at 80 °C, then the solution was stored in the dark prior to use.

4.3.4 Silver nanoparticle synthesis

Ag colloids were prepared according to the method of Lee and Meisel.²⁶ Briefly, once 100 ml solution of 0.1 M AgNO₃ reached 95 with vigorous stirring, 2 ml of 1 % sodium citrate solution as a reducing agent was added to the solution. The mixture was heated at 100 for 1 hour, and then cooled down to room temperature. The Ag colloid was stored in a dark place for future use.

4.3.5 UV-vis, SEM and SERS measurement

UV-Vis absorption spectra of the silver colloidal solutions were obtained using a Beckman Coulter DU 640 spectrometer. The silver colloid was diluted at a 1:3 ratio with DI water, and the solution was placed into a quartz cuvette. The solution was scanned from 200 to 800 nm for 0.5 s and the resolution is set at 5 nm.

Scanning electron microscopy (SEM) images of Ag nanoparticles were obtained using a Hitachi S-800 microscope located at the Nanotechnology Research and Education Center (NREC) at the University of South Florida Tampa campus. In order to
obtain the SEM images, about 2 µL of the silver colloidal solutions were dropped onto a silicon wafer and air dried. The wafers were kept away from light because of silver's photosensitivity. The morphologies of nanoparticles were characterized at an accelerating voltage of 25 kV.

Samples were prepared by adding either 300 µL of the Ag colloidal or 10 µL of the core-shell colloidal solution, distilled water, an aggregating agent, and a target molecule (Rhodamine 6 G, 4-aminothiophenol, or COT) into a glass cuvette, and then agitating the mixture with a pipette.

All the SERS experiments were carried out on a Confocal Raman Microscope purchased from Horiba Jovin Yvon, Inc., which was equipped with an argon/krypton laser (Innova 70C series, Coherent). All experiments employed a 514 nm wavelength laser at 42 mW of power, 5 s of exposure time, and 3 accumulations. The spectrum grating was 600 grooves/mm, and a 20X objective was used throughout the experiments. All reported results are derived from triplicate measurements.

4.4 Results and Discussion

4.4.1 SiO₂ nanoparticle synthesis

Experimental conditions have been shown to affect the sizes of SiO₂ nanoparticles including concentrations of reactants, amount of solvent, reaction temperature, and pH. We obtained the desired sizes of SiO₂ nanoparticles by controlling temperature and reaction time. The SEM images in Figure 1 show the synthesized SiO₂ spheres with varied average sizes, which are 111, 76, 53, and 34 nm. SiO₂ nanoparticles of 53, 76, and 111 nm were prepared by keeping reaction time
constant while varying reaction temperatures. To obtain an average size of 34 nm for the SiO$_2$ nanoparticles, 55 °C and a 10 min reaction time were applied to the reaction mixture. Higher temperature and limited reaction time were necessary to obtain smaller SiO$_2$ nanoparticles since their growth is already rapid at room temperature. As shown in Figure 1, the size of SiO$_2$ nanoparticles decreases as the temperature increases. Higher temperatures can accelerate the rate of nucleation of SiO$_2$, and most of the TEOS molecules can be consumed to produce many smaller seeds at those elevated temperatures, which consequently restricts the sizes of the nanoparticles. According to the study by Tan, temperature is the most significant factor for the formation of SiO$_2$ nanoparticles.$^{118}$

4.4.2 Formation of Ag shells and tuning shell thickness

It is possible to control the shell thickness by adjusting the ratio between the amount of SiO$_2$ and AgNO$_3$. As AgNO$_3$ concentration was increased with respect to a fixed mass of SiO$_2$, the Ag shell gradually thickened. Core-shell structures with varying thickness for each of the four differently sized silica cores were synthesized to find the optimal silver thickness for each. As shown in the SEM images for the core-shell nanoparticles (Figure 1i, 1k, 1m, and 1o), the formation of Ag shells on the SiO$_2$ cores was confirmed by the observable size difference from their SiO$_2$ counterparts (Table 1) and with SEM-EDS analysis (supporting information 1). The histograms in Figure 1 of both the SiO$_2$ nanosphere and SiO$_2$@Ag nanoparticles depict the size distributions for all synthesized nanoparticles. The size distribution was based on the analysis of at least one hundred nanoparticles.
Table 0.1 Comparative size differences of SiO$_2$ nanoparticles before and after Ag-shell formation.

<table>
<thead>
<tr>
<th>SiO$_2$ core average size (nm)</th>
<th>SiO$_2$@Ag core-shell average size (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>34 ± 9</td>
<td>41 ± 9</td>
</tr>
<tr>
<td>53 ± 11</td>
<td>68 ± 6</td>
</tr>
<tr>
<td>76 ± 9</td>
<td>99 ± 12</td>
</tr>
<tr>
<td>111 ± 18</td>
<td>124 ± 21</td>
</tr>
</tbody>
</table>

4.4.3 SiO$_2$/Ag for SERS Enhancement

Dielectric core silver shell structures serve as excellent SERS substrates due to the hybridization of plasmons from both the inner and outer regions of the shell.$^{102}$ Since SERS enhancement is sensitive to the nanoparticle size and the relative dimensions between the core and shell, it is worth studying both the size effects of core shell structures and optimal thicknesses of the silver shells for each. Figure 2 depicts how varied silver shell thickness affects SERS intensity on four silica core size ranges. Rhodamine 6G (R6G) was used as a target molecule to directly compare the SERS sensitivities. Figure 2a shows a typical SERS spectrum of R6G using 76 nm SiO$_2$@23 nm Ag nanoparticles (prepared from 11 mM AgNO$_3$) with 30 mM NaBr as an aggregating agent.
An aggregating agent was used because it further enhances the SERS intensity of a target molecule by creating hot spots. (supporting information 2(b)) The strongest Raman peak at 1655 cm$^{-1}$ corresponds to aromatic C–C stretching for R6G (at 100 pM). This peak was chosen for comparing the SERS intensities of silica core silver shell nanoparticles.

Figure 4.2.(b) shows the effect of AgNO$_3$ concentration, hence the Ag shell thickness on the SERS intensity of R6G. Generally, the SERS intensities of R6G for all four silica cores increase initially as AgNO$_3$ concentration and Ag shell thickness increases. For SiO$_2$ core of 34, 53, 76, and 111 nm, the highest SERS intensity was observed when the AgNO$_3$ concentration was 3, 7, 11, 7 mM, respectively. These silver ion concentrations correlate with the formed shell thicknesses that induce the optimal...
surface plasmons from four core-shell nanoparticles. From Figure 4.1, the average shell thickness of Ag can be estimated to be 7, 15, 23, and 13 nm respectively. Further increase of AgNO$_3$ concentration leads to the decrease of SERS intensity.

Figure 4.2.(c) depicts the relationship between SERS intensity and the core size of the core-shell nanoparticles. Among the four studied sizes of core-shell nanoparticles, the highest SERS enhancement of R6G detection is achieved with an 76 nm SiO$_2$@23 nm Ag nanoparticles. This indicates that both the thickness of silver shell and the size of SiO$_2$ core affect SERS activity. Although many research groups have fabricated SiO$_2$ cores to study SERS activity of SiO$_2$ core-Ag shell nanoparticles, the size of the core is larger than 200 nm which is way too big to achieve the optimal SERS activity.$^{44,104-108}$ Interestingly, a theoretical study has been conducted on such optimizations.$^{40,120}$ According to Tanabe, the maximum field enhancement of SiO$_2$ core-Ag shell nanoparticles in water was achievable at a core-shell diameter ratio of 0.87. Our data agrees well with those predictions since the diameter ratios of our 111 nm SiO$_2$@13 nm Ag, 76 nm SiO$_2$@23 nm Ag, 53 nm SiO$_2$@15 nm Ag, and 34 nm SiO$_2$@7 nm Ag core-shell nanoparticles are 0.83, 0.78, 0.77, and 0.9 respectively.$^{40}$ Additionally, Halas theoretically argued that core radii between 65 and 79 nm would produce the maximal SERS response. The results we obtained indicated that 76 nm SiO$_2$ core-Ag shell nanoparticle produce the strongest SERS enhancement, which agree with the theoretical size proposed by Halas.$^{120}$
4.4.4 UV-vis Analysis for Surface Plasmon Resonance

Figure 4.3 shows the UV-vis spectra of core-shell nanoparticles, which indicate the effect of shell and size on surface plasmon resonance. UV-vis spectra of 76 nm SiO$_2$@ Ag nanoparticles were obtained with four concentrations of AgNO$_3$, which
exhibited plasma resonance bands at 406, 425, and 427 nm for 7 mM, 9 mM, and 11 mM AgNO₃, respectively. As Ag shell thickness on the 76 nm SiO₂ surfaces increases (from increased AgNO₃ concentration), the plasmon resonance peak red-shifts from 406 nm to 427 nm, which is commonly observed with core-shell structures.¹⁰⁸,¹²¹ That peak did not appear when 13 mM AgNO₃ was used to synthesize the shell, but a broad band ranging from the ultraviolet to near-infrared regions did appear.⁴⁴,¹⁰⁴,¹⁰⁸,¹²² This broadening of the resonance band is useful in biological applications because radiation of the near-infrared region can penetrate human tissue without damaging cells, hemoglobin, or melatonin.

The core size effects on surface plasmon resonance were also studied, which is presented in Figure 3b. The same concentration of AgNO₃ at 11 mM was used for all four different sizes of SiO₂ cores, and their plasmon resonances were tuned with the SiO₂ core. The plasmon bands of 34, 53, 176, and 113 nm SiO₂@Ag nanoparticles appeared at 435, 430, 425, and 405 nm, respectively. With the increase of the core size, the position of the peak was gradually blue-shifted. These observations indicate the tunability of core-shell nanoparticles which depends on the size of both the core and shell due to the hybridization of the inner and outer shell.

Among the four different sizes of silica cores, the highest SERS enhancement was achieved when 76 nm SiO₂@23 nm Ag nanoparticle were used as the SERS substrates (Figure 4.2). It is also worth mentioning that excellent stability was demonstrated based on UV-vis spectroscopy.
Figure 0-3 UV-vis spectra of core-shell nanoparticles
(a) Ag shells formed on 76 nm SiO$_2$ at concentrations of 7 mM (blue), 9 mM (red), 11 mM (green), and 13 mM (purple) AgNO$_3$, (b) 11 mM AgNO$_3$ applied to 34 nm (purple), 53 nm (green), 76 nm (red), and 111 nm (blue) SiO$_2$ cores.

Figure 4.4 shows the UV-vis spectra of 76 nm SiO$_2$@23 nm Ag over a measurement period of 5 weeks. A decrease in the absorbance of the core-shell solution was not observed during the first 3 weeks. After 5 weeks, the peak intensity gradually decreased, which indicates that the nanoparticles are stable for 1 month when they are stored in a dark place at room temperature.

4.4.5 SERS Enhancement of R6G, 4-ATP, and COT

Since 76 nm SiO$_2$@23 nm Ag nanoparticles exhibited the highest sensitivity, these nanoparticles were tested further to compare its SERS activity with silver nanoparticles. Figure 4.5 shows the SERS spectra of 76 nm SiO$_2$@23 nm Ag nanoparticles and Ag nanoparticle using Rhodamine 6G (R6G), 4-aminothiophenol (4-ATP), and cotinine (COT) as target molecules. Here, Ag colloid was fabricated by the method of Lee and Meisel. The average size of Ag nanoparticles was estimated to be 67 ± 19 on the SEM image (supporting information 3). This diameter of Ag nanoparticle
is expected to produce strong SERS intensity.\textsuperscript{32} In order to achieve the greatest SERS enhancements, sodium bromide was applied for R6G and 4-ATP and magnesium chloride was applied for COT as aggregating agents that create hot spots.\textsuperscript{43} The concentrations of the two aggregating agents were optimized for the highest SERS intensities with respect to each target molecule.

The dye R6G is a commonly used SERS target molecule. In Figure 4.5.(a), four SERS spectra of 100 pM R6G, obtained using core-shell and silver nanoparticles, are presented. The spectra clearly demonstrate that SiO$_2$@Ag nanoparticles exhibit higher SERS intensity than that of silver nanoparticles. The peaks observed in the SERS spectra of R6G agree with the literature.\textsuperscript{107,119} The presence of NaBr significantly enhanced the SERS bands of R6G, and the contribution of the aggregating agent was so critical that no observable peaks corresponding to R6G were produced when it is not present. The strongest peak, occurring at 1655 cm$^{-1}$, is attributed to a symmetric C=C
stretching in the xanthene ring of R6G,\textsuperscript{119,123} and it was used to calculate the SERS enhancement factors (EF) according to the formula.\textsuperscript{10}

The SERS EFs of R6G for core-shell and Ag nanoparticles with NaBr were calculated to be $2.5 \times 10^{11}$ and $1.0 \times 10^{10}$, respectively.\textsuperscript{20} The SERS enhancement generated by core-shell nanoparticles is roughly 25 times higher than those of silver nanoparticles. Additionally, R6G could be detected at concentrations down to sub-picomolar, which indicates our prepared optimized core and shell nanoparticles are more sensitive than those reported previously.\textsuperscript{44,107,124}

The SERS spectra for 4-ATP as the target molecule are shown in Figure 4.5.(b). Core-shell nanoparticle without NaBr exhibited the strongest band of 4-ATP at 1442 cm$^{-1}$, which is assigned to a b2-type mode.\textsuperscript{125} However, when NaBr was added to the colloid solution, all the peaks corresponding to 4-ATP considerably decreased. Clearly, NaBr is not an effective aggregating agent for 4-ATP. This behavior may originate from the inherent ability of 4-ATP to aggregate silver nanoparticles.\textsuperscript{126} It was previously demonstrated that adding aggregating agents beyond their optimal concentrations decreased the SERS signal of analyte. To the contrary, in the case of silver nanoparticles, the intensities of 4-ATP bands were weak without NaBr. The addition of NaBr leads to the considerable increase of SERS enhancement of 4-ATP for the peak at 1078 cm$^{-1}$, which is assigned to an a1-type mode.\textsuperscript{125} This observation can be explained using the adatom mode proposed by Otto.\textsuperscript{95} In this mode, a target molecule adsorbs on the metal surface and forms a surface complex with a metal atom and halide ion (Ag-molecule-anion), where electrostatic interaction occurs. This interaction influences SERS behavior. The electrostatic force of surface complex is mainly affected
by the properties of the molecule and anion, and it is slightly influenced by the cations existing in bulk layer.\textsuperscript{97} In terms of the ions compositions, there was a different magnitude of ion strength for the core-shell and Ag nanoparticles because less sodium and citrate ions were used to reduce silver ions to Ag nanoparticles than employed for the reduction of silver ions to core-shell nanoparticles. Therefore, the Ag nanoparticle solution may have achieved a stronger SERS signal for the detection of 4-ATP because the addition of the aggregating agent simply increased the ionic strength of the solution.

Interestingly, the relative intensities of the SERS peaks for 4-ATP were also affected by the addition of NaBr. This behavior may have resulted from a change in the orientation of 4-ATP on the surfaces of the nanoparticles that was induced by the ions due to the properties of 4-ATP.\textsuperscript{127,128} In order to estimate the SERS EF of 4-ATP, two strong peaks, at 1442 cm\textsuperscript{-1} and 1078 cm\textsuperscript{-1}, were used. The core-shell SERS EFs were 1.2 × 10\textsuperscript{9} at 1078 cm\textsuperscript{-1} and 3.5 × 10\textsuperscript{9} at 1442 cm\textsuperscript{-1}, and those of Ag nanoparticle were 1.7 × 10\textsuperscript{9} at 1079 cm\textsuperscript{-1} and 2.6 × 10\textsuperscript{8} at 1442 cm\textsuperscript{-1}. The highest SERS EF was exhibited for the 1142 cm\textsuperscript{-1} peak of 4-ATP using core-shell nanoparticles without an aggregating agent, which is 2 times greater than the 1079 cm\textsuperscript{-1} using Ag nanoparticles with NaBr.

To further understand the impact of core-shell nanoparticles on SERS sensitivity, we compared our results for 4-ATP to that of previous studies. Wu’s group reported that they were able to detect 4-ATP down to 5 × 10\textsuperscript{6} M using their silica core and silver shell nanoparticle.\textsuperscript{104} Our optimized core-shell nanoparticles can be used to detect 4-ATP at concentrations below one nanomolar as demonstrated in Figure 5b, which is 200 times lower than what has been reported previously.\textsuperscript{104}
Figure 4.5.(c) shows the SERS spectra of COT as a target molecule. COT is known as a biomarker for metabolized nicotine. During our previous study, COT was observed to be SERS-active due to the presence of pyridine ring, and MgCl$_2$ served as an optimal aggregating agent. Using the same aggregating agent, a strong peak at 1035 cm$^{-1}$ was observed for both core-shell and silver nanoparticle, which is assigned to the symmetric ring breathing mode. Since the band at 1035 cm$^{-1}$ was not observed for either nanoparticle when MgCl$_2$ was excluded, its addition and optimization were necessary to compare the SERS enhancements of core-shell and silver nanoparticles. The SERS intensity of COT with the core-shell substrates demonstrated an EF nearly 3 times greater than that of the silver substrates ($1.3 \times 10^4$ for core-shell and $5 \times 10^3$ for silver nanoparticle).

By comparing SERS sensitivity of core-shell to that of silver nanoparticle using three target molecules, 76 nm SiO$_2$@23 nm Ag nanoparticles are consistently better SERS substrates than silver nanoparticles. For two of the target molecules, R6G and 4-ATP, we compared 76 nm SiO$_2$@23 nm Ag nanoparticles were also compared with core-shell structures reported in previous studies. Not surprisingly, our optimized nanoparticles show greater SERS enhancement than those reported with 200 nm sized silica cores, which signifies that the core size for core-shell nanoparticles is an important factor that affects SERS enhancement in addition to the diameter ratio between core and shell. This phenomenon can be explained by the differences in electron density on metal surfaces of varying nanoparticle size. As the core size increases beyond 76 nm, the shell thickness also increases, which can result in a decreased electron
density on the metal surface. The reduced electron density may lower SERS enhancement for those corresponding nanoparticles.

In addition, 76 nm SiO$_2$@Ag (13 mM AgNO$_3$) nanoparticle showing broad UV-spectrum in Figure 4.3. (a) was examined on SERS enhancement of R6G with different excitation lasers (514 and 647nm), and compared their SERS EFs to that of Ag nanoparticle (Table 4.2). Although this result does not directly mean that UV-vis absorption is not a primary factor to dominate SERS EFs of substrates,$^{129}$ the decrease of SERS EFs of the core-shell nanoparticle from 514 nm to 647 nm is less than that of Ag nanoparticle, which indicates the applicable wavelength range of 76 nm SiO$_2$@Ag (13 mM AgNO$_3$) is wider than Ag nanoparticle.

<table>
<thead>
<tr>
<th></th>
<th>514 nm</th>
<th>647 nm</th>
</tr>
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<tbody>
<tr>
<td>76 nm SiO$_2$@Ag</td>
<td>5.1×10$^9$</td>
<td>1.0×10$^8$</td>
</tr>
<tr>
<td>Ag</td>
<td>3.4×10$^9$</td>
<td>5.8×10$^7$</td>
</tr>
</tbody>
</table>
Figure 0-5 SERS spectra of R6G, 4-ATP and COT
(a) 100 pM R6G, (b) 100 nM 4-ATP, and (c) 10 uM COT using 81 nm SiO$_2$@16 nm Ag core-shell and Ag nanoparticles both with and without aggregating agents.
4.5 Conclusions

To maximize the SERS enhancement of the dielectric core and silver shell nanoparticle, we synthesized silica core with four different sizes, 34, 53, 76, and 111 nm, and then coated various thicknesses of silver shells on each core by controlling the AgNO$_3$ concentration. To achieve the highest SERS enhancement the AgNO$_3$ concentration was further optimized: 34, 52, 81, and 113 nm SiO$_2$ cores required 3, 7, 11, and 7 mM AgNO$_3$ respectively. The diameter ratios for the core-shell nanoparticles was estimated based on SEM: 0.90 for 34 nm SiO$_2$@7 nm Ag, 0.77 for 53 nm SiO$_2$@15 nm Ag, 0.78 for 76 nm SiO$_2$@23 nm Ag, and 0.83 for 111 nm SiO$_2$@13 nm Ag. Our data agree well with the computational results performed by Tanabe that the field enhancement factor of SiO$_2$/Ag in aqueous media is strongest when the core to shell diameter is around 0.87.\textsuperscript{40} UV-vis extinction measurements confirmed that surface plasmon resonance can be tuned by changing either diameter for the core-shell structures. The surface plasmon resonance of 76 nm SiO$_2$@Ag (13 mM AgNO$_3$) nanoparticle was extended to the near-infrared region, which makes it useful for biomedical applications. However, the highest SERS enhancement was achieved using 76 nm SiO$_2$@23 nm Ag nanoparticles, which produced observably stronger SERS signals for the three target molecules tested here (R6G, 4-ATP, and COT) than silver nanoparticles, the most commonly used SERS substrates. More importantly, the optimized SiO$_2$@Ag nanoparticles can be used to R6G down to nanomoles, and shows greater SERS intensities of R6G and 4-ATP than those reported in previous studies. Our study demonstrated the crucial effect of the size and relative diameters of core-shell structures on the SERS enhancement.
CHAPTER FIVE: Application of Raman spectroscopy in Material science

5.1 Raman Study of the Encapsulation of Microperoxidase 11 (MP-11) in Tb-mesoMOFs

Note to the Reader:

Portions of this chapter consisting of Raman experiments has been previously published (Yao Chen, Sungyub Han, Xiao Li, Zhenjie Zhang, and Shengqian Ma)


Author contributions: SH and XL designed the experimental research for the Raman experiments; SH performed research, analyzed data and wrote the initial draft; XL improvised the draft.

5.1.1 Introduction

Microperoxidase 11 (MP-11) is a product of proteolytic digestion of the heme protein cytochrome C. MP-11 possess Fe²⁺ (ferrous) ion located in the center of porphyrin of MP-11 which is called heme and includes 11 polypeptides covalently bound through two thioether bonds of heme from Cys-14 and Cys-17. The imidazole group of His-18 in polypeptide groups is involved to form an axial bond to ferrous ion
resulting in 5 coordination bonds oriented to ferrous ion of heme group. MP-11 is produced by the proteolytic digestion of the electron transfer heme protein cytochrome c. Due to the peroxidase activity and the ability to hydroxylate organic compounds, the MPs can be used as models for enzymatic peroxidase function.\textsuperscript{131,132} Enzyme is well known to be catalysis designed by nature with environmentally friendly and high effectivity. This has allowed enzyme to apply in chemical, pharmaceutical, and food industries.\textsuperscript{133,134} However, the application of enzyme in these fields is often limited by their low stability, difficult recovery and lack of reusability.\textsuperscript{133,135,136} In order to overcome their limitation, solid supports are adopted to immobilize enzyme into their insides and should maintain the activity, stability, ease of separation, and facial recovery of enzyme for reuse.\textsuperscript{137} Metal-organic frameworks (MOFs) are crystalline matrix that consists of organic ligands bound to metal ions. Their pore size of MOFs can be controlled for the encapsulation of the enzyme of interest from microporous to mesoporous. According to the previous study, Mesoporous metal-organic frameworks (mesoMOFs) show better immobilization of enzyme MP-11 and more efficient enzymatic catalysis than mesoporous silica materials.\textsuperscript{138} This result implies that the organic ligands of meso MOFs play an important role in retaining MP-11 molecules to create stronger interaction between them. As Figure 5. 1. 1 is shown, there is a strong $\pi\cdots\pi$ interactions between TATB and the heme of MP-11. This interaction is a force to retention of MP-11 molecules inside the pores of the MOFs
Here, Raman spectroscopy is used to explore the interaction of MOFs to MP-11. Raman spectroscopy, as one of the most convenient vibrational spectroscopies, has been widely used for various fields due to its advantages such as non-destructive, quick sample preparation and simple data analysis. The Raman studies on chemical compounds allow for both quantitative and qualitative analysis of the chemical structures, and have been extensively applied to study intermolecular interactions of various biomolecules including complicated systems involving cancer cells, hemoglobin and hormones. It was also demonstrated to successfully investigate the reaction mechanism and dynamic in hemo-proteions, and detect molecules adsorbed on porous materials. Recently, Raman spectroscopy has been utilized to study gas molecules adsorbed in MOFs. The success of those studies encouraged us to employ Raman spectroscopy to investigate the interactions between biomolecules and MOF.

5.1.2 Experimental

In order to obtain optimal intensities of the samples, different exposure times were applied for the Raman measurements 5 s for buffer solution and Tb-mesoMOF, 10 s for MP-11@Tb-mesoMOF, 15 s for MP-11 sample, and 30 s for MP-11@MCM-41. All
the samples were tested in the same HEPES buffer, therefore, the effect of solvation in HEPES on the vibrational modes were taken into consideration. Before measuring MP-11@Tb-mesoMOF and MP-11@MCM-41, the Raman spectra of HEPES buffer (red) and MP-11 dissolved in HEPES buffer (black) were collected between 400 and 1800 cm$^{-1}$.

5.1.3 Results and Discussion

As shown in Figure 5.1.2, strong Raman bands belonging to the buffer solution are observed in the spectrum of MP-11 solution. In addition, the fingerprint vibrational modes of MP-11 can be clearly identified at 1172 cm$^{-1}$ (v30 of heme), 1317 cm$^{-1}$ (v21 of heme), 1374 cm$^{-1}$ (v4 of heme), 1567 cm$^{-1}$ (v19 of heme), 1596 cm$^{-1}$ (v37 of heme), and 1644 cm$^{-1}$ (v10 of heme).

![Raman spectra of 50 μM MP-11 dissolved in HEPES buffer (black) and the HEPES buffer solution (red).](image)

Figure 0-1-2 Raman spectra of 50 μM MP-11 dissolved in HEPES buffer (black) and the HEPES buffer solution (red).
Figure 5.1.3 shows the Raman spectra of MP-11@Tb-mesoMOF, Tb-mesoMOF and MP-11 in the HEPES buffer. It is notable that since confocal Raman microscopy was used during the measurements, the laser was focused on the MP-11@Tb-mesoMOF crystal at the bottom of cuvette. Therefore, even with the presence of MP-11@Tb-mesoMOF and Tb-mesoMOF crystals in the pH 7.5 HEPES buffer, only little amount of HEPES solution was within the focus volume of the laser. Thus, no strong
signal was observed from HEPES buffer. It can be clearly seen that the Raman spectrum of MP-11@Tb-mesoMOF exhibits the characteristic bands from both MP-11 and Tb-mesoMOF, indicating the presence of MP-11 in Tb-mesoMOF. For example, the peak at 1371 cm\(^{-1}\) (dotted line in Figure 1a) assigned to the v4 (C-N) of heme of MP-11 was observed in the spectra of both MP-11 and MP-11@Tb-mesoMOF. Interestingly, significant energy shifts of Raman bands of MP-11 were found in MP-11@Tb-mesoMOF. As shown in Figure 1b, the six vibrational bands of MP-11 at 1172 cm\(^{-1}\), 1317 cm\(^{-1}\), 1374 cm\(^{-1}\), 1567 cm\(^{-1}\), 1596 cm\(^{-1}\), and 1644 cm\(^{-1}\) were red shifted to 1167 cm\(^{-1}\), 1311 cm\(^{-1}\), 1371 cm\(^{-1}\), 1556 cm\(^{-1}\), 1577 cm\(^{-1}\), and 1636 cm\(^{-1}\) respectively for MP-11@Tb-mesoMOF. The red shift of the last two peaks at 1577 and 1636 cm\(^{-1}\) was observed previously \(^{13}\) and is due to the disruption of the dimer or oligomer structure of MP-11, which exists in high concentration when encapsulated into the pores. Considering the dimensions of MP-11 are ca. 3.3 × 1.7 ×1.1 nm and the sizes of two types of cages in Tb-mesoMOF are 3.9 nm and 4.7 nm, this suggests that only the monomer form of MP-11 should be accommodated within Tb-mesoMOF. The red shifts observed for other four peaks that are associated with the vibrational modes of either the heme structure of MP-11 suggest that MP-11 molecules interact strongly with the framework of Tb-mesoMOF through either the heme moieties.

In addition, obvious energy shifts of Raman bands of Tb-mesoMOF are also observed with the presence of MP-11 within its pores as shown in Figure 5.1.3 ©. The four vibrational bands of Tb-mesoMOF at 993 cm\(^{-1}\), 1057 cm\(^{-1}\), 1414 cm\(^{-1}\) and 1613 cm\(^{-1}\) are red shifted to 990, 1054, 1409, and 1610 cm\(^{-1}\) respectively for MP-11@Tb-mesoMOF. The Raman bands at 993 and 1414 cm\(^{-1}\) of Tb-mesoMOF originate from the
vibration of triazine of TATB,14 and the band at 1613 cm\(^{-1}\) of Tb-mesoMOF is as-signed to C=C stretching mode of benzene ring of TATB ligand.15 It is noteworthy that the band at 1414 cm\(^{-1}\) associated with the vibration of triazine of TATB undergoes a large shift of 5 cm\(^{-1}\), whereas in the case of MP-11 (Figure 5.4.(b)), the largest band shifts are observed in the peaks at 1317 cm\(^{-1}\) (a shift of 6 cm\(^{-1}\)) and 1567 cm\(^{-1}\) (a shift of 11 cm\(^{-1}\)), which are assigned to C-H and C=C of heme respectively.12 This thus suggests that the triazine in the TATB ligand of Tb-mesoMOF interacts strongly with heme group in MP-11.

These Raman spectroscopic data support a mechanism for MP-11 immobilized inside Tb-mesoMOF (Figure 5.1.1) in which there is also strong π···π interactions between the conjugated systems (including triazine and benzene rings) of TATB and the heme of MP-11. These strong interactions facilitate the retention of MP-11 molecules inside the cages of the MOFs framework.

![Raman spectra](image.png)

Figure 5-1-4 Figure 0-4 Raman spectra of MP-11@MCM-41 (black) and 0.8 mM MP-11 in HEPES buffer solution (red).
We also measured the Raman spectra for MP-11@MCM-41, which experienced severe leaching during catalytic assays. Figure 5.1.4 shows the Raman spectra of MP-11 and MP-11@MCM-41. Despite high concentration of MP-11 used during the immobilization process, the Raman signal of MP-11 encapsulated in MCM-41 is much weaker than in Tb-mesoMOF. This agrees well with the observation that MCM-41 has a much lower loading capacity of MP-11 compared to Tb-mesoMOF.6a Two peaks at 1559 and 1636 cm\(^{-1}\) that are associated with MP-11 are observed for MP-11@MCM-41, confirming the presence of MP-11 in MCM-41. Interestingly, only the peak at 1640 cm\(^{-1}\) in the Raman spectrum was observed to shift position, with a red shift of 4 cm\(^{-1}\), which was also observed for MP-11@Tb-mesoMOF and is due to the disruption of the dimer or oligomer structure of MP-11. No additional peak shift was observed for all other bands. These data suggests that there is no strong specific interaction between MP-11 and MCM-41, which should account for the severe leaching of MP-11 during and after its immobilization into MCM-41.

5.1.4 Conclusion

Raman study of MP-11@Tb-mesoMOFs and MP-11@MCM-41 prove that there is a strong interaction between the framework of Tb-mesoMOFs and MP-11 protein through hydrogen bond involved between amide III of polypeptide of MP-11 and trizaine in the TATB ligand of Tb-meso MOFs as well as \(\pi\cdots\pi\) interaction between the heme of MP-11 and the conjugated triazine and benzene rings of TATB ligand of Tb-mesoMOFs. These interactions make MP-11 retained inside of the pores of the MOFs, whereas Raman study confirmed that there is week interaction between silica material of MCM-41 and MP-11, which may result in the leaching of MP-11 out of the MCM-41.
5.2 Raman study of poly(methyl methacrylate) composites of copper-4,4’-trimethylenediprydein

Note to the Reader:

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5.2.1 Introduction

Coordination polymers (CPs), having repeating units of coordination complex, are composed of metal ions and ligand to produce one, two, and three dimension structures, which is an organic-inorganic hybrid compound. CPs has attracted much attention due to high nanometer-sized porosities, regularity of the framework, high thermal and mechanical stability as well as extremely high surface areas. These advantages are useful for the application of CPs material in gas purification, gas separation, gas storage, and catalysis. As polymer units, poly-methylmethacrylate (PMMA) has been used for synthesis of CPs since it is durable and highly transparent with excellent resistance to ultraviolet radiation and weathering. For the inorganic units of CPs, transition metal ions such as Co (II), Cu (II), Ni (II), Zn (II), and Mn (II) have been commonly used to create metal-organic ligand complex. However, copper
(II) is the desirable materials because it is not expensive and characteristic of flexible coordination sphere.\textsuperscript{153,154} This metal containing polymer has advantages compared to traditional carbon -based polymer: manipulate the oxidation states, facilitate electron flies and add functional groups.\textsuperscript{155} Raman spectroscopy is a powerful technique for polymer analysis, since the vibration of backbone of polymer is in modes of Raman active and no sample preparation is required for Raman measurement of polymer. These advantages have attracted researchers to Raman technique to apply in polymer field.\textsuperscript{156-158} There is a Raman study to find an interaction between PMMA and carbon nanotube as a substitute.\textsuperscript{156} They fabricated single wall carbon nanotube (SWCNT) modified with PMMA polymer and applied Raman technique to discover that there was covalent bound involved between PMMA and SWCNT when acid treated SWCNT was used. In addition, Takahashi et al, have devolved a new method to monitor sorption kinetic of CO\textsubscript{2} in PMMA-CO\textsubscript{2} system.\textsuperscript{159} In this project, in situ polymerization method was utilized to synthesis CPs using copper-4,4'- trimethylenedipyridine (CU-TMDP) and PMMA composites. Raman spectroscopy was used to characterize the interaction between two composites.

\textbf{5.2.2 Experimental}

All Raman experiments were performed using a confocal Raman Microscope equipped with a notch Rayleigh rejection filter, a 600 lines mm\textsuperscript{-1} diffraction grating and a cooled CCD detector. For all experiments, radiation at 514 nm from Argon and Krypton laser was applied with a power of 40 mW to minimize possible damage to the samples. A 20X objective was used throughout the experiments, which yields a spot diameter of less than 5 mm of the sample. In order to obtain high quality Raman spectra, both the
exposure time and accumulation time were varied. To confirm the homogeneity of the polymer samples, Raman spectra were collected at different spots of the sample.

5.2.3 Results and Discussion

![Raman Spectra Diagram]

Figure 0-2-1 Typical Raman spectra of CU-TMDP, PMMA, and 0.5 % CU-TMDP-PMMA composites in two wavenumber ranges: 200-1800 cm\(^{-1}\) (left panel) and 2700-3300 cm\(^{-1}\) (right panel). The exposure times of CU-TMDP, 0.5 % CU-TMDP-PMMA and PMMA are 10, 8, and 15 s in the left panel and 15, 3, and 3 seconds in the right panel, respectively.

The typical Raman spectra of CU-TMDP, PMMA, and 0.5 % CU-TMDP-PMMA complex are shown in Figure 5.2.1. Two regions of spectra were shown: 200-1800 cm\(^{-1}\) and 2700-300 cm\(^{-1}\). In the second region, CU-TMDP shows two vibrational peaks at 2933 cm\(^{-1}\) and 3075 cm\(^{-1}\) which can be assigned to the stretching mode of CH\(_3\) and CH, respectively.\(^{160,161}\) Pure PMMA polymer exhibits three strong peaks at 2846 cm\(^{-1}\), 2954 cm\(^{-1}\) and 3002 cm\(^{-1}\) with a small shoulder at 2925 cm\(^{-1}\) which can be assigned to the combination band of O-CH\(_3\), symmetric stretching mode of CH\(_3\), asymmetric stretching
mode of CH\textsubscript{3} and symmetric stretching mode of CH\textsubscript{2}, respectively.\textsuperscript{162-164} The spectrum of the 0.5 % CU-TMDP-PMMA complex in this region is almost identical to that of PMMA, which can be explained by the relatively low concentration of CU-TMDP in the complex and its low cross section indicated by the low intensity of pure CU-TMDP.

In the range from 200 to 1800 cm\textsuperscript{-1}, a weak peak at 250 cm\textsuperscript{-1} was observed in CU-TMDP spectra, which is the characteristic Cu-N mode of the framework structure.\textsuperscript{165} Also, the peaks observed at 979 cm\textsuperscript{-1}, 1076 cm\textsuperscript{-1}, and 1384 cm\textsuperscript{-1} can be assigned to the rocking mode of CH\textsubscript{3}, stretching mode of the ring and symmetric scissoring mode of CH\textsubscript{3}, respectively.\textsuperscript{160,161} Several strong peaks were observed for PMMA which agree well with previous reports and the peak assignment for those peaks was enlisted.\textsuperscript{162,164,166} One characteristic band of PMMA is the one observed at 1730 cm\textsuperscript{-1} which is attributed to the stretching mode of C=O.\textsuperscript{166} The energy of this peak does not change when CU-TMDP is added to the polymer.

With the addition of CU-TMDP into the PMMA matrix, changes were observed in the Raman spectra as indicated by the arrows in Figure 5.2.1 when compared with that of pure CU-TMDP and PMMA. The four peaks at 664 cm\textsuperscript{-1}, 1208 cm\textsuperscript{-1}, 1560 cm\textsuperscript{-1} and 1621 cm\textsuperscript{-1} are of interest. The peak at 669 cm\textsuperscript{-1} of CU-TMDP was observed to red-shift to 664 cm\textsuperscript{-1} in the CU-TMDP-PMMA complex which belongs to the deformation mode of the ring structure.\textsuperscript{160,161} The peak at 1213 cm\textsuperscript{-1} of the CU-TMDP was observed to red-shift to 1208 cm\textsuperscript{-1} for the CU-TMDP-PMMA complex. It was assigned to the stretching mode of the ring-CH\textsubscript{3} with certain contribution from ring stretching too. For both peaks, a red-shift of 5 cm\textsuperscript{-1} was observed when CU-TMDP-PMMA interacts with PMMA in the complex. Further, a new peak was observed at 1560 cm\textsuperscript{-1} for the CU-TMDP-PMMA
complex which is absent in the spectra of both PMMA and the CU-TMDP. This peak has similar energy as the ring stretching and deformation mode of 4-methylpyridine at 1565 cm$^{-1}$. The absence of this peak in pure the CU-TMDP might be due to the ring scattering background of the solid sample. The peak of the CU-TMDP at 1628 cm$^{-1}$, attributed to the ring stretching and CH deformation mode of pyridine ring structure, was observed to red-shift to 1521 m$^{-1}$ for the CU-TMDP-PMMA. Interestingly, all the changes observed when the CU-TMDP was dispersed in the PMMA matrix are form vibrational peaks associated with the ring structure of the PCP. Such observations indicate that pyridine ring structure is involved when the CU-TMDP interacts with the PMMA matrix. Since the energy shift of those peaks is relatively small, a weak interaction between the ring structure and PMMA is expected such as a dipole-dipole interaction or London dispersion force.

5.2.4 Conclusion

Raman measurements of CU-TMDP, PMMA, and CU-TMDP-PMMA complex unveils a red-shift of several vibrational peaks involved with the ring of the PCP when CU-TMDP is dispersed in the polymer and the weak interaction is discovered to be dipole-dipole interaction or London dispersion force. Raman technique is effective and simple analysis tool to characterize polymer materials.

5.3 Raman study of surfactant-free TiO2 surface hydroxyl groups
Note to the Reader:

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Author contributions: SH and XL designed the experimental research for the Raman experiments; SH performed research, analyzed data and wrote the initial draft; XL improvised the draft.

5.3.1 Introduction

Titanium dioxide (TiO$_2$) is one of promising materials for environmentally friendly renewable energy applications due to its photoactivity, stability against both photo and chemical, low cost and non-toxicity.$^{167-169}$ Behind these properties of TiO$_2$, a research has discovered the other characteristics to use its wide band-gap of TiO$_2$ as semiconductor to produce self-cleaning.$^{170,171}$ The self-cleaning mechanism of TiO$_2$ is mainly laid on photocatalysis of TiO$_2$, which electron holes induced by photo energy catalyze the reaction on the surface. This light induced self-cleaning process is applicable to window glass cleaning, solar panel cleaning, and cements to textiles. Self-cleaning are categorized into hydrophilic and hydrophobic. In a hydrophilic, when the water is spread over the surface, the dirt and impurities is carried away from the surface. Whereas, in hydrophobic, water droplets slide and roll over the surface thereby cleaning them.$^{172}$ Traditionally, glass is the main substrates that TiO$_2$ have been coated.
It is known that the application of TiO$_2$ film to polymer has shown higher rate of photocatalytic activity over the glass.$^{173}$ From many types of polymer materials, polycarbonate (CP) is a prominent polymer to achieve an effective self-cleaning property with TiO$_2$.\textsuperscript{174} CPs has many merits over glass due to its applications in earthquake prone areas as window in skyscrapers as alternative material of glass and in the auto industry for headlight enclosures. The self-cleaning activity of TiO$_2$ is critically dependent on its crystal phase. There are three types of crystal phases: anatase, rutile, and brookite. These different phases are subjective to the density of hydroxyl groups on TiO$_2$ surface produced by the pH environment of reaction medium. In this project, Raman spectroscopy is adopted to unveiling the crystalline transformation of TiO$_2$ at various pH ranges. Raman technique has been proved to be an outstanding analytical method to detect the structural differences of TiO$_2$ crystals because of high sensitivity of Raman scattering cross-sections for different crystalline phase.$^{175,176}$ According to the researches by Shen, Raman spectroscopy has achieved the quantitative measurement of rutile phases from the mixture of rutile and anatase.$^{176}$ This result indicates Raman technique has a potential ability for quantitative and qualitative measurement of a phase contents on the phase transformation as a high sensitive analytical method. Additionally, Raman spectroscopy has succeed to characterize both a large single crystalline of TiO$_2$ and nano-crystalline anatase of TiO$_2$.\textsuperscript{177} There is one of interesting researches of TiO$_2$ nanoparticle using SERS technique done by Zhao et al. they fabricated a free-standing electrospun nanofibrous mat containing TiO$_2$ nanoparticles and decorate them again with Ag nanoparticles. This SERS substrate was used for SERS measurement with target molecules. Later, the
target proves were removed by UV irradiation using the self-cleaning property of the TiO₂, the removal of the molecules was confirmed again using SERS measurement. As these studies are shown, Raman technique has an extraordinary potential to apply in inorganic field. Herein, the analysis approach of Raman spectroscopy for this project is considerably practical.

5.3.2 Experiment

All Raman experiments were carried out using a confocal Raman microscope from Horiba Jovin Yvon, equipped with an Argon and Krypton laser. An excitation laser wavelength 514 nm was used with a power of 78 mW at room temperature. Three accumulations and 5 s exposure times were employed for all the samples. The spectrograph grating was 600 grooves mm⁻¹ and a 20 X objective was used. Raman spectra ranging from 50 to 4000 cm⁻¹ were collected. However, only the region with interesting peaks from 100 to 1000 cm⁻¹ is shown and discussed here. To show the role of hydroxyl groups on the TiO₂ nanoparticles, the Raman spectra of the solvent was obtained by isolating it from the nanoparticles using an electrophoresis technique.

5.3.3 Results and discussion

Raman spectroscopic investigations of phonons of different symmetries in TiO₂ nanocrystals synthesized by the sol-gel method were carried out. Figure 5.3.1 (A) shows Raman spectra of crystalline TiO₂ solutions with different pH conditions. To verify that the appearance of the bands is caused by TiO₂ nanoparticles and not the solvent (water), the nanoparticles were isolated with the previously mentioned electrophoresis technique.
There are a total of six Raman bands which are absent in the spectra of the aqueous solution without the presence of TiO$_2$ nanoparticles (Figure 5.3.1 (b)).

Figure 5.3.1 (A) presents five major bands at ~ 150, 285, 508, 678, and 906 cm$^{-1}$ and another weak band at 822 cm$^{-1}$. Two Raman bands of the anatase phase of TiO$_2$ were observed at ~ 150 and ~ 508 cm$^{-1}$. These bands are assigned to $E_g$ and $B_{1g}/A_{1g}$ photonic modes, respectively.$^{178,179}$ The $E_g$ mode centered around 150 cm$^{-1}$ is the most commonly used anatase Raman band to probe phonon confinement effects.$^{180}$ One can see from figure 5.3.1 (A) that a change in the pH of peroxotitanium complex from 7.3 to 8.3 induces a shift (toward lower wavenumbers) of the ~150 cm$^{-1}$ $E_g$ phonon mode occurs for crystalline nanoparticles of larger sizes. This is in line with the previous DLS observations which showed larger particles size for the sample pH 8.3 vs. pH 7.3 and will be further confirmed in XRD section. As it can be seen, Raman active phonons of rutile at 442 ($E_g$), 607 ($A_{1g}$), and 692 cm$^{-1}$ are absent in the presented Raman spectra in Figure 5.3.1 (A).$^{181}$ Conversely, further structural analysis by XRD later shows the
presence of rutile peaks in the pattern. One notes that the appearance of rutile peaks in XRD patterns is most likely due to the post-annealing of the crystalline TiO_2 nanoparticles at 90 °C, which give rise to the formation of rutile. The Raman band at 668 cm\(^{-1}\) could be interpreted as Ti–O–Ti crystal phonons,\(^{179-181}\) which is shifted to 678 cm\(^{-1}\) in the presented Raman spectra figure 5.3.1 (A). Several factors including non-homogeneity of the size distribution,\(^{182,183}\) as well as defects and nonstoichiometry may contribute to the changes in the peak position, line-width, and the shape of the Raman modes.\(^{184}\) It was demonstrated that the Raman lines become also weak and broad when the samples have local lattice imperfections, which might be related to the oxygen stoichiometry of the material and crystallinity.\(^{184,185}\) Hence, absence of the observed strong peak at 150 cm\(^{-1}\) at pH 9.3 and 10.3 as well as the change in the line-width and the shape of Raman band at 678 cm\(^{-1}\), can be described by the increase in the amount of noncrystalline phase as well as increase in the heterogeneity of size distribution. The weak band at 822 cm\(^{-1}\) is related to a covalent Ti–O–H bond,\(^{179,186}\) while the vibration band at 906 cm\(^{-1}\) could also be attributed to Ti–O–H bonds.\(^{186}\) It has already been shown for TiO_2 nanotubes that the Raman band at 922 cm\(^{-1}\) is related to Ti–O–H vibrations.\(^{186,187}\) In this study, the effect of pH of peroxotitanium complex on the structure of crystalline TiO_2 was mainly shown in the intensity change of the band at 906 cm\(^{-1}\). The pH acts on the protonation and de-protonation equilibrium of surface hydroxylated groups. The use of NH_4OH to adjust the pH levels of the samples can promote the formation of Ti–O–H. As pH increases during the refluxing process, TiO_2 can preferably bind to the –OH– groups surrounding the crystalline phase which results in interrupting the formation of the anatase. The increase in pH seems to cause this
peak at 906 cm\(^{-1}\) to become gradually enhanced, while simultaneously causing the strong peak at 150 cm\(^{-1}\) to fade away. One observes that by increasing the pH of peroxotitanium complex the intensity of the weak band at 822 cm\(^{-1}\) (covalent Ti–O–H) increases slightly, as well. The Raman bands at 285 and 678 cm\(^{-1}\) have been previously observed from the TiO\(_2\) anatase dried at 373 K.\(^{186}\) High temperatures can induce the transform of the TiO\(_2\) structure.\(^{186}\) Hence, the presence of the Raman peaks at 285 and 678 cm\(^{-1}\) suggest that a higher pH environment (in the peroxotitanium complex) might play the same role as high temperature annealing in the structural transformation of crystalline TiO\(_2\) since these peaks at 285 and 678 cm\(^{-1}\) are intensified with increasing the pH of peroxotitanium complex. The above mentioned Raman study regarding the TiO\(_2\) amorphous samples treated with different pH levels indicates that the pH environment affects the formation of the anatase phase of TiO\(_2\). It can be deduced that as the pH level increases, the formation of the anatase phase of TiO\(_2\) is interrupted which transforms the TiO\(_2\) structures.

**5.3.4 Conclusion**

The work reveals that Raman spectroscopy successfully characterizes the transformations of TiO\(_2\) crystalline when TiO\(_2\) synthesis is processed at various pH levels.
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1. SILVER NANOPARTICLES SYNTHESIS

Silver nanoparticle solutions was prepared by Lee and Meisel method. To put it concretely, weight out 0.017 g of silver nitrate (AgNO\textsubscript{3}) (Fisher Scientific, S486-100) and transfer quantitatively it into 150 ml of Erlenmeyer flask previously treated by acid wash, then fill it up 100 ml of Millipore water to make 1 mM AgNO\textsubscript{3}. As a reducing agent, 0.1 g of trisodium citrate (Na\textsubscript{3}C\textsubscript{6}H\textsubscript{5}H\textsubscript{5}O\textsubscript{7}·2H\textsubscript{2}O)(Fisher Scientific, BP327-500) is placed into a 50 ml of glass ware and pour 10 ml of Millipore water to make 1 % (w/w) trisodium citrate solution. In order to hit up AgNO\textsubscript{3} solution, put a clean stirring bar into the Erlenmeyer flask and place it on the hot plat. Since it is necessary to monitor the temperature of the AgNO\textsubscript{3} solution, make sure the bottom of thermometer is completely immersed into the AgNO\textsubscript{3} solution. Apply heat to the AgNO\textsubscript{3} solution up to 95 °C while the solution is vigorously stirred, and then turn off all of lights in the room. Once the temperature is reached, 2 ml of 1 % trisodium citrate solution is immediately added into the AgNO\textsubscript{3} solution using a micro pepette. After then, it is observed that the color of AgNO\textsubscript{3} solution is turned to gray within several minutes. The reaction continues for 1 hours by keeping the temperature between 95 and 100 °C. After the reaction is done, cool down silver nanoparticle solution to room temperature in where there is no light.
2. SILICA CORE AND SILVER SHELL NANOPARTICLES SYNTHESIS

2.1 Silica nanoparticle synthesis

Silica sphere nanoparticles are prepared using the Stöber method. Firstly, 45 ml of ethanol (C\textsubscript{2}H\textsubscript{6}O) (Fisher Scientific, A405F-1GAL) is transferred into 150 ml of clean Erlenmeyer flask, and put a stirring in there. Secondly prepare a water bath on the hot plat and set a temperature of 55 °C for the water bath. When the temperature is stable, the 45 ml of ethanol-contained flask is placed in a water bath and add 3.12 ml of tetraethyl orthosilicate (TEOS, C\textsubscript{8}H\textsubscript{20}O\textsubscript{4}Si) (Aldrich, BCBM1319L) using a micro pipet while the solution is mixed. Finally, add 3 ml of ammonium hydroxide (NH\textsubscript{4}OH) (Sigma-Aldrich, 38796KM) into the solution and let the reaction for 10 minutes. After the reaction, the solution indicates transparent white color. To remove ethanol and unreacted chemicals, immediately separate 34 nm silica nanoparticle from the solvent using a centrifuge (LW Scientific, Inc., LWS-Combo-V24 centrifuge). Once it is confirmed that the nanoparticle is completely collected at the bottom of the tubes, use a pipet to suck the solution up and refill the tubes with fresh ethanol. Redisperse silica nanoparticle using sonicator (VWR, B1500A-DTH), and repeat the separation step to obtain pure silica nanoparticles. The fresh ethanol-containd nanoparticle solution is transferred to a clean beaker and placed on the hot plat. Apply gentle heat to the solution untill all of the ethanol are evaporated and collect the 34 nm silica powder in the glass vial for the next use. Figure A.1 shows the appearance of silica colloid (A) and silica podwer (B).

To synthesis the other sizes of silica nanoaprticles (53 nm, 76 nm and 111 nm), different temperatures are required with 8 hours of reaction time while the rest of
experimental conditions are identical with 34 nm silica synthesis above. 53 nm, 76 nm, 111 nm of silica nanoparticles are prepared using 60 °C, 55 °C, and room temperature to the solution mixture in the water bath, respectively.

Figure A-1 SiO₂ colloid solution (A) and SiO₂ powdewr (B)

2.2 Silver seeds attachment on the surface of silica nanoparticles

In order to cover the surface of silica nanoparticles with complete silver shells, it is necessary to affix Ag seeds to the silica surface, which serve as nucleation sites during shell growth. This method is proposed by Kim. 20 ml of ethanol is transferred into 50 ml of Erlenmeyer flask. 0.1 g of silica powder was dissolved into 20 ml of ethanol using the sonicator and put a stirring bar into the flask. Prepare 1 M AgNO₃ by dissolving 1.7 g of AgNO₃ into 10 ml of Milipore water. 0.1 M butylamine solution is prepared by transferring 0.1 ml of butylamine to 9.9 ml of Milipore water. The flask containing silica powder in the ethanol is placed in the water bath on the hot plat and use a micro pipet to deliver 100 ul of 1 M AgNO₃ to the flask while the solution is stirred. The solution mixture is heated to reach 65 °C and use a syringe pump (new era pump systems, Inc., 253978) to add 2 ml of 0.1 M butylamine (Sigma-Aldrich, 55496BPV) to
the reaction vessel with 60 ul/min injection rate. The temperature of reaction is kept at 65 °C for 2 hours without light. As the reaction is developed, it will be observed the solution is turned to gray tint as shown in Figure A. 2. After the reaction reaches an endpoint, the Ag-coated silica nanoparticles are centrifuged to remove ethanol, then dispersed into 20 ml of Milipore water. Repeat this separation step two times.

Figure A-2 Ag seeds decorated SiO₂ nanoparticle solution

2.3 Silver shell formation on silver coated-silica nanoparticle

Secondary reduction of Ag⁺ was conducted using various concentrations of AgNO₃ to control the final thicknesses of silver shells on silica nanoparticles. 1M AgNO₃ solution is prepared by dissolving 1.7 g of AgNO₃ to 10 ml of Milipore water. Also as a reducing agent, prepare 1 M of trisodium citrate by dissolving 2.58 g of trisodium citrate into 10 ml of Milipore water. 10 ml of Ag seed-coated silica solution is added to 10 ml of Milipore water where 50 ml of Erlenmeyer flask contains. The mixture flask is placed in the water bath. First desired amount of 1 M AgNO₃ solution is introduced to the diluted
Ag seed-coated silica solution under continuous stirring. Heat it up to 80 °C, then deliver 1.5 times higher volume of 1 M trisodium citrate solution than that of AgNO₃ to the flask. For example, In case of synthesis of 76 nm SiO₂@23 nm Ag nanoparticle that shows the highest SERS intensity of target molecules, 220 ul of 1 M AgNO₃ and 330 ul of 1 M trisodium citrate solutions are consumed at this secondary reduction step. The reaction is permitted to occur for 1 hour at 80 °C and turned to dark gray color as shown in Fig A.3. The solution should be stored in the dark prior to use.

Figure A-3 SiO₂ core @ Ag shell nanoparticle solution.

3. Assembly of Silica core and silver shell nanoparticle on glass slides for SERS measurement

Assembly of nanoparticles on substrates is one of ways to prepare to observe SERS enhancements of metallic nanoparticles. It is important to obtain uniform film composed of closely packed nanoparticles on substrates since high reproducibility and
intensitis of SERS analysis are produced by the uniformy packed arrangement of nanoparticles. Here, oil/water interfacial assembly method is used to obtain nanoparticles layers on glass substrates, which is proposed by Vanmaekelbergh and co-workers.

2 ml of silica core and silver shell nanoparticles solution is tranfered into a clean container and add 1 ml of hexan in the container. It will be observed that organic layer is formed above the colloid solution. Deliver 1 ml of ethanol to the hexane layer. When ethanol is dropped, nanoparticles are assembled at the interface between hexane and water layers. Figure A. 4 (a) shows the formation of shiny nanoparticle film in the middle of the container. After hexan layer is evaporated at the room temperature, the particles are compactly formed on the water layer. Prepare clean quarz cuvettes for Raman measurement (3 cm X 3cm) by acid wash treatment. The dry cuvette is dipped into the solution with a small angle and place it underneath the film as shown in Figure A. 4 (b). Lift up the cuvette sild carefully to collect the particle layer. Figure A. 4 (c) indicates that the nanoparticles film assembled on the slide has dark gray color compared to the clean slide. The nanoparicle film is dried in an oven and drops R6G solution on the slid for SERS measurement.

10 µM R6G is dried on the slide assemble with 76 nm SiO₂@23 nm Ag nanoparticles film, and collects SERS spectra from five different spots on the slide. However, SERS measureemnt on cuvette slide requires to adjust Z axis of microscope for focusing laser radition on the the film. In this SERS anaysis, 600 um of Z axie is set for the optimal condition.
Figure A-4 The assemble procedure of 76 nm SiO$_2$@23 nm Ag nanoparticles on thg glass slides. (a) the formation of nanoparticles film between colloid solution and hexane layers after the addition of ethanol, (b) loading the film on the cuvette slide, (c) cuvette slides assembled with nanoparticle film (right) and without nanoparticles film (left).

Figure A. 6 indicates the SERS spectra of 10 µM R6G collected from 5 different spots on the nanoparticles film. The SERS intensities of them are relatively much lower than that of R6G measured from colloid solution with the addition of aggregating agent showing at Figure 4.2. The peak intensities differ from each of different spots. These results are orginated from uncontrollable assembly process of nanoparticles on the slide. Especially, this assembly methode has a trouble to get uniform gaps between each of nanoparticles on the slide. As mentioned before, strong SERS enhancements are observed at hot spots created by the adjuctions of nanoparticles. If the gaps
between nanoparticles is not uniformed, it does not expect reproducible SERS data. In SEM in Figure A.5, it is observed the nanoparticles film on the cuvette slide has emptied spaces among the aggregated nanoparticles. Additionally, 2 dimensional arrangement of nanoparticles on the slide can produces less hot spots than 3 dimensional one formed by colloidal nanoparticle solution with the addition of aggregating agent, which results in low SERS intensity of R6G from the slide substrate.

Figure A-5 SEM image of 76 nm SiO$_2$@23 nm Ag nanoparticles film.
Figure A-6 SERS spectra of 10 µM R6G with 76 nm SiO$_2$@23 nm Ag nanoparticles assembled on glass slide. Excitation laser 514 nm, 78 mW, Exposure time 5, and Accumulation time 3.