Optimization, Modification and Application of Gold Nanoparticles as the Substrates of Surface Enhanced Raman Spectroscopy

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Optimization, Modification and Applications of Gold Nanoparticles as the Substrates of Surface Enhanced Raman Spectroscopy

by

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

First of all, I would like to dedicate this work to my parents who have been supporting me mentally and financially when I needed help. Also, I would like to thank God for giving me this courage and comforted me when I was struggling with things. I would like to thank my aunt and uncle who provided me an opportunity to study here at USF. Last but not least, I thank my wife being there for me very patiently every step of the way and putting up with all those frustrations. Without your help I would not be here.
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ABSTRACT

Surface enhanced Raman spectroscopy (SERS) is one of the techniques that overcomes the poor intensity of Raman scattering by utilizing the metal surface to enhance the Raman scattering. So far, silver (Ag), gold (Au) and copper (Cu) have been demonstrated to provide good enhancement for Raman signals. Many studies have proved that SERS is a powerful technique. However, the origin of the enhancement still needs clarification. More importantly, how to further improve the SERS enhancement through optimization of the SERS substrates and technique is a long and enduring challenge.

Chapter 3 is dedicated to find out the optimal size of AuNPs. Au NPs with different sizes were synthesized from 17 nm to 80 nm. The SERS activities of AuNPs were tested using target molecules of 4-aminothiophenol and 4-nitrothiophenol. The experiments were performed under three different conditions: same number of AuNPs, same surface area of AuNPs, and same concentration of AuNPs. For the same number of AuNPs, it showed linear relationship between the enhancement factor and sizes of AuNPs. However, in case of same surface area and concentration, the maximum enhancement was achieved around 50 nm AuNPs. These results were identical for both molecules, which indicate that the conclusions might be also applicable to other analytes. More importantly, the highest SERS enhancement can be achieved with AuNPs of 50 nm while they introduce minimum toxicity to the biological samples.

Once the optimal size of AuNPs was found, the work in chapter 4 was dedicated to find the way to improve the SERS enhancement of molecules that do not have strong affinity toward the surface of AuNPs. The affinity was improved by using 2-mercaptoethanol as a linking
molecule (2MELM) since the thiol group of 2ME can be strongly adsorbed on the surface of AuNPs, and the other end of 2ME has a hydroxyl group that can induce intermolecular forces toward the molecules of interest. Three molecules were chosen as the target molecule (TM): benzoic acid, cyclohexanol and 1,3-cyclohexanediol. When the results were compared between the substrates with LM and without LM, the spectra of benzoic acid did not display any difference. However, the spectra of the other two TMs show higher enhancement with presence of LM than that without. The results from this simple modification method shine light on the possible characterization or detection of molecules that have low affinity toward the metal surface using SERS.

SERS was also applied to study the intermolecular interaction in the field of Material Science. For the first part of the chapter 5, the interaction between vitamin B$_{12}$ and metal organic frame (MOF) was studied. The results confirmed the encapsulation and also, strong interactions between the two components were observed from the shift of vibrational modes of both VB$_{12}$ and Tb-MOF upon encapsulation. Second part of the chapter 5 was dedicated to study the functionalization of single wall carbon nanotubes in polymer network. The results confirm the successfully functionalized carbon nanotube.

SERS was used to detect different biomarkers through collaborations and the results are shown in Chapter 6. The first part was neurotransmitters, dopamin, melatonin and serotonin. All of them showed low µM range as their detection limit. Other two parts are the preliminary results from studying caffeine and nicotine. The results were promising exhibiting detection limits in low nM to sub nM range. None of these results were performed with optimized condition, therefore, when the conditions are optimized, it is very probable to have even lower detection limits.
CHAPTER ONE

INTRODUCTION

1.1 History of Raman and SERS

Raman spectroscopy or scattering was named after Sir Chandrasekhara Venkata Raman, an Indian scientist, who is the first one to discover the inelastic scattering of sunlight in 1928.\(^1\) He filtered down sunlight into monochromatic light then blocked this monochromatic light. From this experiment, he observed a small amount of light was scattered with different frequency. This inelastic scattering from the incident light is now called “Raman Scattering”. Figure 1.1 describes the difference between Raman scatterings and Rayleigh scattering. A molecule that is located in the ground electronic states (\(V = 0\)) or excited states (\(V = 1\)), can absorb the energy of a photon (\(E_{\text{ex}}\)) and gets excited to the virtual state of an energy level, which are indicated as dotted line in Figure 1.1. Then the excited molecule reemits a photon of energy indicated as down arrow. When this absorption and reemission of photon of energy happens, there are three possible ways. The scattered radiation can have a lower frequency than the initial radiation, which is called “Stoke scattering” shown as (A). When the scattered radiation has a higher frequency than the excited radiation, it is called “Anti-Stoke scattering” shown as (C). Another possibility is an elastic scattering when the initial and scattered radiations have the same energy, which is called “Rayleigh scattering” shown as (B). In most of the Raman spectra, since the Anti-stoke scattering occurs at higher energy, Stoke scattering is more desirable because the
ground vibrational level \((V = 0)\) is more highly populated than the excited state \((V = 1)\). Therefore, the Stokes scatterings are used more often than Anti-stokes in the Raman spectrum.

There are many advantage of Raman spectroscopy.(2-5) One of the most well-known advantages is the detection is not interfered by water, which opens up many biological applications. Also, it does not require sample preparation and samples can be either liquids or solid. Simply, place any sample on the stage is only step needed. Once sample is placed on stage, it only takes seconds to acquire the results. Raman spectrum provides highly specific chemical finger print of a molecule. Despite all these advantages mentioned above, there are couples of disadvantages. If the sample matrix is complicated, it suffers from the fluorescence. Also, if the laser is too intense, sample can be heated and destroyed. Previously mentioned disadvantages can be avoided by purifying sample and control the intensity of the laser. However, there is one
disadvantage cannot be avoided that is the signals from Raman scattering is very weak. (6; 7) Therefore, Raman spectroscopic detection needs to be combined with a sensitive and highly optimized instrumentation.

Surface enhanced Raman spectroscopy (SERS) is a vibrational analysis technique that enhances the inelastic scattering of incident light by employing coinage metals like silver, gold and copper. (4; 5; 8) SERS was discovered in 1974 by Fleischmann et al. Originally, he was trying to develop a method that can provide the specific spectroscopic information of different chemicals. This could help to study the electrochemical process of the chemical of interest. Fleischmann believed that in order to get better information of the chemical of interest, more chemicals need to exist on the surface of the electrode. The way he chose to increase the amount of chemical is to roughen the surface of electrode. As a result, the first SERS phenomenon was discovered when intense Raman scattering was observed from adsorbed pyridine on a roughened silver electrode surface in aqueous condition. (9-11) This tremendous discovery intrigues many researchers to find out what causes SERS phenomenon for the promising futures. By mid 1980s, the experimental observations and facts were somewhat established and lead to the crucial features of the mechanisms. Nowadays there are two well accepted theories that could help to understand the SERS phenomenon.

1.2 Theories of SERS

In general, two enhancement mechanisms are well accepted by the science community so far: electromagnetic enhancement (EME) (12-14) and chemical enhancement (CE) (15-17). Electromagnetic enhancement theory is the one that is predominantly responsible for the enhancement and also known to contribute most of the SERS enhancement, which is at or above
10^6. EME happens when the surface plasmon, which is the collective excitation of the electrons on metal surface, becomes excited by the incident laser light. This excited surface plasmon aids in great enhancement of the electromagnetic field on the metal surface. The enhanced electromagnetic field then amplifies both the incident laser and the scattered Raman. By using a simplified model of a metal sphere in an external electric field, the fundamental physics that underlies the electromagnetic enhancement mechanism can be well explained. Study shows that when the spherical particle has a smaller radius than the wavelength of light (laser), an uniform electric field forms around the particle and the induced field on the surface of that sphere is proportional to the incident laser field. In short, when the external field (E_0) is applied to the sphere, it causes the polarization (P), which leads to the dipole moment inside of the sphere. The polarization and dielectric constant of vacuum are the two components to express the magnitude of the induced field (E_{ind}) inside of the sphere. Equation 1.1 below expresses the magnitude of induced field.

\[
E_{ind} = \frac{P}{3\varepsilon_0} \quad (1.1)
\]

Equation 1.2 describes the polarization of sphere (P) that defines the sum of the dipole moment (\mu_{ind}) per unit volume. While \(a\) is the radius of the sphere.

\[
P = \frac{\mu_{ind}}{\frac{4}{3} \pi a^3} \quad (1.2)
\]

As mentioned earlier, the induced field is proportional to the incident laser field. This helps to establish the correlation, \(\mu_{ind} = \alpha E_0 = \alpha\). The magnitude of the external field and polarizability
(α) of the sphere are both proportional to the induced dipole moment. The polarizability (α) can be expressed as the equation 1.3.

\[ \alpha = 4\pi\varepsilon_0 \left| \frac{\varepsilon_i(\omega) - \varepsilon_m}{\varepsilon_i(\omega) - 2\varepsilon_m} \right| a^3 \] (1.3)

The dielectric constant of the surrounding and the metal medium are expressed as \(\varepsilon_i\) and \(\varepsilon_m\) respectively, representing the radius of the sphere. When the equation 1.2 and 1.3 are inserted into the equation 1.1, it can be simplified as equation 1.4, which expresses the magnitude of the induced field including dielectric constant of sphere and the surrounding medium with the external field applied.

\[ E_{ind} = \left| \frac{\varepsilon_i(\omega) - \varepsilon_m}{\varepsilon_i(\omega) - 2\varepsilon_m} \right| E_0 \] (1.4)

As mentioned earlier, excited surface plasmon can amplify intensity of both incident field and scattered field as well. When these factors are considered, the SERS enhancement factor (EF<sub>EM</sub>) from the electromagnetic field can be derived as shown below.

\[ EF_{EM} = \left| \frac{\varepsilon_i(\omega_{inc}) - \varepsilon_m}{\varepsilon_i(\omega_{inc}) - 2\varepsilon_m} \right|^2 \left| \frac{\varepsilon_i(\omega_{scat}) - \varepsilon_m}{\varepsilon_i(\omega_{scat}) - 2\varepsilon_m} \right|^2 \] (1.5)

\(\varepsilon_i(\omega_{inc})\) and \(\varepsilon_i(\omega_{scat})\) are the dielectric constant of the surroundings at the wavelength of the incident laser light and the Raman scattering, respectively. The equation shows that when both \(\varepsilon_i(\omega_{inc})\) and \(\varepsilon_i(\omega_{scat})\) are equal or close to \(2\varepsilon_m\), the induced and scattering field are at the maximum value, which leads to the highest \(EF_{EM}\). Hence, only coinagemetals including Ag, Au and Cu are good substrate for SERS.
In general, there are many factors that can influence the magnitude of the enhancement factor from the electromagnetic field such as shape and size of the metal nanoparticles. (14; 19) Also, in case of the non-spherical metal nanoparticles, the relative orientation of incident field also plays an important role.

Even though the electromagnetic enhancement could explain and support majority of the SERS enhancement. There were experimental results that could not be explained by the EME by itself. One example is when the two chemicals, N₂ and CO, that have nearly the same polarizability were tested under the exactly same condition. The results were astonishing because there was about 200 times enhancement factor difference between the two chemicals.(10) Since when the molecules are adsorbed on the surface, the EME should provide the amplification of Raman scattering without any discrimination. It was suggested that there was another factor that contributes to the SERS enhancement that works independently of EME. There are two possible explanations. The first one is when the adsorbate is interacting with the surface, there is shifting or broadening of electronic state of the adsorbate. The second explanation is when chemisorption happens between the adsorbate and the surface, there is a new electronic state that serves as a resonant intermediate state in Raman scattering. The latter case is more convincing since it is commonly observed that the interaction between highest occupied molecular orbital and the lowest unoccupied molecular orbital of adsorbates are equally distributed in energy with respect to the Fermi level of the metal. This chemical enhancement is known to contribute the SERS enhancement up to 10².

Understanding the two enhancement theories also helps us in calculating the SERS enhancement factors. The most commonly known way to calculate the EF is called, substrate enhancement factor (SEF). The equation 1.6 expresses the SEF.(20-24)
\[ SEF = \frac{I_{\text{SERS}}/N_{\text{Surf}}}{I_{\text{RS}}/N_{\text{Vol}}} \] (1.6)

The \( I_{\text{SERS}} \) and \( I_{\text{RS}} \) are the intensities of SERS and normal Raman peaks, respectively. The \( N_{\text{Vol}} \) is the average number of molecules in the scattering volume in normal Raman. The \( N_{\text{Surf}} \) is the average number of molecules that are adsorbed on the surface in the scattering volume for the SERS experiment.

Another way to calculate the SERS EF is called, analytical enhancement factor (AEF) and can be expressed as the equation 1.7. (25)

\[ AEF = \frac{I_{\text{SERS}}/c_{\text{SERS}}}{I_{\text{RS}}/c_{\text{RS}}} \] (1.7)

The \( c_{\text{SERS}} \) and \( c_{\text{RS}} \) are the concentrations of molecule in SERS and normal Raman experiment respectively. The AEF ignores the fact that SERS is a surface sensitive technique. As it is seen in equation 1.7, the entire concentration of the analyte is counted into the EF calculation when there is only so many molecules that are adsorbed on or near by the surface of substrate.(26; 27) So, the concentration of analyte should be low enough that only a monolayer or sub-monolayer forms on the substrates in order to deliver the accurate results.

1.3 SERS Substrates

Ever since the SERS technique has been discovered, there were many studies focusing on improving SERS enhancement. Therefore, numerous experiments have been performed to examine the contributions of metal nanosubstrates, especially, to investigate the correlation between the SERS enhancement and the size, geometry and compositions of the metal substrates. (28-34) It was concluded that the nano-scaled metal substrates are essential in amplifying the
Raman signals. Based on the electromagnetic enhancement, the plasmon frequency of metal substrate has to be in resonance with the frequency of the incident laser light to achieve the maximum SERS enhancement. The reason that coinage metals, especially, silver (Au) and gold (Ag), are most commonly used SERS substrates because of their plasmon frequency is located in or near by the commercially available laser frequency.

Ag nanoparticles (AgNPs) are known for providing higher SERS enhancement compare to gold nanoparticles (AuNPs). It is because Ag has d-s band gap in the UV region, which causes less damping of the plasmon mode. The most widely used synthesis method is called Lee and Meisel method. This process requires sodium citrate to reduce AgNO$_3$ under the heat.

Another common synthesis method is called Leopold method, which uses hydroxylamine to reduce AgNO$_3$ in room temperature. Even if the AgNPs can provide high SERS enhancement, it is hard to control their size and always results in wide size distribution. Also, AgNPs are only

Figure 1.2. UV-Vis spectra showing red shift which indicating increase of size. SEM image of average size 50 nm Au NPs. Red bar is inserted to show the size of Au NPs.
stable for a short period of time and not biocompatible, which limits their applications in biological systems.

To synthesize the AuNPs, the similar reduction method can be carried out by using sodium citrate to reduce the HAuCl$_4$ under the heat.(38) Even though the AuNPs do not provide as high enhancement as AgNPs do, it is desirable because of benefits like, easy size control, narrow size distribution, high stability and biocompatibility. In case of the biocompatibility of AuNPs, it opened up the SERS into many different biological applications.(39-41) Synthesized AuNPs and AgNPs are usually characterized by employing UV-Vis, Scanning electron microscope (SEM) or Transmission electron microscope (TEM). The UV-Vis spectrum is used to find out the relative size and size distribution. The SEM and TEM are used to figure out the shape and average size of the nanoparticles.(8) When those reduction methods were employed, the NPs have sphere shapes and AgNPs have average size of 19 nm and AuNPs have average size of 90 nm. Figure 1.2 is shows the UV-Vis spectroscopy of the average size of 50 nm AuNPs. The maximum absorption is at 530 nm, which has been red shifted from 520 nm, which belongs to the average size 19 nm AuNPs. The SEM image of synthesized AuNPs can help you to determine the overall shape of the AuNPs, which is sphere. As mentioned previously, the size of NPs plays an important role in the SERS enhancement. Many factors need be considered to achieve the highest SERS enhancement. The volume of the NPs needs to be large enough since enhancement is generated from the number of excited electrons on NPs surface. However, when the volume gets too large, the enhancement can suffer from the larger radiation damping effect. Many research results agree that the optimal size of Au and AgNPs lies between 30 to 100 nm.(9; 11) Different shapes, such as, nanorods, nanostars, nanoflowers or gold lace shells, NPs also have been synthesized and studied since they can provide different optical properties than just
sphere shapes. Figure 1.3 shows some of the characteristics of the nanorods. Such nanostructure provides benefits of having two different SPR bands. The one is from the short axis (w) that is a weak transverse band, which is similar to the nanosphere. Another one is located along the long axis (L) that is a strong longitudinal band in the longer wavelength region. Also, by controlling the amount of AgNO₃ during the synthesis, the aspect ratio between long and short axis can be controlled. This controllable nature of the nanorod can also manipulated the longitudinal plasmon resonance shift from the visible to the near IR region.

Other examples mentioned above, nanostars and nanoflowers also have their benefits. Figure 1.4 shows the TEM images of nanostars and nanoflowers. They are grown on top of the sphere shaped NPs, so their surface is rougher than the regular sphere shapes. This tip of the roughened surface can provide sharp tip effect, which provides stronger electromagnetic field that lead higher SERS enhancement. Also the surface plasmon originated from the core (sphere)
and tips around can further enhance the electromagnetic field. Lastly, the roughened surface can provide more surface area where more molecules of interest can interact than smoother surface. However, the reproducibility is remaining as one of the issues.

Employing different composition to synthesize the bimetallic, also known as core-shell, nanoparticles attracted many researchers because of the benefits from composition dependent physical and chemical properties. One example could be $\text{Au}_{\text{core}}\text{Ag}_{\text{shell}}$ as it shows in Figure 1.5. This bimetallic nanoparticle can be synthesized by creating the silver core then coat the surface of the silver core with thin layer of gold. This way the molecules adsorbed on the surface of the $\text{Ag}_{\text{shell}}$ can be influence by electromagnetic field of both Au and Ag. The TEM images in figure 1.5 are one way to confirm the formation of core-shell nanoparticles. Another way is to utilize UV-Vis spectroscopy. In figure 1.5 it clearly shows that the formation of core-shell nanoparticles

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Figure 1.4. (A) TEM image of Nanostars, the inserted image is zoomed in image to show the detail shapes of star like substrate. (B) TEM image of flower shaped nanoparticles. Scale bars are inserted in images to help to figure out the size of nanoparticles. Reproduced with permission from ref 46 and 47. Copyright 2008 American Chemical Society.
by observing the absorbance peak between AuNPs and AgNPs absorbance peak. There has been several results showing that bimetallic substrate generate higher SERS enhancement than the monometallic substrate. Another example is $\text{Ag}_{\text{core}}\text{Au}_{\text{shell}}$. This substrate does not provide as high SERS enhancement of AgNPs itself, but higher than AuNPs itself. The most important benefit is this $\text{Ag}_{\text{core}}\text{Au}_{\text{shell}}$ substrate can be used in biological applications. Since the toxic silver core is coated with the thin layer of the gold, it can remove or minimize the toxicity from the silver. One of the reasons that this bimetallic substrate is not in common use is because of the reproducibility and hard to control the thickness of the shell since the distance of the molecule is crucial from core metal. (48-56)

Figure 1.5. (a) TEM image of Ag core Au shell nanoparticle. (b) Overall images of core shell nanoparticles. (c) the orange dotted line is UV-Vis spectrum from AgNPs, and the green dotted line is UV-Vis spectrum from AuNPs. The blue line is UV-Vis spectrum of Ag@AuNPs. Reproduced with permission from ref 56. Copyright 2013 American Chemical Society.
So far, the focus was only on the examples of optimization from single substrate point of view. However, the most intense electromagnetic field enhancement is coming from the junctions where two or more NPs are clustered, which is called a hot spot. (57-59) This hot spot can generate the enhancement factor larger than \(10^{10}\), which makes it possible to have a single-molecule detection. (60) It is known that when AuNPs cluster together, the absorption band is shifting toward the longer wavelength range, 700 to 900 nm, where the \textit{in vivo} investigation is possible. These benefits from hot spots triggered many researchers to explore the aggregation of the NPs. The most common way to induce the aggregation of the nanoparticles is using salts. (61; 62) Previously, it was believed that the halide ions of salts are the only ones influence the aggregating of NPs and SERS enhancement. However, recently, Han et al. published work that proves the importance of the cations in SERS enhancement. (63) His work was to compare the multiple salt aggregating agents to find out the highest SERS enhancement of one molecule. The concentration and types of aggregating agents were varied while keeping the rest of the experimental set up the same. The results showed that the best aggregating agent is highly dependent on the analytes. For example, when 0.1 mM of MgCl\(_2\) was used, the highest SERS enhancement was delivered for cotinine.

There is another way to create an aggregation of the NPs by utilizing the silica, polystyrene or polymer beads. This way requires several steps to create the aggregation, however, the aggregation is more reproducible and homogeneous than the common method mentioned earlier. One way to create this aggregation is to deposit desired NPs on the surface of the supporting beads. In order for the NPs to be deposited on the surface, the supporting beads need to be functionalized by being treated with sulfuric acid or 3-mercaptopropyltrimethoxysilane. (62; 64; 65) In Figure 1.6, successful synthesis of NPs has been characterized by color change, UV-
Vis and TEM. The UV-Vis spectra are showing the maximum absorbance shift from bare silica supporting bead (a) to largest size of AgNPs on the surface of the supporting beads (e). As the size of AgNPs on the surface of supporting beads increases, the maximum absorbance peak red shifts that indicate the size increase, and the peaks become broader, which indicates the broader size distribution. TEM image confirms the successful synthesis of AgNPs functionalized supporting beads. This fictionalization will induce the adsorption of metal NPs, which is synthesized separately. The density of the metal NPs on functionalized supporting bead surface can be controlled by adjusting the amount of metal NPs and reaction time.

1.4 Applications

The most common way to apply SERS technique is direct detection of a molecule of interest in solution. In the past few decades, the researchers experimented many different methods, such as, shapes and sizes, to improve the enhancement and sensitivity of the SERS
techniques. Recent trend though, as it was mentioned earlier, the biocompatibility of the gold-nanoparticles and development in core-shell nanoparticles have opened up the variety of applications in biological field. The benefits are not only with the substrates. SERS can be ideal in many biological applications because high SERS signals can be generated from the low power laser (IR region), which minimizes or removes the invasiveness of the laser on the live cell. Also, the detection time can be manipulated. This makes the real time detection of the biological process possible. Lastly, most of the Raman spectroscopy is equipped with microscope that can be focused in a micrometer scale, which make it possible to get a high resolution results from the microenvironment in cells.

The predominant SERS use in biological application is using tagging methods.(50; 66-71) The idea behind this tagging method is when the molecule of interest is not an active SERS molecule; it is attached to another molecule that is highly sensitive in SERS detection. So, by detecting the tagged SERS active molecule, it was possible to have qualitative information of the molecule of interest. One of the most common examples is using antigen and antibody. Figure 1.7 is one of the illustrations of antigen and antibody use by Xie et al.(19) The analytes of interest are mixed with antibody and binds to one end of the antibody. The metallic nanoparticle that has a Raman reporter adsorbed on the surface is attached to the other end of the antibody. When these two solutions are mixed, the antibody that is attached to metal nanoparticles with the Raman reporter caps off the analytes. During the measurements, the Raman reporters on the metal nanoparticles surface give out SERS signals to confirm the successful interaction between the antibody and the analytes. Based on this tagging method, there have been reports of successful application of SERS in detecting pathogens,(72-77) cancer maker detection,(78-80) imaging live cell and tissue.(81-83)
SERS also can serve as biosensors and it has been utilized to detect various biological samples and diseases. One of the most intriguing examples is SERS-based in vivo glucose sensor. Diabetic patients constantly, 3-10 times per day, deal with drawing their blood to check their blood glucose level. Patient’s life can be greatly improved if they do not have to draw their blood to check on glucose level. This work was done by the Van Duyne and his group. (84-86) This device has thin silver film that covers the Si nanosphere. Then the silver film surface is functionalized with a decanethiol and mercaptohexanol self-assembled monolayer. This functionalized silver film device then coupled with specially offset Raman spectroscopy. The results from the study provide accurate and consistent glucose reading even measuring lower concentration than what International Organization Standard requirement. Another interesting
area of application of SERS techniques is to determine the types of dye used in the art works. Many valuable art works get damaged or faded by natural sunlight with time. According to the Casadio et al., (87) SERS can deliver the information of dye by only using small amount of sample in short period of time. This can help restoring the valuable art work without worrying about using wrong dye. There also have been reports that SERS technique was successfully applied to detect the explosive materials like half-mustard agent and dinitrobenzenethiols. (88; 89) Detection of these explosive materials could be challenging but converting gas phase molecules to liquid phase molecule can be coupled with microfluidics-SERS sensor. There are companies, Thermo Scientific, Inc, and Intavec, Inc, who makes portable Raman spectrometer, makes real-time detection in the field much easier. SERS technique continuously delivering impressive results in many different fields of science and it looks very promising in future.
2.1 Preparing Glassware for the Experiments

2.1.1 Sulfuric Acid and Nitric Acid Wash

All the glassware that is used for the experiments needs to be cleaned by acid wash. Two different types of acid wash baths are created. The first one is composed of mainly sulfuric acid. This bath has a concentration around 10 M of sulfuric acid. Commercially available ones (in stock) have a concentration of 18 M (Fisher Scienfic, A300C-212). So, Stock sulfuric acid need to be diluted to around 10 M by mixing with Millipore water (Cascada, Pall Corporation). The glassware after experiments needs to be first rinsed clean with large amount of deionized water, then fully immersed into the sulfuric acid bath and left in bath for about 4 hours. Once 4 hours has passed, take the glassware out and rinse with deionized water. Make sure when concentrated acid are used, perform all procedure in a container covered with sodium bicarbonate and water mixture. Neutralize all the washing solution using sodium bicarbonate before dumping into the sink. When thorough rinse is over, get rid of most of the water on the glassware. Then, fully immerse them into nitric acid bath.

The concentration of nitric acid in the bath is around 8 M. This concentration of stock nitric acid is around 16 M (Fisher Scienfic, A200C-212). Therefore, stock nitric acid need to be
diluted to 8 M by mixing with deionized water. The glassware needs to stay at least for 2 hours before being taken out. In order to wash the nitric acid off of the glassware effectively, hot or boiling water is recommended. So, 20 to 30 minutes before taking the glassware out of the nitric acid bath, you need to boil Millipore water. Like the sulfuric acid, all washing and rinsing procedure needs to done in a container with sodium bicarbonate and water. Once the rinse is over, the glassware can be air dried or placed in the oven.

2.1.2. Nitric Acid with Hydrochloric Acid Wash

It is a recent addition to our acid wash. This acid wash is created by mixing stock nitric acid with stock hydrochloric acid (Fisher Scienfici, SA9225-1) in 3 to 1 ratio. This acid wash is commonly used to wash off the metal ion moieties. So, in case you are working with gold or silver nanoparticles, this acid wash is preferred for the simpler washing process. Once the glassware is immersed into bath, it needs to be in for two hours. The same procedure is followed as that of the nitric acid bath. This acid wash has a distinctive color. When it first created, it is yellow. As it is in use, the color becomes orange. Then it goes back to yellow color. So, when faint yellow color is observed, the acid bath needs to be changed.

2.2 Gold Nanoparticles Synthesis

2.2.1 Synthesis of 17 nm Gold Nanoparticles

To synthesize gold nanoparticles with the average size of 17 nm, simple reduction process by Cyrankiewicz et al. was employed.(8; 36; 38) First accurately measure 0.1970 g of HAuCl4 (Acros Organic, 16961-25-4) in weighing boat. No metal spatula should be used when dealing with HAuCl4 because of easy oxidation. So, to handle the HAuCl4, a teflon stick was
carved into shape of spatula and used. Then carefully rinse the HAuCl₄ off the weighing boat into a 100 mL volumetric flask. Use Millipore water to fill up the volumetric flask up to the meniscus. Pour all 100 mL of 0.5 mM HAuCl₄ solution into a 125 mL Erlenmeyer flask and add a clean Teflon bar. Place it on a hotplate (Corning, ANYT-0175) and place a thermometer inside of the Erlenmeyer flask. Make sure the mercury tip is fully immersed into the HAuCl₄ solution. Heat up the hot plate (place the dial at 4 or 5) and turn the stir bar vigorously (place the dial at 5), and watch the temperature rises up to around 93 degree Celsius (°C). Then turn the heat down to the lowest. Temperature will keep rising. When it reaches close to 98 °C, add 4 mL of 1% (w/w) sodium citrate solution. A micropipette can be used for easy control. In a few seconds, the color change should be observed from clear to really dark purple. The heat needs to be controlled and the temperature should be down to 95 °C. Once the temperature reaches around 96 °C, the heat dial can be turned up to 3 or 4. If the temperature rises, turn the dial down to the lowest or second again. This process might need to be repeated for several times. Heat and stir the solution continuously for 30 minutes. Once the reaction is over, turn the heat off and leave the stir bar on until the solution cools down to warm or near the room temperature. The final solution color is dark red purple as show in Figure 2.1. Take the Erlenmeyer flask off of the hot plate and

![Figure 2.1. 17 nm AuNPs solution.](image-url)
cover the flask with a parafilm and place it in the drawer or somewhere dark. In order to avoid any possibilities of photosensitivity, the hot plate set up should be located where there is minimal amount of light.

2.2.2 Synthesis of 30 nm Gold Nanoparticles

To synthesize the average size of 30 nm gold nanoparticles, the 17 nm AuNPs solution is used as seeds. A further reduction using HAuCl₄ and hydroxylamine is carried out. In detail, 30 mL of seed (17 nm AuNPs) solution is transferred into a 500 mL Erlenmeyer flask. Then, 270 mL of Millipore water is added to have a total volume of 300 mL. Now, place the 500 mL Erlenmeyer flask on as stir plate with a stir bar. Set the dial at 5 or above. While the solution is vigorously stirred at a room temperature, 3 mL of 0.2 M hydroxylamine (Sigma Aldrich, 161136) reducing agent is added to the solution. Then, 2.5 mL of 25.4 mM HAuCl₄ is added drop by drop using a micropipette into the solution. If constant rate is desired, a flow meter should be used. Keep stirring for 10 to 15 minutes. Once time is over, cover the mouth of the 500 mL Erlenmeyer flask with a parafilm, and then store it in the drawer or dark. The figure 2.2 shows the color of the 30 nm AuNPs solution when the synthesis is completed.

Figure 2.2. 30 nm AuNPs solution.
2.2.3 Synthesis of 40 nm Gold Nanoparticles

To synthesize 40 nm AuNPs, transfer 30 mL of 30 nm AuNPs solutions into a 500 mL Erlenmeyer flask. Then, add 270 mL of Millipore water to make the total volume of 300 mL. Place the flask on a stir plate with a stir bar in. Set the dial above 5. While the solution is vigorously mixed at room temperature, add 0.7 mL of 0.2 M hydroxylamine and then add 0.45 mL of 25.4 mM HAuCl₄ drop by drop into the solution using a micropipette. Once everything is added, keep stir for another 10 to 15 minutes. When it is done, cover the mouth of flask with a parafilm then store the solution in the drawer or dark place. The figure 2.3 shows the color of the 40 nm AuNPs solution after the synthesis.

![Figure 2.3. 40 nm AuNPs solution.](image)

2.2.4 Synthesis of 50 nm Gold Nanoparticles

Synthesizing 50 nm AuNPs follows almost same steps as those of synthesizing 40 nm AuNPs. Transfer 30 mL of 30 nm AuNPs solutions into a 500 mL Erlenmeyer flask and add 270 mL Millipore water to make the final volume of 300 mL. Place the flask on the stir plate with a
stir bar in and set the dial at 5 or above for vigorous mix. While the solution is mixed at the room temperature, add 1.5 mL of 0.2 M hydroxylamine is added then 0.8 mL of 25.4 mM HAuCl₄ solution is added drop by drop using a micropipette. Once everything is added, keep stir for 10 to 15 minutes. Then cover the mouth of flask with a parefilm and place it in the drawer or dark. Figure 2.4 shows the color of 50 nm AuNP solution after the synthesis.

2.2.5 Synthesis of 60 nm Gold Nanoparticles

Transfer 30 mL of 30 nm AuNPs into a 500 mL Erlenmeyer flask and add 270 mL of Millipore water to make the final volume of 300 mL. Carefully drop the stir bar into the flask...
and place the flask on the stir plate. Set the stir speed at 5 or above for vigorous mix. While the solution is vigorously mixed at the room temperature, add 2 mL of 0.2 M hydroxylamine solution, then add 1.3 mL of 25.4 mM HAuCl₄ drop by drop using a micropipette. Once everything is added, keep stir for 10 to 15 minutes. Then cover the mouth of flask with a parafilm and place it in the drawer or dark. Figure 2.5 shows the color of 60 nm AuNP solution after the synthesis.

2.2.6 Synthesis of 80 nm Gold Nanoparticles

To synthesize 80 nm AuNPs, transfer 30 mL of 60 nm AuNPs solutions into the 500 mL Erlenmeyer flask. Then, add 270 mL of Millipore water to make the total volume of 300 mL. Place the flask on to the stir plate with a stir bar in. Set the dial above 5. While the solution is vigorously mixed at room temperature, add 0.67 mL of 0.2 M hydroxylamine solution and then add 0.43 mL of 25.4 mM HAuCl₄ drop by drop into the solution using a micropipette. Once everything is added, keep stir for 10 to 15 minutes. When it is done, cover the mouth of flask with a parafilm then store the solution in the drawer or dark place. The figure 2.6 shows the color of the 80 nm AuNPs solution after the synthesis.
2.3 Experimental Procedures

2.3.1 Normal Raman Measurements

Raman spectroscopy equipment was purchased from Horiba Jovin Yvon, equipped with two different types of lasers. One is an Argon and Krypton laser (Coherent, Innova 70C series) to produce 514 nm and 642 nm of wavelengths, and another one is a diode laser (Coherent, Diode Cube) to produce 785 nm of wavelength. All the SERS experiments were carried out using a Confocal Raman Microscopy (Olympus, IX71) purchased from Horiba JovinYvon. There are two spectrum gratings, 600 and 1200. The spectrum grating 600 was used throughout the experiment. Microscope is equipped with three different objectives, 20 X, 40 X, and 100 X. All the experiments were carried out using 20X microscopic objective lens.

For normal Raman spectroscopy, sample preparation does not require many steps. Any samples that can be placed on the top of the microscope stage can be tested. For example, if the desired sample is in solid form, place the solid sample on a clean glass slide. If the sample is powder, grind it into fine particles and press it in a dense pallet on a glad slide. Once placed, set the microscope in camera view. Adjust the axis (X, Y and Z) to get a clear image of the sample. Switch the camera view to the laser. If the sample is corrected under focused, there should be white spot on the center or near the center of the screen. Once the focus is confirmed, take the spectrum. It is always better to have a spectrum with a wide wavenumber range (0 – 3600 cm\(^{-1}\)) taken at first if no information is known about where the expected or highest peak is located. The exposure time, accumulation number and even slight adjustment of Z-axis can be varied to get a good spectrum with higher signal/noise ratio.
If the sample is in liquid form, it can be placed in a 1 mL quartz cell (Starna Cells Inc, 3-Q-10). Place the special cut sample holder for the cell on the stage then hold it with clips around the stage. This special cut sample holder has a hole in the middle, so the cell can be placed on top of the hole. Liquid sample is hard to be distinguished by camera view. So, the optimal height of Z-axis needs to be tested beforehand. Turn the coarse knob that controls Z-axis all the way to the top. Then, change the number of Z-axis to zero on computer screen (you can do this either by change the number to zero, or by click on the red lightening mark on right side of the axis). Once the Z-axis is set to zero, type any number between 400 and 500 into the Z-axis then you will hear and see the stage is moving down to wherever the number you set. The unit of Z-axis is in micrometer. Place the sample on the holder and the laser should be focused on the liquid sample. Just like the solid sample, It is always better to have a spectrum with a wide wavenumber range (0 – 3600 cm⁻¹) taken at first if it is the first time dealing with such chemicals. The exposure time, accumulation number and even slight adjustment of Z-axis can be optimized to get the best spectrum.

2.3.2 Surface Enhanced Raman Spectroscopy

Preparing samples for the SERS experiment follows similar steps as the liquid samples. The difference is SERS samples require metal nanoparticles substrates. Transfer 1 mL of metal nanoparticles solution into the 2 mL quartz cell then add samples. Use a micropipette to mix the solutions several times. Place the cell on the top of the hole in the sample holder. Z-axis should be already at the optimal position (between 400 and 500 from the top). Take the spectrum. Some samples provide excellent information without any additional work. However, if the spectroscopy does not provide enough information, aggregating agents can be used to help to get better spectrum. Optimal types and amount might be different for each sample. The most
commonly used aggregating agent is 1 M NaCl. Simply, add 1 mL of 1 M NaCl into the solution that contains sample and metal nanoparticles. Like mentioned above, it is always better to have a spectrum with a wide wavenumber range (0 – 3600 cm⁻¹) taken at first if we do not know where the expected or highest peak is located. The exposure time, accumulation number and even slight adjustment of Z-axis can be controlled to get the better spectrum.

2.3.3 UV-Vis Spectroscopy

UV-Vis spectroscopy (Beckman Coulter DU 640 spectrometer) is used to figure out the relative size of the AuNPs in the solution. This was done by comparing the maximum absorptions of synthesized AuNPs. In order to achieve accurate results, samples should have absorbance reading below 1.0. So, when preparing the samples for the UV-Vis measurements, equal amount of Millipore water is added with AuNPs solution into the quartz cuvette. Once both solutions are added, cover the cuvette using a parafilm. Rock them gently to mix the solution or use a micropipette to mix as well. The quartz cuvette used for UV-Vis has two clear walls and three frosted wall. Once the solution is prepared, set the zero line and back ground of UV-Vis. Setting up the zero line is done by inserting a black block into the sample holder. Then, back ground is set by inserting the quartz cuvette that has only the solvent of the sample solution. When the cuvette is inserted into the sample holder, two clear walls should be facing the light path. After the back ground is set, insert the cuvette that contains the sample like (AuNPs) solution. Run scan to collect the results. All the UV-Vis spectra can be normalized using the program, so the concentrations of solution do not have to be exactly the same. Also, quartz cuvette needs to be thoroughly rinsed between trials to avoid possible contamination. All the data need to be converted into “.cvs” form in order to transfer into other computers to plot them. The wavelength range is usually set between 200 nm to 800 nm, this window usually covers our
sample. Also, the resolution is usually set at 5 nm. But for our purpose, setting up at or below 1.0 nm resolution usually provides better results.

2.3.4 SEM

SEM is used to measure the size and shape of AuNPs. Hitachi microscope for SEM imaging is located in Nanotechnology Research and Education Center (NREC) on the University of South Florida Tampa campus. To prepare a sample for the SEM imaging, a silicon wafer is used. It can be purchased from NREC. The silicon wafer needs to be cleaned using ethanol or methanol before use. These wafers can be used many times, by simply wash off the sample residues off of the surface with ethanol or methanol. Drop 1 or 2 µL of AuNPs solution using a micropipette on the edge of the silicon wafer. Or, it does not have to be on the edge, but try to find some distinctive area that you can easily recognize because once it goes into the SEM sample chamber; it is very hard to figure out where the sample is. So, instead, locate a distinctive area then look for the sample. Once the sample is dropped on the wafer surface, place it into the Petri dish and put it in the drawer. It usually takes less than an hour for your samples to dry. You do not have to drop only one sample on silicon wafer, if the wafer surface provides more than one distinctive area then you can drop more than one sample on it.
CHAPTER THREE

OPTIMAL SIZE OF GOLD NANOPARTICLES FOR SURFACE-ENHANCED RAMAN SPECTROSCOPY UNDER DIFFERENT CONDITIONS

Note to the reader

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Author contributions. Seongmin Hong and Xiao Li designed the research; Seongmin Hong performed research, analyzed data and wrote the initial draft. Xiao Li helped in improvising the draft.

3.1 Abstract

Gold nanoparticles have been used as effective surface-enhanced Raman spectroscopy (SERS) substrates for decades. However, the origin of the enhancement and the effect of the size of nanoparticles still need clarification. Here, gold nanoparticles with different sizes from 17 to 80 nm were synthesized and characterized, and their SERS enhancement toward both 4-aminothiophenol and 4-nitrothiophenol was examined. For the same number of nanoparticles, the enhancement factor generated from the gold nanoparticles increases as the size of nanoparticles increases. Interestingly, when the concentration of gold or the total surface area of gold nanoparticles was kept the same, the optimal size of gold nanoparticles was found out to be around 50 nm when the enhancement factor reaches maximum. This conclusion is critical when
Gold nanoparticles are used as SERS substrates in biological systems so that minimum toxicity is introduced while highest enhancement is achieved. The same size effect was observed for both 4-aminothiophenol and 4-nitrothiophenol which suggests that the conclusions drawn in this study might also be applicable to other adsorbates during SERS measurements.

3.2 Introduction

Surface enhanced Raman spectroscopy (SERS) is a surface sensitive technique that provides high Raman scattering enhancement of molecules adsorbing on a rough metal surface, such as silver, gold and copper.(4; 5) Presently, there are two well-accepted theories describing the mechanism of SERS amplification: electromagnetic enhancement and chemical enhancement. Electromagnetic enhancement (EME) is responsible for up to $10^6$-$10^7$ increase of Raman scattering which occurs as the surface plasmon gets excited by incident light and amplifies the electromagnetic field of the metal surface.(4; 5) Chemical enhancement, on the other hand, provides up to $10^2$ increase of Raman scattering and it happens when the molecule adsorbs strongly on the surface of the metal which leads to changes of its polarizability. Due to the high enhancement, SERS technique has thus been studied extensively for last few decades and it provides many advantages including nondestructive nature, low detection limit, high sensitivity and easy sample preparation. SERS has been widely applied in many different areas.(2; 3; 90-95)

Metal nanoparticles (NPs) especially Ag and Au NPs have been widely employed in SERS because of their unique physical properties that depend on size and shape of the nanoparticles.(32; 33) Among the metals mentioned above, silver usually exhibits the highest SERS activity. It was reported that the optimal Ag nanoparticles size is 15 nm which generates the strongest SERS activity in solution.(32) Interestingly, the optimal size of Ag NPs was
observed ca. 50 nm when off-resonance SERS is adopted. On the other hand, gold has captured the most interests among recent studies. Gold metals known to be biocompatible and show a strong excitation close to the IR region of light, which has attracted considerable interests in its use in biotechnological systems. Various research groups have studied and reported the relationship between the SERS enhancement and the shape and size of the immobilized gold NPs on a substrate using different analytes like 4-aminothiophenol (4-ATP) and 5,5’-dithiobis (2-nitrobenzoi acid) (DNBA). The results indicate that the enhancement is highly dependent on many factors including the size of gold NPs, however with controversial conclusions. When 4-ATP adsorbed on immobilized gold NPs, the SERS intensity from gold NPs with the size of 30 nm was lower than that from gold NPs with the size of 18 nm. When 4-ATP was sandwiched between Au NPs and a smooth Au substrate, the SERS intensity was found out to increase as the size of the gold NPs increases. When labeled gold NPs was immobilized at a gold smooth surface, 60-nm NPs result in the largest SERS enhancement. In addition, the SERS intensity from 4-mercaptobenzoic acid on gold nanoparticles was reported to increase as the size of the NPs increases in solution when the total number of nanoparticles was kept the same.

Therefore, in this study, we investigated and compared the SERS enhancement of gold NPs of different sizes using both 4-nitrothiophenol (4-NTP) and 4-aminothiophenol (4-ATP) as the target molecules to better compare our results with previous ones. The SERS intensities of target molecules were probed by adsorbing them onto gold nanoparticles of the average size of 17, 30, 40, 50, 60 and 80 nm created using further reduction method. SERS spectra were recorded by using 647 nm Argon-Krypton lasers on a confocal Raman microscope. Furthermore, we determined the SERS enhancement and size correlation of nanoparticles under three
conditions including the same number of nanoparticles, the same total surface area of the nanoparticles and the same concentration of gold in solution. Interestingly, different conclusions of the relationship were drawn under various conditions. The optimal size of gold NPs that provides the highest enhancement factor was found out while the concentration, the total surface area and total number of the gold NPs were kept the same. This is the first time that the optimal size of gold NPs was established under various conditions in solution.

3.3 Experimental Methods

Hydrogen Tetrachloroaurate (III) trihydrate (HAuCl₄·3H₂O, 99.9+%), trisodium citrate (C₆H₅Na₃O₇·2H₂O, 99.9%) and hydroxylamine Hydrochloride (H₃NO·HCl, 99+%) were purchased from Fisher Scientific and used as received. SERS tested sample, 4-nitrothiophenol (80%) and 4-aminothiophenol (97%) were purchased from Sigma-Aldrich and used as received. All glassware was acid washed using sulfuric acid and nitric acid, and all the solutions were prepared using deionized water (18.2 MΩ·cm) from a Cascada BIO and AN Lab Water System.

3.3.1 Synthesis of gold nanoparticles

Gold nanoparticles (NPs) of different sizes were synthesized by multiple reduction processes based on the work of Haiss et al.(38; 101). First, gold nanoparticles were synthesized by reduction of 0.5 mM HAuCl₄ with 1% trisodium citrate in aqueous solution.(38) After this step, gold NPs of the average size of 17 nm were obtained. Next, 2.50 mL of 25.4 mM HAuCl₄ and 3.00 mL of 0.20 M Hydroxylamine were added into the 17 nm gold seed solution to synthesize gold NPs with a larger size. After this step, gold NPs of the average size of 30 nm were obtained. Using 30 nm gold NPs as the seeds, various amount of 25.4 mM HAuCl₄ and 0.20 M Hydroxylamine were added to the solution to synthesize larger gold NPs of different sizes.(101) In detail, 0.45 mL, 0.80 mL and 1.3 mL HAuCl₄ and 0.70 mL, 1.5 mL and 2.0 mL of
Hydroxylamine were used to synthesize gold NPs with the average size of 40, 50 and 60 nm, respectively. To synthesize 80 nm gold NPs, 60 nm Au NPs were used as seed and 0.43 mL HAuCl₄ and 0.67 mL of Hydroxylamine were added. An extra amount of reducing agent was added to ensure complete reduction of gold. All the solutions were dark red at the end. Colloidal solutions were kept in the dark during storage because of its photosensitivity concern.(102)

3.3.2 Sample preparation

In order to create calibration plots for 4-ATP and 4-NTP, samples were prepared by first pipette mixing 1.00 mL of gold colloidal solutions with different amount of 4-ATP or 4-NTP, then for 4-NTP, 1.00 mL of 1 M NaCl was added into a glass cuvette. All the measurements were done using exactly the same experimental conditions. Prepared samples were placed in the dark for 10 minutes before the measurement. This step helps to minimize the fluctuations of SERS spectra by allowing the colloidal solution to reach the equilibrium state.

3.3.3 UV-Vis, SEM and SERS measurements

UV-Vis absorption spectra of the colloidal solution were obtained with a Beckman Coulter DU 640 spectrometer. The UV-Vis spectra were used to elucidate the relative size of the particles in the solutions by comparing the location of the maximum peak wavelength in the spectra. Gold colloidal solutions were mixed with equal parts of deionized water and placed into quartz cuvette. The concentrations of gold colloidal solutions were adjusted to offset the UV-Vis spectrum for easy viewing. The concentrations were 0.083 mM, 0.040 mM, 0.015 mM, 0.017 mM, 0.019 mM and 0.028 mM for gold NPs of the average size from 17 nm to 80 nm. The wavenumber range was set between 200 nm to 800 nm and 0.5 s was used to scan the gold
colloidal solutions. UV-Vis spectra of the 17 nm to 80 nm gold colloidal solutions have been collected over a 4-week period of time to test the stability of the nanoparticles.

To measure the size of gold nanoparticles, Scanning Electron Microscopy (SEM) images were obtained using the Hitachi microscope (Hitachi S-800) located at Nanotechnology Research and Education Center on the University of South Florida’s Tampa campus. Before the SEM measurements, the gold colloidal solutions were dropped (ca. 2 µL) on top of the silicon wafer and air dried. The wafers were kept away from light because of the photosensitivity concerns. By imaging the particles using SEM,(103) the size and shape of individual particles of gold were characterized as well as the size distribution of the particles.

All the SERS experiments were carried out using a Confocal Raman Microscopy (Olympus, IX71) purchased from Horiba JovinYvon, equipped with an Argon and Krypton laser (Coherent, Innova 70C series) producing 514 nm and 647 nm of wavelengths. For all experiments, an excitation laser with the wavelength at 647 nm has been applied with 40 mW of power, 3 s of exposure time and 3 accumulations. The spectrum grating was 600 and the 20X microscopic objective lens was used throughout the experiments. All the results that were reported have been repeated independently for three times for the reproducibility. To examine the effect of nanoparticle size on the enhancement, either the concentration of gold, the total number or the total surface area of the gold NPs was kept the same in the final sample solutions.

3.4. Results/Discussion

3.4.1 SEM images of different-sized Au nanoparticles

To measure the size and its distribution of gold NPs, SEM technique was applied to get image of the gold NPs. The size of gold NPs in each image was measured individually. At least
100 nanoparticles were counted for each sample to estimate the mean diameter and the relative standard deviation of the gold nanoparticles.

Figure 3.1 shows typical SEM images and the histograms of size distribution of gold nanoparticles with the mean diameter of 17, 30, 40, 50, 60 and 80 nm, respectively. The particle shape was nearly spherical for the nanoparticles of all sizes. The statistical analysis results of the Au nanoparticles including the mean size, standard deviation and relative standard deviation are listed in Table 3.1. The standard deviation increases as the size of the gold NPs increases. However, the relative standard deviation values for the sizes around 40, 50, 60 and 80 nm of gold

Figure 3.1. SEM images of the gold NPs with an average size of a) 17 nm, b) 30 nm, c) 40 nm, d) 50 nm, e) 60 nm and f) 80 nm. The size histograms of the gold NPs with an average size of g) 17 nm, h) 30 nm, i) 40 nm, j) 50 nm.
NPs are apparently lower than others. The size distribution of the gold nanoparticles is comparable to previous studies,(100) which allow us to correlate the size of the nanoparticles with the SERS properties of the nanoparticles in solution.

Table 3.1. Size distribution, maximum peak wavelength in UV-Vis absorption spectra and calculated concentration, number and surface area of different gold colloidal solutions

<table>
<thead>
<tr>
<th>Size (nm)</th>
<th>Standard Deviation (nm)</th>
<th>Relative Standard Deviation (%)</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>Concentration of Gold (mM)</th>
<th>Number of Gold NPs (/L)</th>
<th>Surface Area of Gold NPs (m$^2$/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17</td>
<td>5</td>
<td>29</td>
<td>520</td>
<td>0.25</td>
<td>$(2.1 \pm 0.9) \times 10^{16}$</td>
<td>19± 5</td>
</tr>
<tr>
<td>30</td>
<td>7</td>
<td>23</td>
<td>525</td>
<td>0.12</td>
<td>$(5.5 \pm 1.9) \times 10^{15}$</td>
<td>15± 3</td>
</tr>
<tr>
<td>40</td>
<td>6</td>
<td>15</td>
<td>526</td>
<td>0.045</td>
<td>$(8.0 \pm 1.8) \times 10^{14}$</td>
<td>4.1± 0.6</td>
</tr>
<tr>
<td>50</td>
<td>8</td>
<td>16</td>
<td>528</td>
<td>0.050</td>
<td>$(4.4 \pm 1.1) \times 10^{14}$</td>
<td>3.6± 0.6</td>
</tr>
<tr>
<td>60</td>
<td>8</td>
<td>13</td>
<td>530</td>
<td>0.056</td>
<td>$(3.1 \pm 0.6) \times 10^{14}$</td>
<td>3.5± 0.5</td>
</tr>
<tr>
<td>83</td>
<td>13</td>
<td>16</td>
<td>555</td>
<td>0.084</td>
<td>$(5.8 \pm 2.6) \times 10^{13}$</td>
<td>1.2± 0.4</td>
</tr>
</tbody>
</table>

3.4.2 UV-Vis absorption spectra of gold nanoparticles

Figure 3.2 (A) shows the UV-Vis absorption spectra of gold nanoparticles of average size of 17, 30, 40, 50, 60 and 80 nm, respectively. The wavelength with maximum absorbance $\lambda_{\text{max}}$ was found out to increase from 520 nm to 555 nm, as shown in Table 3.1. As the size of Au NPs increases, the $\lambda_{\text{max}}$ increases which agrees well with the previous conclusion that the maximum peak wavelength red shifts as the relative particle size gets bigger.(38; 104)

To test the stability of those gold nanoparticles, UV-Vis absorption spectra of the colloidal solutions were collected over 4-week period of time. Figure 3.2 (B) shows the change of the maximum absorbance of different gold colloidal solutions with time. Clearly, the absorption intensity of those gold colloidal solutions does not change much over a month, which indicates that gold NPs are stable within that time frame. This phenomenon is also approved by
SERS measurements as the SERS-activity of those gold nanoparticles changes little within the period of one month.

Once the average size and its distribution of gold NPs were determined using SEM and UV-Vis techniques, the concentration, number and surface area of gold nanoparticles in the colloidal solutions with different sizes were calculated. Since more than enough reducing agent was added during synthesis, total reduction of gold is expected. Therefore, the number of gold NPs in the colloidal solution was estimated by equation 1 through dividing the total mass of gold in HAuCl₄ used to synthesize the gold NPs by the individual mass of a gold NP.

![Graph](image)

Figure 3.2. A) The UV-Vis spectra of gold NPs of different size. The concentrations were 0.083 mM for 17 nm, 0.040 mM for 30 nm, 0.015 mM for 40 nm, 0.017 mM for 50 nm, 0.019 mM for 60 nm and 0.028 mM for 80 nm. B) The change of maximum absorbance of gold NPs solution with different size over 4 weeks.
\[ n = \frac{m_t}{m_i} = \frac{m_t}{DV} = \frac{m_t}{D 4\pi r^3/3} = \frac{6m_t}{D\pi d^3} \quad (1) \]

In equation 1, \( n \) is the number of nanoparticles; \( m_t \) is total mass of Au in the solution; \( m_i \) is the mass of one nanoparticle; \( D \) is density of Au assuming that the density does not change with size of the nanoparticles; \( r \) is radius of the nanoparticle and \( d \) is the diameter of the nanoparticle.

Similarly, surface area of gold nanoparticles with certain size is calculated using equation (2) assuming that all nanoparticles are spherical.

\[ A = \pi d^2 n = \frac{6\pi d^2 m_t}{D\pi d^3} = \frac{6m_t}{DD} \quad (2) \]

Table 1 shows the distribution of size, calculated concentration, number and surface area of the gold nanoparticles in different colloidal solutions when synthesized.

3.4.3 SERS studies of 4-Aminothiophenol (4-ATP) to test the effect of concentration, number and surface area of nanoparticles on the enhancement

SERS technique was applied to compare the enhancement of gold NPs with different sizes. Figure 3.3(a) shows the SERS spectra of 4-ATP collected from gold NPs with the size of 17, 30, 40, 50, 60 and 80 nm respectively. The observed peak positions of 4-ATP agree with the literature values.(106; 107) In detail, the peaks at 1079 and 1587 cm\(^{-1}\) are assigned to the stretching vibrations of C-S and C-C, respectively. Another peak at 390 cm\(^{-1}\) represents the bending vibration of C-S. The peak at 1079 cm\(^{-1}\), as indicated by the arrow in Figure 3.3(a), was used to compare the intensity of the different gold solution because it exhibits the highest intensity and is characteristic of 4-ATP. The rest of the assignment of SERS peak of 4-ATP is summarized in Table 2.
Table 3.2. SERS Spectral Peak Assignment of 4-ATP \(^a\) and 4-NTP \(^b\)

<table>
<thead>
<tr>
<th>SERS peak (cm(^{-1})) of 4-ATP</th>
<th>Assignment</th>
<th>SERS peak (cm(^{-1})) of 4-NTP</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>390</td>
<td>(\delta) (CS)</td>
<td>332</td>
<td>(\delta) (CS)</td>
</tr>
<tr>
<td>635</td>
<td>(\gamma) (CCC)</td>
<td>723</td>
<td>(\pi) (CH) + (\pi) (CS) + (\pi) (CC)</td>
</tr>
<tr>
<td>1005</td>
<td>(\gamma) (CC) + (\gamma) (CCC)</td>
<td>854</td>
<td>(\pi) (CH)</td>
</tr>
<tr>
<td>1079</td>
<td>(v) (CS)</td>
<td>1081</td>
<td>(v) (CS)</td>
</tr>
<tr>
<td>1177</td>
<td>(v) (CH)</td>
<td>1110</td>
<td>(\delta) (CH)</td>
</tr>
<tr>
<td>1485</td>
<td>(v) (CC) + (\delta) (CH)</td>
<td>1340</td>
<td>(v) (NO(_2))</td>
</tr>
<tr>
<td>1587</td>
<td>(v) (CC)</td>
<td>1569</td>
<td>(v) (CC)</td>
</tr>
</tbody>
</table>

\(^a\) the peak assignment of 4-ATP is from reference 106 and 107.

\(^b\) the peak assignment of 4-NTP is from reference 107.

Figure 3.3. SERS spectra of (a) 0.25 µM 4-ATP and (b) 0.044 µM 4-NTP aqueous solution using gold nanoparticles with the size of 17, 30, 40, 50, 60 and 80 nm, respectively. The chemical structure of 4-ATP and 4-NTP is shown in the inset of spectra. The arrow of 4-ATP (1079 cm\(^{-1}\)) and 4-NTP (1340 cm\(^{-1}\)) indicates the peak used for the calculations of the enhancement factor.
Figure 3.4. Experimental enhancement factor of 4-ATP, 1079 cm$^{-1}$, as a function of size of gold nanoparticles a) when the number of gold NPs was kept the same, b) when the surface area of all gold NPs was kept the same, and c) when the concentration of gold was kept the same. The solid lines are just guiding of the eye. The error bar in x-axis is the standard deviation of the size of the nanoparticles while the error bar in y-axis is the standard deviation of enhancement factors from three experiments.

Figure 3.4 (a-c) shows the change of SERS analytical enhancement factor (AEF) of the vibration peak at 1079 cm$^{-1}$ with the size of gold nanoparticles when (a) the number of gold NPs, (b) the total surface area of NPs and (c) the concentration of gold were kept the same. All experiments have been repeated at least three times to calculate the standard deviation in AEF. The AEF shown in Figure 3.4 was calculated using Equation 3, where $I_{\text{SERS}}$ and $I_{\text{NR}}$ are the intensity of the vibrational peak in SERS and normal Raman (NR) measurements, respectively, $c_{\text{NR}}$ and $c_{\text{SERS}}$ are the concentration of 4-ATP or 4-NTP in NR measurements and the SERS measurements, respectively.(25; 108; 109)
\[
EF = \frac{I_{\text{SERS}^{\text{NR}}}}{I_{\text{NR}^{\text{SERS}}}}
\]  

(3)

Since SERS enhancement is mainly influenced by the surface adsorbed molecules, 0.18 \(\mu\)M, which was located in dynamic linear range of calibration plots of 4-ATP and 4-NTP, was used for the SERS measurements for more accurate analytical enhancement factor (AEF) measurement. The dynamic linear range of the analytes for the SERS measurements is determined using calibration plots for gold NPs with different size. When the total number of gold NPs in the sample were kept the same, the SERS intensity increases as the size of the gold NPs increases, as shown in Figure 3.4 (a). This observation can be easily understood due to the fact that as the size of the gold NPs increases, the surface area of the NPs increases, hence the amount of 4-ATP molecules adsorbed on the surface increases, and so does the SERS intensity. Clearly, surface area of the SERS substrates is directly related to the enhancement achieved for a SERS measurement.

However, is surface area the only reason for achieving high SERS enhancement? To answer this question, the spectra of 4-ATP from different colloidal solutions were collected by keeping the total surface area of the gold NPs the same. The results were shown in Figure 3.4 (b). Interestingly, the highest enhancement was achieved from gold nanoparticles with a size ca. 50 nm. This result undoubtedly indicates that the SERS enhancement depends not only on the surface area of the SERS substrates but also on other factors such as the enhanced electromagnetic field generated from the surface plasma. It is known that the local electromagnetic enhancement increases with the increasing particle size.(31) But, as the nanoparticles size gets bigger, the convex shape of the surface becomes flatter, the particles absorb less light and less inelastic scattering occurs on the surface, which leads to weakening of electromagnetic field on the surface and the overall SERS intensity.(97; 110-112) Moreover,
previous study of the correlation between the surface plasmon resonance (SPR) properties of gold NPs and SERS spectra has revealed that the SP band red-shifts with increasing particles size. The largest SERS EF was found for the gold NPs with an SPR maximum between the wavelengths for laser excitation and for the vibrational band under study. Therefore, as the size of the nanoparticles increases, the SPR moves close to the excitation wavelength of the laser (647 nm) and eventually away from that of the vibrational mode (696 nm). This explains our observations that the SERS EF generated from gold nanoparticles maximizes when the size of the gold NPS is around 50 nm. Interestingly, this conclusion agrees well with previous report when Au nanoparticles were immobilized on substrate.

Substrate enhancement factors (SEF) is another way of calculating enhancement factor, which is shown in Equation 4, where \( I_{\text{SERS}} \) and \( I_{\text{NR}} \) are the intensity of the vibrational peak in SERS and normal Raman (NR) measurements, respectively, \( N_{\text{NR}} \) and \( N_{\text{SERS}} \) are the total number of 4-ATP and 4-NTP in NR measurements and the surface concentration of the analyte in SERS measurements, respectively.

\[
EF = \frac{I_{\text{SERS}}N_{\text{NR}}}{I_{\text{NR}}N_{\text{SERS}}} \tag{4}
\]

SEF considers the actual concentration of molecules and surface area of NP substrates that contribute to the SERS enhancement. This SEF can compromise the disadvantage of AEF where you have more than a monolayer of molecules present in sample. In this experiment, SEF was calculated for the size of gold NPs that showed highest AEF. For 4-ATP, the highest AEF was found size around 50 nm of gold for both condition where the concentration and surface area of gold were kept the same. The SEF for gold NPs with size around 50 nm was estimated to be \(2.67 \times 10^4\).
Employing gold NPs in SERS experiment opened up more opportunities to study biological samples either *in vivo* or *in vitro* because of their better biocompatibility than Ag ones. Also, gold nanoparticles have attracted more and more attention as promising drug delivery means directly into biological systems.(95; 110) To minimize possible toxicity effect of injecting gold NPs into the biological samples, the smallest possible amount of gold should be used for each measurement. Therefore, the effect of the gold NPs’ size on their SERS enhancement was tested by keeping the concentration of the gold in the sample the same. The results were shown in Figure 3.4 (c). The highest enhancement was achieved from gold nanoparticles with a size ca. 50 nm. According to the recent study by Chen and his colleagues, gold nanoparticles with the size bigger than 8 nm and smaller than 50 nm are toxic to the biological samples that they tested.(113) Therefore, using gold nanoparticle ca. 50 nm can not only minimize the possible toxic effect on biological samples but also maximize the SERS enhancement factor generated from the NPs.

3.4.4 SERS studies of 4-Nitrothiophenol (4-NTP) to test the effect of concentration, number and surface area of nanoparticles on the enhancement

To test whether the conclusions drawn above are specific to 4-ATP and also to compare our observations with previous reports, we performed the same study using 4-NTP as the target molecule. Figure 3.3 (b) shows the SERS spectra of 4-NTP collecting from gold NPs with the size of 17, 30, 40, 50, 60 and 80 nm, respectively.(106; 107) The peaks at 1081, 1340 and 1587 cm\(^{-1}\) are due to the stretching vibrations of C-S, N-O and C-C, respectively. The peak at 854 cm\(^{-1}\) represents the wagging vibration of C-H while the peak at 1110 cm\(^{-1}\) is assigned to the bending vibration of C-H. The peak at 1340 cm\(^{-1}\), as indicated by the arrow in Figure 3.3 (b) was used to
compare the intensity of the different gold solution because it exhibits the highest intensity. The assignment of the SERS peaks of 4-NTP is summarized in Table 2.

Figure 3.5 (a-c) shows the change of SERS AEF of the vibration peak at 1340 cm\(^{-1}\) with the size of gold nanoparticles when (a) the number of gold NPs was kept the same, (b) when the surface area of all gold NPs was kept the same, and (c) when the concentration of gold was kept the same. The solid lines are just guiding of the eye. The error bar in x-axis is the standard deviation of the size of the nanoparticles while the error bar in y-axis is the standard deviation of enhancement factors from three experiments.

Figure 3.5 (a-c) shows the change of SERS AEF of the vibration peak at 1340 cm\(^{-1}\) with the size of gold nanoparticles when (a) the number of gold NPs, (b) the total surface area of NPs and (c) the concentration of gold were kept the same. Generally, despite of their similar chemical structure, big differences were observed between the SERS spectra and the EF of 4-NTP and those of 4-ATP even though the same experimental conditions including the SERS substrates
were used. This was observed before and might be explained by the difference in their chemical structure between the nitro group of 4-NTP and the amine group of 4-ATP. (106; 107) As shown in Figure 3.5 (a), when the number of gold nanoparticles was kept the same, the SERS EF increases as the size of the gold particle increases. Furthermore, Figure 3.5 (b) and (c) show that the highest EF was achieved from gold nanoparticles around 50 nm when the surface area and the concentration of gold were kept the same, respectively. The SEF for 4-NTP for size 50 nm gold NPs were both $1.27 \times 10^4$. (25; 108; 109) All these results were nearly identical with those from 4-ATP. This clearly indicates that the conclusions drawn from 4-ATP and 4-NTP might also be applicable to other adsorbates when using Au nanoparticles as the SERS substrates for the detection.

3.5 Conclusions

Different sizes of gold nanoparticles were synthesized based on the work of Cyrankiewicz et al and Haiss et al.(38; 101) SEM technique was applied to determine the size and shape of the gold NPs. The average size of gold nanoparticles was found out to be 17, 30, 40, 50, 60 and 80 nm, respectively with spherical shape. Gold NPs with the size of 50 nm exhibit the largest standard deviation. The red shift of $\lambda_{\text{max}}$ value from UV-Vis absorption spectra of the colloidal solutions also indicates the size increment of gold NPs. Both the SERS activity and the UV-Vis absorption of gold NPs were observed to be stable for at least one month.

Using gold NPs with different sizes, the SERS spectra of 4-ATP and 4-NTP were collected. In order to find out the optimal size of the gold NPs that provides the highest enhancement factor, either the concentration of gold, the number or the surface area of the gold NPs was kept the same. When the number of gold NPs was kept constantly, there was a positive
linear relationship between the size of the NPs and the EF based on the SERS measurements. This phenomenon can be explained by the fact that surface area increases as the size of the gold NPs increases when the total number of NPs is the same, which leads to the higher SERS intensity. However, interesting phenomena were observed when keeping the total surface area of gold NPs or the concentration of gold constant. As shown in Figure 3.4 and 3.5 (b and c), the highest intensity was observed when the size of gold NPs was around 50 nm when either the surface area of gold NPs or the concentration of gold was the same. Such phenomena might be explained by the relationship between the SERS enhancement and the size or surface area of the NPs.

More importantly, the same correlations were obtained for both 4-ATP and 4-NTP. This indicates that such conclusions might not be highly sensitive to the chemical structure of the target molecules. Instead, the conclusions might also be applicable to other adsorbates. Generally, gold NPs of the size around 50 nm are optimal when the surface area or the concentration is critical. In addition, gold NPs with size around 50 nm shows minimum toxicity effect on biological samples. This conclusion is essential when SERS is used to detect important biomolecules in biological samples so that minimum amount of gold can be introduced into the biological system to achieve lowest toxicity possible while the highest SERS sensitivity can be reached at the same time.
CHAPTER FOUR

ONE STEP SURFACE MODIFICATION OF GOLD NANOPARTICLES FOR SURFACE-ENHANCED RAMAN SPECTROSCOPY

Note to the reader

This chapter has been published as Seongmin Hong and Xiao Li. One Step Surface Modification of Gold Nanoparticles for Surface-Enhanced Raman Spectroscopy. Applied Surface Science.

4.1 Abstract

One of the drawbacks of surface enhanced Raman spectroscopy is that molecule of interest needs to be adsorbed or at least present near the surface of the substrates in order to achieve high enhancement. Unfortunately, majority molecules have no suitable functional groups interacting with the surface of the substrate and suffer poor SERS enhancement. In this work, one-step surface modification of gold nanoparticles (AuNPs) using linking molecules (LM) was demonstrated to attract the target molecules (TM) closer to the surface of AuNPs, hence lead to higher SERS enhancement. Here, 2-mercaptoethanol was employed as the linking molecule for its strong adsorption on AuNPs surface through thiol group on one end and the intermolecular interaction between the LM and the TM through the hydroxyl group at the other end. Three target molecules were tested. For benzoic acid, no intensity difference was observed on the AuNPs modified with LM compared with the non-modified original ones. Interestingly, two times higher enhancement was observed from cyclohexanol TM on the modified surface. As much as four times higher enhancement was achieved with the modified Au surface from 1,3-
cyclohexanediol. The stronger the interaction between the LM and TM is, the higher the SERS enhancement factor is. Also, the enhancement is highly dependent on the surface coverage of the LM. This simple modification method is important for most molecules which do not strongly interact with commonly used SERS substrates like Au or Ag nanoparticles and enables their possible characterization or detection using SERS.

4.2 Introduction

Surface enhanced Raman spectroscopy (SERS) is a surface sensitive technique that provides high inelastic scattering enhancement of molecule by adsorbing on a coinage metal surfaces, such as silver, gold and copper. Electromagnetic enhancement (EME) and chemical enhancement (CE) are two well accepted theories describing the mechanism of SERS enhancement. EME is responsible for $10^6$-$10^7$ increase of Raman scattering and it happens when the electromagnetic field of the metal surface becomes amplified through excitation of surface Plasmon by incident light. CE provides up to $10^2$ increase of Raman scattering and it occurs when the adsorbed molecule on the surface of the metal to form a bond and changes its polarizability. SERS technique has been under focus for last few decades and became more and more promising with its unique advantages like low detection limit, high sensitivity, easy sample preparation and wide possibility of biological applications.

In order to get high SERS intensity, either electromagnetic or chemical enhancement can be optimized using various methods. On one hand, researchers have explored extensively the effect of metal or bimetals composition, the shapes and sizes of those nanoparticles and nanostructures to improve the SERS activity of the substrate through EME. On the other hand, studies focusing on modification of nanostructured surface have been carried out to achieve better selectivity and SERS enhancement of the target molecules through CM
especially in biological sensing or detection. The main approach of modifying surface was to link one end of complex on the fixed metal or roughen metal surface of the SERS substrates while the other end of the complex attracts the molecules of interest. Employing antibody and antigen due to their high affinity is one of the examples. One end of the antibody contains a thiol group which can strongly adsorb to the surface of the gold nanoparticle while the other end of the antibody will attract the antigen for detection. Another example is to anchor one end of a molecule on gold or silver NPs or film. Then the functional group at the other end attracts the target molecule. Or, one can place gold or silver NPs or film on the functional groups to form a sandwich complex to create a hot spot. However, all these examples share common drawbacks that they require time consuming and complicated modification procedure. Also, they are very target specific, which can only be used for the certain molecule detection.

In this work, a simple one-step surface modification of gold nanoparticles (AuNPs) using linking molecule (LM) was investigated to provide higher selectivity and enhancement towards the target molecules (TM). To prove the effectiveness of the LM, the TM itself needs to exhibit poor SERS enhancement. So, the molecules that contain thiol or nitro groups are eliminated to avoid their direct adsorption on the AuNPs surface. However, the TM also needs to have functional groups that firstly aid in solubility of the molecules in aqueous solution and secondly induce the intermolecular forces between the LM and TM. Therefore, a hydroxyl group is chosen for our target molecules. Hence, cyclohexanol and 1,3-cyclohexanediol (1,3 CHD) are chosen for they contain one or two hydroxyl groups and are expected to show different affinity to the LM. As a comparison, the benzoic acid is also tested as TM which is expected to have weak interaction due to its steric hindrance. The three TM molecules have simple chemical structure
and do not exhibit strong SERS intensity themselves. However, they are basic constitutes for many important biomolecules of wide interests.

2-mercaptoethanol (2ME) was chosen as the linking molecule. First, it has a thiol group which can secure strong adsorption of thiol group on AuNPs surface and a hydroxyl functional group which provides the intermolecular forces between linking molecule and the target molecules. Second, the length of carbon chain for 2ME is short since the distance between target molecule and the AuNPs surface is critical for the EM enhancement. The one-step AuNPs surface modification was done by simply adding the 2ME linking molecules in Au colloidal solution and mixed vigorously for a few seconds. Then, the effect of such modified AuNPs was examined on the SERS intensity of the target molecules.

4.3 Experimental Methods

Hydrogen tetrachloroaurate (III) trihydrate (HAuCl₄·3H₂O, 99.9+ %), trisodium citrate (C₆H₅Na₃O₇·2H₂O, 99.9%) and hydroxylamine hydrochloride (H₃NO·HCl, 99+ %) were purchased from Fisher Scientific and used as received. SERS tested samples, 2-mercaptoethanol, benzoic acid, cyclohexanol and 1,3-cyclohexanediol were purchased from Sigma-Aldrich and used as received. All glassware was acid washed using sulfuric acid and nitric acid, and all the solutions were prepared using deionized water (18.2 MΩ•cm, a Cascada BIO and AN Lab Water System).

4.3.1 Synthesis of gold nanoparticles

Gold nanoparticles average size of 50 nm was synthesized through multiple reduction processes based on the work of Cyrankiewicz et al. and Haiss et al. First, gold NPs of the average size of 17 nm were synthesized by reduction of 0.50 mM HAuCl₄ with 1% trisodium citrate in aqueous solution. After this step, 2.50 mL of 25.4 mM HAuCl₄ and 3.00 mL of 0.20 M
Hydroxylamine were added into the 17 nm gold seed solution to synthesize gold NPs with the average size of 30 nm. Using 30 nm gold NPs as the seeds, 0.45 mL of HAuCl₄ and 0.70 mL of 0.20 M hydroxylamine were added to synthesize average size of 50 nm AuNPs. In order to achieve complete reduction of gold, the amount of reducing agents were in excess. All the solutions were dark red at the end. Colloidal solutions were kept in the dark during storage because of its photosensitivity concern.(102)

4.3.2 Sample preparation

First, certain concentrations of 2-mercaptoethanol were added to 1 mL AuNPs with 100 μL of aggregating agent 1 M NaCl. Then, the target molecules benzoic acid, cyclohexanol or 1,3-cyclohexanediol were added to test the SERS enhancement. Aggregating agents were used for the measurements of benzoic acid and 1,3-cyclohexanediol, but not cyclohexanol which leads to lower SERS intensity.

4.3.3 UV-Vis, SEM and SERS measurement

The UV-Vis absorption spectra of the colloidal solution were obtained with a Beckman Coulter DU 640 spectrometer. Gold colloidal solutions were mixed with equal parts of deionized water and placed into quartz cuvette. The concentration was 0.083 mM, 0.040 mM, and 0.017 mM for gold NPs of the average size from 17 nm, 30 nm and 50 nm. The wavenumber range was set between 300 nm to 800 nm and 0.5 s was used to scan the gold colloidal solutions.

Scanning Electron Microscopy (SEM) images were obtained using the Hitachi microscope (Hitachi S-800) located at Nanotechnology Research and Education Center on the University of South Florida’s Tampa campus. Samples for the SEM measurements were
prepared by dropping about 2 µL of the gold colloidal solutions on top of the silicon wafer and air dried. The wafers were kept away from light because of the photosensitivity concerns.

All the SERS experiment were performed using a Confocal Raman Microscopy (Olympus, IX71) purchased from Horiba JovinYvon, equipped with an Argon and Krypton laser (Coherent, Innova 70C series) producing 514 nm and 647 nm of wavelengths. Laser settings for all experiments were kept the same. An excitation laser with the wavelength at 647 nm has been applied with 40 mW of power, 5 s of exposure time and 10 accumulations. The spectrum grating was 600 grooves/mm and a 20X microscopic objective lens was used. All the results that were reported have been repeated independently for at least three times for the reproducibility.

4.4 Results

4.4.1 Characteristics of Gold Nanoparticles (AuNPs)

Figure 4.1 shows a typical SEM image and UV-Vis spectrum of the AuNPs. The SEM

![UV-Vis spectrum and SEM image of AuNPs](image)

Figure 4.1. A typical UV-Vis spectrum and SEM image of AuNPs with an average size of 50 nm.
image shows the overall shape of the AuNPs was sphere. To estimate the average size and the standard deviation of AuNPs, over 200 nanoparticles were measured individually. The average size of the AuNPs was 50± 8 nm which has been demonstrated to be the optimal size for high SERS activity.(8) The UV-Vis spectroscopy shows the maximum absorption, \( \lambda_{\text{max}} \), of AuNPs was at 528 nm, which agrees well with the previous studies.(8)

4.4.2 Normal Raman and SERS of 1,3-Cyclohexanediol

Figure 4.2 shows the spectra of 1,3-Cyclohexanediol (1,3-CHD). The peak at 1038 cm\(^{-1}\), assign to the vibrational mode of ring breathing of the cyclohexane, is used for calculating the SERS enhancement factor, as indicted by the arrow.(137) The more intense peak at 996 cm\(^{-1}\) adjacent to the one at 1038 cm\(^{-1}\) overlaps with that of the liking molecule (LM) 2ME. So, this peak is not chosen for further calculation. The normal Raman spectrum of 1,3-CHD (Figure

![Figure 4.2](image)

Figure 4.2. a) normal Raman spectrum of 5.6 mM 1,3-cyclohexanediol. b) SERS spectrum of 5.6 mM 1,3-cyclohexanediol. The arrows indicate the peaks at 1038 cm\(^{-1}\) that is used for the detection and calculating the enhancement.
4.2(a)) was taken with a concentration of 5.6 mM and exhibits low intensity ca. 15 counts. SERS spectrum of 1,3-CHD (Figure 4.2(b)) was obtained with the same concentration and the intensity is about 100 counts. The analytical SERS enhancement factor (AEF) can be calculated to be 6.7. This extremely low enhancement factor clearly indicates that 1,3-CHD is poorly enhanced for SERS based on bare AuNPs.(25; 108; 109) In other words, 1,3-CHD interacts very weakly with the AuNPs surface and the molecules only present in the surrounding of the NPs instead of direct adsorption.

4.4.3 Dynamic Range of linking molecule 2-Mercaptoethanol on AuNPs

Figure 4.3 shows the calibration curve and SERS spectrum of the linking molecule 2ME. The SERS spectrum of 2ME exhibit a strong peak at 1008 cm\(^{-1}\) which is assigned to the vibrational mode of out of phase C-C-O stretching.(138) This peak does not interfere with the

![Figure 4.3](image)

Figure 4.3. Calibration curve of the linking molecule, 2-mercaptoethanol (2-ME) by SERS. Inserted is a SERS spectrum of 0.24 \(\mu\)M 2ME. The arrow indicates the peak at 1008 cm\(^{-1}\) used to create this curve. All experiments have been repeated three times.
characteristic peak of the TM at 1038 cm\(^{-1}\), therefore was chosen to indicate the presence of the LM. The concentration range of 2ME was examined between 36 nM to 7.1 µM. When the concentration reaches around 240 nM, the intensity reaches its plateau indicating formation of a full monolayer of 2MELM on the AuNPs surface. Further increase of the concentration of 2MELM has negligible effect on its SERS intensity. Hence, the surface coverage of the LM on the AuNPs can be estimated by compare the SERS intensity of the peak with the saturated one of a full monolayer.

4.4.4 Optimization of 2MELM coverage for the detection of 1,3-CHD

To achieve higher enhancement, the effect of surface coverage of LM was examined on

Figure 4.4. A) SERS spectra of 1,3-CHD TM with varied concentration of LM at (a) 7.1 µM, (b) 710 nM, (c) 240 nM, (d) 140 nM, (e) 100 nM, (f) 86 nM, (g) 71 nM, (h) 65 nM, (i) 59 nM, (j) 47 nM and (k) 36 nM. The inserted blue arrow indicates the peak of 1,3-CHD TM that was used to calculate the intensities for the B). The inserted red arrow indicated the peak of 2ME LM. B) The relationship between SERS intensity of TM (diamond, blue) or LM (square, red) and the concentration of LM. All experiments have been repeated three times.
the SERS intensity of the TM. Figure 4.4 A) shows the SERS spectra of 1,3-CHDTM with
different concentrations of 2ME LM. The concentration of 1,3-CHDTM was 5.6mM since it
exhibits a minimal but still recognizable normal Raman intensity as shown in Figure 4.2 (a). The
concentrations of LM were varied within the dynamic range from 36 nM (spectrum k) to 240 nM
(spectrum a). The black and red arrows indicate the peak used to signpost the SERS intensity of
the TM and LM, respectively. Figure 4.4 B) shows the dependence of SERS intensity of both
TM and LM on the concentration of the LM. As indicated by the solid line, the SERS intensities
of 2ME LM increases almost linearly as the concentration increases. This observation is easily
explained since the concentration range tested is well within the linear dynamic range. Clearly,
the presence of TM has little effect on the SERS spectra of the LM or the interaction between the
LM and the AuNPs. This is reasonable since LMs exhibit much stronger interaction with the
AuNPs than TM does.

The dotted line represents the intensities from 1,3-CHDTM. When the concentration of
LM reaches at 71 nM, shown as spectrum f, the SERS intensity of 1,3-CHDTM reaches its
maximum. Interestingly, when the concentration of LM is low, the intensity of TM is lower than
that from non-modified AuNPs surface. This is because the presence of the LM blocking the
possible adsorption sites for the TM. As the concentration of LM increases, the surface coverage
of LM increases resulting in attracting more TM to the surface through intermolecular interaction
between LM and TM. Hence, high SERS intensity from LM was observed. However, as the
concentration of LM further increases above 71 nM, the surface coverage of the LM rises beyond
50%. The steric effect of the excess amount of LM on the AuNPs surface inhibits the approach
of TM close to the surface, resulting in low SERS intensity of the TM. The optimal condition is
achieved when the surface coverage of the LM is around 50% that can attract TM close to the surface instead of blocking.

When the surface coverage of the LM is ca. 50%, the SERS EF of 1,3-CHD TM is calculated to be 27. This number is about 4 times that from bare AuNPs without 2ME LM. This supports the idea that the optimized coverage of 2ME LM can attract the 1,3-CHD TM through intermolecular force, and improve the enhancement from TM of interests.

4.4.5 Application of optimized 2ME LM to Cyclohexanol and Benzoic Acid Target Molecule

The effect of LM on the SERS intensity of other two TM, cyclohexanol and benzoic acid were tested. Figure 4.5 shows the normal Raman and SERS spectra of cyclohexanol with and without the LM. The peak at 837 cm\(^{-1}\), assigned to the breathing mode of cyclohexane ring,\(^\text{139}\) was used for further calculation. For regular SERS based on bare AuNPs, the AEF is calculated

![Figure 4.5](image)

Figure 4.5. a) Normal Raman spectrum of cyclohexanol TM. b) SERS spectrum of cyclohexanol TM without 2ME LM. c) SERS spectrum of cyclohexanol TM using 2ME LM. The arrow inserted shows the peak of interest from cyclohexanol which was used to calculate the enhancement.
to be 3. With the aid of LM on the AuNPs, an AEF of 7 is achieved. Therefore, the enhancement factor from utilizing optimized 2ME LM was slightly more than 2 times higher than that without 2ME LM. Clearly, the presence of LM can attract cyclohexanol to the Au surface and a higher SERS intensity was obtained.

Figure 4.6 shows the normal Raman and SERS spectra of benzoic acid with and without the LM. For bare AuNPs, the AEF can be calculated to be 5. The peak at 1005 cm\(^{-1}\), assigned to the ring breathing mode and was used to calculate the enhancement.(140) The SERS intensities with and without the LM shows no difference. This result indicated that the presence of 2ME LM on the AuNPs was not able to attract the TM. It is probably the steric hindrance of the products from benzoic acid in provided condition.
4.5 Discussion

As mentioned earlier, the enhancement of SERS is originated from two different mechanisms. One is known as EME providing the majority of the enhancement, $10^6-7$. The other one is CE aiding the enhancement up to $10^2$. In most cases, CE does not generate strong enhancement. Therefore, we are focusing on conditions that can benefit mainly from EME. When the molecule of interest is close to the amplified electromagnetic field of metal substrate, improved SERS enhancement from the molecule can be achieved. Hence, a one-step modification of AuNPs surface using liking molecules (LM) was investigated here to obtain high SERS activity.

The purpose of employing linking molecule is to attract the target molecules close to the surface of AuNPs. Hence, there are several factors need to be considered in order to have properly functioning LM. First, one end of the LM should have a functional group that can strongly adsorb on the surface of AuNPs. A thiol group was selected for this purpose. Second, another end of the LM needs to have a functional group that can induce intermolecular force toward the TM. There are three commonly known intermolecular forces, hydrogen bonding, dipole-dipole, and London dispersion forces. Among them, the hydrogen bond provides the strongest interaction between two molecules. Since the TM contains hydroxyl groups, a hydroxyl group is adopted in LM too. Third, molecules with big cross section such as a benzene ring should be avoided to minimize the interference from LM in the SERS spectrum. Therefore, a simple hydrocarbon chain is chosen. Lastly, the chain that connects two functional groups should be as short as possible to get high EME. Based on all these factors, 2-Mercaptoethanol was chosen. The thiol end can adsorb on the surface of AuNPs, meanwhile, the hydroxyl group can point out and help to form intermolecular interaction between LM and TM. Also, two functional
groups are connected by a carbon chain that contains only 2 carbons which is only about 0.4 nm. The TM, if attracted, can experience the enhanced electromagnetic field of AuNPs.

SERS results show that the effect of the same LM is different for the three TMs, benzoic acid, cyclohexanol, and 1,3-cyclohexanediol. In details, no improvement in SERS intensity was observed for benzoic acid when LM was used to modify the AuNPs surface. Two times improvement in SERS AEF was obtained for cyclohexanol using LM modified AuNPs than bare AuNPs. The highest enhancement of four times was observed for 1,3 CHD after LM modification of the surface. Such phenomena can be explained by the intermolecular interaction between the LM and TM. While cyclohexanol can provide one site to form hydrogen bonding with LM, 1,3-CHD has two possible interaction sites for hydrogen bonding which can interact with two LM simultaneously. In case of benzoic acid, it also has two possible active sites to form a hydrogen bond with 2ME LM. However, no enhancement due to LM was observed. This can be explained by its chemical structure. Unlike in 1,3-CHD, the two hydroxyl groups are distant from each other, the two active sites (carbonyl and hydroxyl) in benzoic acid are right next to each other. The steric effect inhibits the interaction between two possible sites and LM. As the results, little improvement was obtained for the SERS measurement of benzoic acid. In general, the stronger the interaction between the LM and TM is, the higher the SERS EF can be achieved. This conclusion is important for the SERS detection of target molecules which do not interact with the non-modified bare SERS substrate. Even if the TM is not involved in any specific interaction like antibody-antigen, the one-step modification using LM based on common intermolecular forces can be employed to improve its SERS activity.
4.6 Conclusion

The effect of 2-mercaptopethanol as the LM on the SERS intensity of three TM, benzoic acid, cyclohexanol and 1,3-cyclohexandiol were examined. 2-mercaptopethanol was chosen as linking molecule because it has a thiol group that willingly adsorb on the gold surface, a hydroxyl group providing the intermolecular interaction with TM and a short hydrocarbon chain. The highest enhancement for the TM was obtained when the 2ME LM concentration is at 71 nM which is about 50% of surface coverage. Among the three TM, the calculated SERS EF was 4 times for 1,3-cyclohexanediol on AuNPs with LM modification than that without. For cyclohexanol, with less probability of hydrogen bonding, the SERS EF was only 2 times on modified AuNPs than non-modified ones. For benzoic acid, there was no difference in SERS enhancement from AuNPs whether 2ME LM was used or not. This conclusion is essential since existing linking molecule technique in SERS requires either “Lock and key” which is limited to antigen and antibody, or forming chemical bond between linking and target molecules which however needs long and complicated procedure. This new approach offers a simple and quick procedure. Also, it expands the application of SERS to molecules which are not involved in any specific couples. It can be used to improve the SERS intensity of any target molecules as long as suitable LM is chosen which can provide intermolecular interaction with the TM.
CHAPTER FIVE
APPLICATION OF RAMAN SPECTROSCOPY IN MATERIAL SCIENCE

5.1 Raman studies of the Encapsulation of Vitamin in Mesoporous MOFs

5.1.1 Introduction

Recently, many researchers have been focused to study and understand the biomolecule transportation within a porous system. Understanding the transportation-related mechanism in a porous system could be essential to research advances in many different fields like molecular biology, physiology, immunology and biochemistry. This also can contribute to applications in drug delivery, biocatalysis, drug and gene delivery and design and operation of biosensor.(141-144) Metal-organic frameworks (MOFs) are crystalline matrix that consists of metal ions or cluster and organic bridging ligands. One of the main reasons that MOFs became attractive to many researchers is its structural versatility. The functional groups of ligand can be manipulated or designed specific to interact with molecules of interest. Also, the pore size of MOFs can be controlled from microporous to mesoporous.(145-147) Therefore, employing MOFs as a porous system is ideal for the applications like separating or storing gas, and biomedical applications.(148-150)
Commonly, vitamins B are known to play a crucial role in cell metabolism, in particular, regulating the conversion process of protein and other types of nutrition into energy.(151) According to the report, impairment of brain function, heart disease, and also Alzheimer’s disease are related to the deficiencies of B vitamins.(152) Even though vitamins B have different solubility in water, they are all water-soluble. Most of the B vitamins need to be replenished on a regular basis because human body does not store B vitamins but excretes any excess. Therefore, it is common that people could have mild B vitamin deficiency. Among B vitamins, vitamin B$_{12}$ (cobalamin) is most commonly known specie and it plays an important role in brain function, nervous system, and for the formation of blood. Furthermore, it involves in intracellular metabolism, growth of cell and development process.(153; 154) Therefore it was chosen to be a probe molecule. Figure 5.1 shows the molecular structure of the vitamin B$_{12}$ (cobalamin). Raman spectroscopy is the vibrational spectroscopic method that opened up variety of possible applications to different scientific fields.(141-144) One of the many promising applications of Raman spectroscopy is that it provides fine detail information of molecular structure and interaction between molecules.(148-150) It has also been used to reveal the interaction between molecule of interest and a matrix.(148-150) In this study, the interaction between vitamin B12

![Figure 5.1. Molecular structure of vitamin B$_{12}$.](image_url)
and organic metallic or inorganic framework, Tb-MOF or MCM 41, was successfully characterized by Raman spectroscopy.

5.1.2 Experiment

All the Raman experiments were carried out using a Confocal Raman Microscopy (Olympus, IX71) purchased from Horiba JovinYvon, equipped with an Argon and Krypton laser (Coherent, Innova 70C series) producing a laser with the wavelength at 514 nm and 647 nm. In case of studying interaction between Tb-MOF and vitamin B12, an excitation laser with the wavelength at 514 nm was applied with 40 mW of power, 5 s of exposure time and 3

![Figure 5.2. Raman spectra of Tb-MOF (red), VB12 (3 mg/mL aqueous solution, blue) and VB12 in Tb-MOF (green).]
accumulations. To study the interaction between MCM 41 and vitamin B 12, an excitation laser with wavelength at 647 nm was applied with 20 mW of power, 10 s of exposure time and 5 accumulations. The reason for using different laser wavelength is that MCM 41 suffers from fluorescence background interference when the laser at 514 nm is used. The spectrum grating was 600 grooves/mm and an objective of 20X was used for both applications.

5.1.3 Results and Discussion

Figure 5.2 shows the Raman spectra of Tb-MOF (red), vitamin B12 solution (blue) and VB12 in Tb-MOF (green). Characteristic peaks of Tb-MOF were observed in the spectra of Tb-MOF with and without vitamin B12 encapsulation. As indicated by the solid lines in Figure 5.2, the peak at 1447 cm⁻¹ is assigned to the C-O stretching of benzoic acid structure and the peak at 3089 cm⁻¹ is assigned to the C-H stretching mode in Tb-MOF. (155) Not surprisingly, the energy of both peaks remains the same before and after vitamin B12 encapsulation indicating that these vibrational modes are not affected when vitamin VB12 is in the Tb-MOF framework. On the other hand, as illustrated by the dotted lines in Figure 5.2, there are also peaks specific of VB12 presenting in both spectra of VB12 before and after being encapsulated in Tb-MOF. For example, the strongest peak of VB12 at 1496 cm⁻¹ belonging to the in-phase stretching vibration of the Corrin system (156; 157) exhibits exactly the same energy in solution and in Tb-MOF, also shown in Figure 5.3 (C). Also, there is no change observed for all peaks of VB12 above 2000 cm⁻¹ in the hydrogen-involving vibrational region when VB12 is in the Tb-MOF. These identical peaks in both spectra with exactly same energy prove the successful encapsulation of VB12 in Tb-MOF.
Further comparison of the three spectra in detail provides information about possible interaction between Tb-MOF and VB12. Figure 5.3 shows the magnified Raman spectra zoomed in from Figure 5.2 in different ranges. In Figure 5.3 A) from 600 cm⁻¹ to 650 cm⁻¹ of spectrum range, the peak at 627 cm⁻¹ is involved with the deformation of the pyrroline rings and possible bending modes of the corrin ring.(156) Interestingly, when VB12 is encapsulated in Tb-MOF, blue peak shift were observed: the peak at 627 cm⁻¹ is shifted to 630 cm⁻¹. This peak is believed to shift from VB12 since the slight duplex form of peak 630 cm⁻¹ in VB12 encapsulated Tb-MOF spectrum is identical to the peak at 627 cm⁻¹ in VB12. The energy shift of this peak
indicates that such vibrational modes are involved when VB12 is encapsulated in the Tb-MOF. As the stretching mode of the corrin ring at 1496 cm$^{-1}$ does not exhibit any shift, it is suggested that VB12 interacts with the Tb-MOF framework through the pyrroline ring structure.

Figure 5.3 B) shows the change of three peaks belonging to Tb-MOF upon encapsulation of VB12. The three peaks, 993 cm$^{-1}$, 1020 cm$^{-1}$ and 1057 cm$^{-1}$, all red shifted by 8 or 6 cm$^{-1}$ upon VB12 insertion indicating strong interaction between VB12 and Tb-MOF. Among those three peaks, the strongest shift is observed on the one at 993 cm$^{-1}$, which belongs to the C-N stretching vibration of triazine of TATB(158). The peak at 1057 cm$^{-1}$ is assigned to the wagging mode of triazine structure while the peak at 1020 cm$^{-1}$ belongs to the in-plane deformation of the benzoic ring structure (155). In Figure 5.3 C), the peak of Tb-MOF shift from 1612 cm$^{-1}$ to 1607 cm$^{-1}$ upon encapsulation of VB12, which belongs to C=C stretching mode of benzene ring of TATB ligand(159). Clearly, the triazine and benzoic ring structure of Tb-MOF might serve as the active site when interacting with VB12.

Figure 5.4 shows the Raman spectra of VB12 (green), MCM 41 (red) and VB12 in MCM 41 (blue). The peak at 978 cm$^{-1}$ assigned to terminal silanol group of MCM 41, as indicated by a dotted line, is observed in the spectra of both MCM 41 and VB12 in MCM 41. This is the only available peak to identify the presence of MCM 41.(160; 161) Many peaks of VB 12, as indicated by the solid lines are observed in the spectra of both VB12 and VB12 in MCM 41. This proves that VB12 can be successfully placed inside of the MCM 41. Interestingly, detailed examination of the spectra reveals that there is no peak shift, not a single one, of either VB12 or MCM 41 when VB12 is encapsulated in the framework. This observation indicates that there is no strong interaction between VB12 and MCM 41. Such conclusion agrees well with the fact that VB12 can easily be released from the MCM 41 after only one or two days.
5.1.4 Conclusion

Raman study of VB12, Tb-MOF and VB12@Tb-MOF confirmed the encapsulation of VB12 in the pores of Tb-MOF. More importantly, the shift of vibrational modes of both VB12 and Tb-MOF upon encapsulation indicates that there is strong interaction between the two components and possibly between the pyrroline structure of VB12 and the triazine and benzoic ring of Tb-MOF.

The interaction between vitamin B2 (VB2) and MOF or MCM 41 was also examined by Raman spectroscopy. However, VB2 does not provide good Raman signals due to high fluorescence background. In fact, there is no Raman information reported so far about VB2. So, it is not discussed in this paper.
5.2 The Effects of Covalently Functionalized Single Walled Carbon Nanotubes on polymer Properties

5.2.1 Introduction

Polymer nanocomposites are promising material that can be used in different areas, such as, implantable devices and even spaceships.(162-164) These vast areas of applications attracted many researchers to study and control physical properties of polymers to make them more useful. Recently, using carbon nanotubes (CNTs) in polymer has generated attention because of their unique properties like electrical conductivity and high modulus.(165) However, including CNTs in polymer could be challenging because existing van der Waals forces make CNTs have tendency to form into a mass. According to the studies, utilizing sonication, covalent functionalization, in situ polymerization and novel solvent combinations can prevent the agglomerate of CNTs by disperse them. However, it is also known when too strong of sonication is applied, the suspended and dispersed CNTs can fall out of solution or be damaged. Also, when the CNTs are functionalized with covalent bonds, the properties of CNTs can be changed from those of interest.(165; 166)

Suggested by the results from Clayton et al., sonication and functionalization were combined to create well dispersed CNTs in polymer matrix.(165) Poly(4-methyl-1-pentene), PMP, was chosen as the polymer matrix because it is easily available, transparent, and it has relatively high modules. CNTs were functionalized by using reductive alkylation and 1-Iodododecane. Carbon nanotubes were purchased commercially, and it contains 35% to 50% of amorphous carbon and catalyst by weight. There were two possible ways to purify the purchased CNTs. First method is let the metal catalyst and amorphous carbon undergo acid digestion. However, this step may not provide enough CNTs for the experiment. The second method was
using filtration after the functionalization. This method provided high quantity of functionalized CNTs (FCNTs) for the experiment. After this step, the FCNTs were dissolved in carbon tetrachloride along with PMP and then the solvent was removed leaving the FCNTs spread homogenously in the polymer.

Vibrational spectroscopy has long been used to characterize the molecule structure and the interaction among molecules. Employing Raman spectroscopy to study carbon nanotubes with and without functionalization therefore provides desired information about the structure of the carbon nanotubes such as the metallic properties and the degree of disorder. (167; 168) In this study, carbon nanotubes and dodecyl iodide functionalized carbon nanotubes are successfully characterized by Raman spectroscopy.

5.2.2 Experimental Method

All the Raman experiments were carried out using a Confocal Raman Microscopy (Olympus, IX71) purchased from Horiba JovinYvon, equipped with an Argon and Krypton laser (Coherent, Innova 70C series) producing a laser at 514 nm and 647 nm of wavelengths. For all experiments, an excitation laser with the wavelength at 647 nm was applied with 20 mW of power, 10 s of exposure time and 5 accumulations. The spectrum grating was 600 grooves/mm and an objective of 20X was used.
5.2.3 Results and Discussion

Figure 5.5 shows the Raman spectra of carbon nanotubes before and after functionalization. Two characteristic peaks were observed in the Raman spectrum of raw carbon nanotubes, the red one in the figure. Peak I at $1310 \text{ cm}^{-1}$ is the D-band generally founded in the $sp^2$ carbon materials and is usually defined as a disorder induced Raman scattering. Peak II at $1580 \text{ cm}^{-1}$ is the G-band of the tangential modes of carbon nanotubes.\(^{(144; 169)}\) To monitor the structure change of the functionalized carbon nanotube, its Raman spectrum was collected and shown as the blue spectrum. After functionalization, the peak positions of both D and G modes remain the same. However, the peak intensity ratio $I_D/I_G$, relative intensity of D-band to that of the center of G-band, increases from 0.14 to 0.67 as the result of the functionalization. The

![Raman spectrum of carbon nanotubes](image)
increase of this ratio indicates higher structural disorder and smaller crystalline size in the functionalized carbon nanotubes compared with the pure carbon nanotubes. (144; 169)

5.2.4 Conclusion

Vibrational characterization of the carbon nanotubes by Raman spectroscopy confirmed the functionalization of carbon nanotubes by dodecyl iodide. Furthermore, the increase of the relative intensity of D-band vs. G-band indicates that higher disorder of the carbon nanotubes after functionalization.
CHAPTER SIX
SERS DETECTION OF BIOLOGICALLY IMPORTANT MOLECULES

6.1 Detection of Neurotransmitters

6.1.1 Introduction

Neurotransmitters are chemical signalers that are passed from one neuron to another through the process of neurotransmission. Neurotransmission is an electrical movement in synapses caused by a dissemination of nerve impulses to control the human function. Neurotransmitters are located at the end of the neuron called axon or presynaptic terminal. When the electrical impulse, called action potential, is generated from the cell body and reaches the axon, the neurotransmitters in the axon are released into the extracellular space, called synaptic cleft, where extracellular proteins influence the diffusion, binding, and degradation of molecules released from the presynaptic terminal. Then, neurotransmitters are taken up by post synaptic receptors to cause either depolarization or hyperpolarization of the membrane. If the membranes are depolarized by accumulation of neurotransmitters, the action potential will occur. But when the accumulation of neurotransmitter causes hyperpolarization, the action potential will be diminished. (170; 171)

Dopamine is one of the neurotransmitters that play important roles in the brain, such as cognition, motor activity and inhibition of prolactin production. It affects physiological conditions like behavior, motivation, reward, sleep, mood, attention and learning. Many studies
show that deficiency of dopamine is related to Alzheimer's disease, Parkinson's disease and major depressive disorders that still do not have definite cures. (172-174) Despite dopamine being a very important part of brain functions, it is difficult to study using currently available analytical techniques because of its low physiological concentration.

![Molecular structure of dopamine](image)

Figure 6.1. Molecular structure of dopamine.

Dopamine has been studied with many different techniques, such as fluorescence, IR spectroscopy and electrochemistry. (175-177) However, particular obstacles limit the effectiveness of those techniques. In order to detect dopamine using fluorescence, dopamine needs to bind to a fluorescence tag molecule that makes detection possible since dopamine itself does not generate fluorescence. However, dopamine is a small compound with low concentration. If fluorescence is to be used, the attached fluorescence tag molecule is usually much larger than dopamine. Consequently, the tag molecule strongly interferes with the behaviors of dopamine process. Since dopamine needs to be studied in aqueous conditions, IR, which is easily interfered by the presence of water, could not be used for the low-concentration detection of dopamine. Different types of electrochemical methods also have been used to study dopamine; however, there are several problems that have been reported. First of all, dopamine has a concentration \textit{in vivo} at the nanomolar level, and this challenges the sensitivity limitations of electrochemical methods. In addition, the detection of dopamine is usually interfered by other electroactive substances in the fluids, such as homovanillic acid, 3,4-dihydroxyphenylacetic acid and ascorbic
In the case of using fast-scan cyclic voltammetry (FSCV), the background current is relatively larger than the current generated from dopamine. Another major limitation of FSCV is background drift, which limits the use of digital background subtraction techniques to intervals less than 90 seconds before distortion of dopamine signal occurs. The problems for the slow-scan cyclic voltammetry and ellipsometry are that they do not allow detection of dopamine in a sub-second time domain. In the case of real time in vivo studies, these techniques do not provide the high enough selectivity and sensitivity because of its slow scan rate. To overcome these problems, modification is possible, but requires more time and steps that limit the applicability of using electrochemical methods.

Melatonin and serotonin are other neurotransmitters that play important roles in physiological and pathological parts of living organisms, therefore, gained attraction from the researchers in recent years. These neurotransmitters are produced by many tissues and cells in living organisms, such as, retina, bone marrow cells, platelets, gastrointestinal tract, skin, and lymphocytes. However, they are mainly produced by the pineal gland. The existence of neurotransmitter receptors in various locations mentioned above proves how serotonin and melatonin can be involved and play an important role in physiological processes in living cells.

Figure 6.2. Molecular structure of melatonin.  

Figure 6.3. Molecular structure of serotonin.
Similar to the dopamine, these two neurotransmitters also can affect the behavior, sleep, reproduction, immune responses, and energy balance of human by affecting brain function. Therefore, people who have problem with regular sleep and severe mood swing use either melatonin or serotonin to easy their symptoms. According to the recent study, serotonin and melatonin have antioxidant properties that counteract with reactive oxygen and nitrogen species. (182-184)

All those aforementioned importance of melatonin and serotonin resulted in extensive studies of them in many different areas. In fact, the results from those studies have provided most relevant understanding of neurotransmitters in organisms. However, there are problems with existing techniques. For example, labeling and immunohistochemistry are two commonly used methods to study the neurotransmitters. But, labeling creates challenges in health risks and immunohistochemistry have problems with specificity because the sizes of these neurotransmitter molecules are small. It is challenging to distinguish them from its precursors and metabolites when designated antibodies were used for the detection. (182-184) Thus, the development of selective and less hazardous detection methods has become necessary.

The main problems to detect all these neurotransmitters mentioned above, dopamine, melatonin, and serotonin, they are in low concentration and there are many other coexisting endogenous compounds in blood. Appearance of Surface Enhanced Raman Spectroscopy (SERS) became one of the most promising techniques since it provides several advantages: high enhancement and specificity of molecule, many biological applications since there is no significant interference from aqueous solvent, easy sample preparation, one wavelength can be applied to multiple species, and none invasive when near infrared laser is used. Considering the low concentration and selectivity of those neurotransmitters are the main issues. It is promising
that employing SERS can deliver better results in both qualification and quantification of neurotransmitters. Also, utilizing SERS possibly introduce the in vivo study of neurotransmitters.

6.1.2 Results and Discussion

6.1.2.1 SERS detection of neurotransmitters

In order to demonstrate the SERS activity of neurotransmitters, dopamine, melatonin and serotonin, series of concentrations of those neurotransmitters were prepared. Figure 6.4 (A) shows the SERS spectrum of dopamine with the concentration of 95 µM. The experiments were carried out using silver substrates with average size of 90 nm and 647 nm laser with settings of 40 mW of power with 2 seconds of exposure time and 1 accumulation. Series of dopamine

Figure 6.4. (A) SERS spectrum of dopamine with concentration of 95 µM using 647 nm laser. (B) Calibration curve of dopamine.
solutions were prepared with different concentrations, 95 μM, 30 μM, 10 μM, 1 μM, and 0.1 μM. The result is showing in Figure 6.4 (B), which is the calibration plot of dopamine using SERS. The peak at 1300 cm⁻¹ which is from the stretching mode of C-N was used to plot the calibration plot. There is another peak that appears the strongest above 1000 cm⁻¹ was not used considering the possibility of an instrumental error. The lowest concentration used was 0.1 μM and the calibration plot proves detection of sub-micro concentration of dopamine is possible.

Figure 6.5 (A) shows the SERS spectrum of melatonin with concentration of 3.2 μM. The experiments were carried out using gold substrates with average size of 40 nm and 785 nm laser with settings of 40 mW of power with 2 seconds of exposure time and 5 accumulation. Different

Figure 6.5. (A) SERS spectrum of melatonin with concentration of 3.2 μM using 785 nm laser. (B) Calibration curve of melatonin.
concentrations, 16 µM, 8 µM, 3.2 µM, 1.6 µM, and 0.16 µM of melatonin solutions were prepared to investigate the detection limit of the melatonin using SERS. The initial result was showing the hyperbolic trend plot, which was different than what was expected. So, the experiments were repeated 3 times to see whether the trend of plot would be consistent. The result is showing in Figure 6.5 (B). One of the possibilities of poor linearity of calibration plot is the formation of melatonin monolayer on gold nanoparticles surface around the concentration of 2 µM. The peak at 1247 cm\(^{-1}\) which belongs to the stretching mode of C-N was used to plot the calibration plot because simply it has the highest intensity. The lowest concentration used was 0.16 µM and the calibration plot proves detection of sub-micro concentration of melatonin is possible. In order to have better calibration curve, more points need to be added between 0 to 2 µM.

Lastly, serotonin was tested. The experiments were carried out using both silver and gold substrates with average size of 90 nm and 40 nm, respectively. The source of laser and settings were kept the same using 647 nm laser with settings of 35 mW of power with 2 seconds of exposure time and 5 accumulations. However, the results were slightly better when the silver nanoparticles were used as substrates, so only results from AgNPs were used in this text. Figure 6.6 (A) shows the SERS spectrum of serotonin with concentration of 9 µM. The peak at 1560 cm\(^{-1}\) which belongs to the stretching mode of C-C was used to plot the calibration plot because simply it has the highest intensity. Different concentrations, 90 µM, 27 µM, 9 µM, 0.9 µM, and 0.09 µM of serotonin solutions were prepared to examine the detection limit of the serotonin using SERS. The result is showing in Figure 6.6 (B). The lowest concentration used was 0.09 µM and the experiment results prove the melatonin detection of sub-micro concentration of
melatonin is possible. Based on all these preliminary results of neurotransmitters we tested using SERS, it strongly supports the possibility of SERS being used as superior detection method.

6.2 Detection of Caffeine

6.2.1 Introduction

Caffeine, also known as 1,3,7-trimethylxanthine, is a commonly known alkaloid that is one of the purine alkaloids. Figure 6.7 shows the molecular structure of caffeine. More than sixty different plants contain caffeine and many of these have already been consumed by human with different purposes, such as medicine, beverages and ingredients. The most well-known function of caffeine is to stimulate the sympathetic nervous system. Recently there has been increase in numbers of clinical studies of caffeine in obesity, fatigue, anxiety, attention deficit and
Alzheimer’s decease. All these clinical studies were done based on the results of the studies that show effectiveness of caffeine in physical performance, energy expenditure, muscular coordination, and cognitive function. Studies also proved that people might suffer from anxiety, tremor, palpitations, insomnia, gastrointestinal disturbances and high blood pressure from high dose of caffeine. Despite numbers efforts, there are still clear roles of caffeine, such as bioactivity or mechanisms.

Numerous techniques have been developed to study caffeine. However, those existing techniques generally focused on in vitro studies. For example, high performance liquid chromatography (HPLC) is one of the methods to study caffeine. This method could provide information of the active interaction between caffeine and molecules or the coefficients of permeation of caffeine. In order to deeply understand caffeine’s bioactivity or mechanisms, it is required to have experimental set up that is same or at least similar to the living organism condition (in vivo). Employing SERS into studying caffeine in vivo environment can provide highly specific information of the caffeine even if with the low concentration. Also, with the specificity of SERS, it is expected to have a minimal interference from other coexisting molecules in cell.
6.2.2 Results and Discussion

Caffeine was tested using SERS technique and the results are showing in figure 6.8.

Figure 6.8 (A) shows the SERS spectrum of caffeine with concentration of 43 µM. The experiments were carried out using gold substrates with average size of 40 nm. The source of laser and settings were kept the same for all trials using 785 nm laser with settings of 40 mW of power with 2 seconds of exposure time and 10 accumulations. Figure 6.8 (B) is the calibration plot of caffeine with different concentrations, 1.1 mM, 0.43 mM, 86 µM, 43 µM, and 4.3 µM. The plot was created using the peak at 1000 cm⁻¹ which belongs to the stretching mode of C-N-C was used. This peak was chosen to plot the calibration plot since it clearly is the peak that has
highest intensity. The peak intensities were collected from the caffeine SERS spectra of different concentrations then plotted. The lowest concentration used was 4.3 µM, which is slightly higher than other molecules tested so far. However, when the experimental conditions are optimized, such as different laser sources and substrates, detection of sub-micro concentration of caffeine is very probable.

6.3 Detection of Nicotine Using SERS

6.3.1 Introduction

According to the studies, tobacco smokes contain more than 4000 chemicals that are known to be harmful and cause cancers. It was reported by WHO there are more than 5 million people die from smoking and second hand smoking each year worldwide. The medical cost caused by exposure of tobacco smoke is about ten billion dollars in United States alone.

Nicotine is one of the major and most potent chemicals existing in tobacco plants. Nicotine can be used as stimulant drugs and also has anti-herbivore function, so it was used as an insecticide in the past. Originally, the study of nicotine was related to understand smoking behavior and usually used biological fluids to analyze. Extensive studies of harmful effects of chemicals from tobacco smokes lead to develop products like nicotine patches or gums. These replacement

Figure 6.9 Molecular structure of nicotine
products provide minimal or no chemical toxicity from tobacco smokes, therefore, industry has been grown rapidly.

The most common method to quantify the nicotine and its metabolites is using HPLC coupled with UV-Vis spectroscopy. It was reported that the detection limit was as low as 0.99 ppm. The problem employing HPLC/UV-Vis is this technique requires pretreatment of samples and only limited number of sample can be tested per day because of time consuming preparation. Alternative methods, such as, GC-MS, and radioimmunoassay, have been used to test the nicotine. However, they enclose similar problems of HPLC/UV-Vis sample preparation.

![SERS spectrum of nicotine with concentration of 9 nM using 514 nm laser.](image1)

![Calibration curve of nicotine.](image2)

Figure 6.10. (A) SERS spectrum of nicotine with concentration of 9 nM using 514 nm laser. (B) Calibration curve of nicotine.
Advantages like sensitivity and molecular specificity, SERS has been attracted researchers to study many different molecules. Results from studies suggest that utilizing SERS to study nicotine can provide inexpensive, rapid and simple sample preparation with minimal or no fluorescence interferences.

6.3.2 Results and Discussion

Figure 6.10 is the result from SERS experiment of nicotine. Figure 6.10 (A) shows one of the examples of the SERS spectra of nicotine with the concentration of 3 nM. Figure 6.10 (B) is the calibration plot of nicotine. Different concentrations, 0.36 mM, 7.2 µM, 0.14 µM, 3 nM, 60 pM, and 1.2 pM, of nicotine solutions were prepared for SERS experiment. The experiment setups were kept the same throughout the experiment. Silver nanoparticles with average size of 90 nm were used as substrates. The 514 nm laser was used with settings of 40 mW of power with 2 seconds of exposure time and 2 accumulations. The calibration plot was created using the peak at 1031 cm\(^{-1}\) which belongs to the stretching mode of C-N was used. The peak intensities were measured and collected from the nicotine SERS spectra of different concentrations then plotted. The peak at 1031 cm\(^{-1}\) was chosen to plot the calibration curve since it clearly is the peak that has highest intensity. The lowest concentration used was 1.2 pM, which is inarguably lower than other molecules tested so far. Considering the experimental conditions were not optimized, detecting caffeine with pM concentration proves how powerful the SERS can be. If the experimental conditions are optimized using different substrates, such as bimetallic nanoparticles, detection of nicotine that has lower than pM of concentration is possible.
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APPENDIX (A)
SILVER NANOPARTICLE SYNTHESIS

Silver colloidal solutions can be prepared by using four different methods, Lee and Meisel method, Alternative Lee and Meisel method, Creighton method and Leopold and Lendl method. The synthesis methods were explained in detail below.

1. Lee and Meisel method

- Accurately measure 0.017 g of silver nitrate (AgNO₃) (Fisher Scientific, S486-100) and place it into an acid washed 100 mL volumetric flask, then fill up Millipore water to make 1 mM of AgNO₃. Also, weight 1 g of trisodium citrate (Na₃C₆H₅O₇·2H₂O) (Fisher Scientific, BP327-500) and dissolve into 100 mL Millipore water to make 1% (w/w)
trisodium citrate solution. Transfer the AgNO₃ solution from the 100 mL volumetric flask to a 125 mL Erlenmeyer flask then put the stir bar into the flask. Place it on the hot plate to heat the solution up to 93 °C while the solution is vigorously mixed. When the temperature is reached, 2 mL of 1% w/w sodium citrate is added using a micro pipette. The temperature of the mixture should be kept between 93 °C to less than 100 °C for an hour, then cool down to room temperature. During the heating, a grey color mixture was obtained. Figure A.1 shows the SEM image of synthesized silver nanoparticles and the bar graph indicates the overall size distribution of them.

2. Alternative Lee and Meisel method

- This method follow exactly same step up to transferring 1 mM AgNO₃ into 125 mL Erlenmeyer flask. The only difference is how and when to add the 1% w/w trisodium citrate solution. Accurately measure 0.017 g of silver nitrate (AgNO₃) and place it into

![SEM image and overall size distribution of silver nanoparticles synthesize with alternative Lee and Meisel method.](image)

Figure A.2. SEM image and overall size distribution of silver nanoparticles synthesize with alternative Lee and Meisel method.
acid washed 100 mL volumetric flask, then fill up Millipore water to make 1 mM of AgNO₃. Also, weight 1 g of trisodium citrate (Na₃C₆H₅O₇·2H₂O) and dissolve into 100 mL deionized water to make 1% w/w trisodium citrate solution. Transfer the AgNO₃ solution from the 100 mL volumetric flask to a 125 mL Erlenmeyer flask then put the stir bar into the flask. Place it on the hot plate to heat the solution up to 90 °C while the solution is vigorously mixed. First portion, 0.2 mL, of 1% trisodium citrate solution is added at that temperature. Then, bring the temperature up to 95 °C. The next three portions, 0.6 mL, are going to be added in every 15 minutes. When all the reducing agent is added, the solution is stirred for another 30 minutes then cool down to the room temperature. Figure A.2 shows the SEM image of synthesized silver nanoparticles and the bar graph indicates the overall size distribution of them.

3. Creighton Method

-Prepare 25 mL of 1 mM AgNO₃ by dissolving 0.0043 g of AgNO₃ into the 25 mL volumetric flask with deionized water. Also, 75 mL of 2 mM sodium borohydride

Figure A.3. SEM image and overall size distribution of silver nanoparticles synthesize with Creighton method.
(NaBH₄) is prepared by dissolving 0.0057 g in Millipore water. Transfer 75 mL of NaBH₄ into 125 mL of Erlenmeyer flask with stir bar. Then, add 25 mL of 1 mM AgNO₃ drop wise. There is no heat required in this process. The experiment can be performed at room temperature. While the 1 mM AgNO₃ is added, the color change into brownish grey is observed. Keep stirring for another 30 minutes. Figure A.3 shows the SEM image of synthesized silver nanoparticles and the bar graph indicates the overall size distribution of them.

4. Leopold and Lendl Method

-1 mM AgNO₃ is prepared by dissolving 0.017g of AgNO₃ into 100 mL of volumetric flask with deionized water. Also, prepare 0.10 M sodium hydroxide by dissolving 4 g of NaOH into a 100 mL volumetric flask with Millipore water, and 0.06 M hydroxylamine is prepared by dissolving 0.198 g of NH₂OH into a 100 mL volumetric flaks with Millipore water. Transfer 1 mM AgNO₃ into a 125 mL Erlenmeyer flask. Take 4.5 mL of

![Figure A.4. SEM image and overall size distribution of silver nanoparticles synthesize with Leopold and Lendl method.](image-url)
0.1 M NaOH and 5 mL of 0.06 M NH₂OH, then rapidly added them into 1 mM AgNO₃ solution. Keep stirring the mixture until the grey milk color is observed. Once color change observed, continue stir for several minutes. Figure A.4 shows the SEM image of synthesized silver nanoparticles and the bar graph indicates the overall size distribution of them.

For all methods, a small Teflon stir bar was used for homogeneous and vigorous mixing of the reagents. A standard thermometer was used to monitor the temperature change. Placement of the thermometer should allow only the tip to be submerged in solution to avoid losing reduced silver and gold particles due to coating the surface of the thermometer. Once the solutions were made, they were kept inside of the drawers to avoid contact with any light sources due to photosensitivity. All glassware was acid washed prior to use to avoid any kind of contamination.
APPENDIX (B)

LASER

1. How to turn the laser on

There are several steps to turn the laser on properly. Steps are simply, but it is crucial to keep the procedure in the right order since the laser can be easily broken or damaged. So, in this chapter, the steps are explained step by step with pictures.

- Step 1. First, the power switch had to be turned on. It is located on the wall. As seen on the picture below, the lever should be on off position at all time when the laser is not in use. To turn the main power on, the lever has to be pushed up to where the on sign is. The arrow indicates the direction where the lever should move to turn the power on.
- Step 2. After the main power is on, the cooling water has to be turned on. The lever is located right side of the main power unit on the wall. As seen on picture below, the yellow lever positioned perpendicular to the water line. This means the water is blocked. When the lever is turned down as shown in the arrow direction, the water is opened. It can be easily heard by water running sound. The water has to run constantly while the laser is in use. Even after the laser is turned off, it has to be in open position for 10 – 15 minutes to make sure that the laser body and controller box cools down to room temperature.

![Image of water lever](image.png)

- Step 3. Next step is to turn on the laser controller box. On the front face of the box, there is a key which is in off position. When you turn that key into on position, the system will make noise with fan spinning inside of the controller box. The key can be either taken out be placed somewhere safe or remaining inserted in the box.
- Step 4. On top of the controller box there is a laser controller. It has many different buttons that can change the setting of laser, such as, power and wavelength. When the controller box is on, this controller will turn on together and display the laser setting. The laser is at off since the laser itself has not been turned on yet.

- Step 5. The next step to take is push the on/off button on the controller. When the on/off button is pressed, it will make quite loud clicking sound, it is normal. Then, the display shows the count down from 50 seconds to 0 second. When it gets to the near 10 seconds, there will be another loud clicking sound.
- Step 6. When the count down is over, you should see the laser light coming out from the head of the laser box and display should show the power of the laser with rest of the settings.

- Step 7. If you do not see the power on display or laser light coming out at the head of the laser box, the laser is probably blocked by the slit. This slit can be opened by turning the knob, which is indicated by the arrow, to open position. It is located on the top of the laser head. Then now the laser light should come out of the laser box and display shows the power of the laser.
- Step 8. The sample stage has to be checked to make sure the laser is coming through the objective and reaching the sample. If the laser light does not reach to the sample stage, the optics has to be re-aligned.

2. How to Calibrate the Laser

The laser needs to be calibrated in order to get the reliable experimental results. When the laser is not calibrated properly, it can cause the severe error in the results. The silicon is used as a reference target when the laser is calibrated. Here are the steps to calibrate the laser properly.
- Step 1) After the laser is on, place the silicon reference target on the sample stage. The picture below show what the reference looks like. The white arrow indicated the silicon reference target that is attached to the surface of the glass slide. When the reference is placed on the sample stage, the face where the silicon is attached need to face down. There are four pins that can secure the reference. The glass slides usually fits right into the grove on the sample stage, but if the slides need extra security, those pins can be used.

![Image of reference target on sample stage]

- Step 2) Open the program call “lab spec5” first. When it opened, click on the camera icon, which is located on the top menu bar. Then, the computer screen will appear as black. Turn the video switch on which is located left behind of sample stage. The arrow in the picture below indicates the video switch.
- Step 3) Once the video switch is on, the computer screen is going to change into either green (514 nm laser) or red (647 nm laser). Now the silicon reference target needs to be focused. The focus is done by moving sample stage into X,Y, and Z direction with coarse knob and sample stage remote controller. The left picture below shows the coarse knob indicated by arrow. The right picture below shows the stage remote controller. Coarse knob can be used only to adjust Z direction. The stage remote controller can control all X,Y and Z directions.

- Step 4) Once the silicon reference target is focused using coarse knob and stage remote controller, the computer screen should show the focus as white circular spot as shown in picture below. The arrow is indicating the focus. Now your sample is focused.
- Step 5) When the focus is done, change the X-axis unit to nm by clicking on the "Option", which is located on the top left of the screen. Then click on the "Unit" then click on "nm" now your X-axis unit is changed to nm.

- Step 6) The spectrometer now need to be set to zero order. On the bottom of the screen, there is a cartoon called "Spectrometer". Click on the arrow that is pointed to the left side. This will set the zero order. This step takes couple of minutes. Also, be careful not to use the box at the bottom. If user writes "0" into that box, the result will not be a zero order.

- Step 7) Once the spectrometer shows the zero order, click on the "Spectral Real Time Display" on top of the screen. This item is appeared as a blue clock shape cartoon. Once clicked, it will make clicking noise and spectrum of Rayleigh scattering spectrum will appear on the screen. Check the zero position of Rayleigh peak.

- Step 8) If the Rayleigh peak is off zero, click on "Set up" then click on "Instrument Calibration". Then there will be new small window on screen. There is a box at the bottom on the small window called "Zero". Change the numbers, so the Rayleigh peak is centered at zero. This step (changing numbers in the box) probably needs to be repeated several times.
- Step 9) Once zero position is set, the X-axis needs to be changed to 1 cm$^{-1}$. This is done by repeating step 5) “Option” $\rightarrow$ “Unit” $\rightarrow$ “1 cm$^{-1}$” (Only the last step is different)

- Step 10) Now the spectrometer bottom of the screen needs to be set to 520 cm$^{-1}$, where the silicon reference peak should be located. This time the exact number should be written in the box. This step also takes minute or two to reach that wavenumber.

- Step 11) Click on the “Spectral Real Time Display” to check the location of silicon reference peak.

- Step 12) If the position of the peak is exactly at 520 cm$^{-1}$, the calibration is completed. However, if the peak is off position, the step 8) needs to be repeated. Click on the “Setup” then click on the “Instrument Calibration”. New small window should appear. The bottom of the small window, there is a box called “Koeff”. Play with the numbers until the silicon peak is centered at 520 cm$^{-1}$. This step (changing numbers in the box) probably needs to be repeated several times. When user change numbers, start out with small change in numbers. If the number changed dramatically, the center position will be way away from the silicon peak, it will be hard to find that peak and bring back to the screen.

3. How to switch the lasers

The lab is currently equipped with three different laser sources, 514 nm, 647 nm, and 785 nm. The first two are from Argon/Krypton laser (shown in picture below on picture left) and the third one is from small argon laser (shown in picture below on right). Argon/Krypton laser can generate high power (up to 3 W) laser and the laser is stable. However the small argon laser only produce maximum of 40 mW of power and does not produce stable laser in the current lab environment. So, the specially designed plastic box
was needed to minimize the vibration from the other equipments around. Here are the detail procedures how to change the source of lasers.

- Step 1) Change the filters to whatever the wavelength is needed. The exchangeable filters are located on top part of the main Raman instrument box. The picture below is showing the top part of the instrument box and the arrow is indicating the location of the filters.

To open the panel, grab a black knob and slides it to the right side. Once the panel is opened, you will need to pull out the rubber that is another layer of filter protection. Picture below shows what the inside looks like. Two arrows indicate the filter holder. The top left one is for the filter that allows only desired wavelength and the bottom left one is
for the notch filter. The rest of the parts are fixed, so they do not need to be touched. The filters that are not in use can be stored in the free spaces like top right corner area (shown in circle).

![Notch filter setup](image)

Picture below is showing 3 pairs of filters starting from the right, 514 nm, 647 nm and 785 nm. The picture below the filters shows the one examples of when one pair of the filter has been inserted. The filter that appears taller inserts into the top left holder and shorter one is going into bottom right holder. There are two holes in the filters, so they will slide right in. However, it is always recommended to push the filter down to make sure that the filters are in all the way. Especially, 647 nm filters need extra push in order for it to slide in completely. Once filters are in, place the rubber mat back on top then slide the panel back to seal the area.

![Filter insertion examples](image)
Step 2) In case of using 514 nm or 647 nm, follow the how to turn the power on procedure mentioned in previous section. Then depending on which source of laser is needed, the setting in the laser controller has to be changed to the desired laser source. Then the knobs, which are located the opposite side of the laser head, need to be manipulated. Picture below is showing the two knobs. When the source of laser is changed, only top left knob is manipulated. If the knob is turning left, it will lead to the 647 nm. Or, if the knob is turned right, it will lead to the 514 nm. It is always better to have high power output when the laser source is changed because it is very easy to lose the light and hard to get it back to proper align. Once the top left knob is reached desired laser’s maximum output, the knob on the bottom right is used to further maximize the laser output. Slightly adjusting both knobs back and forth is recommended to get the maximum output.
- Step 1) In case of using 785 nm laser, it requires different procedures. Turning power on this laser is rather easy. The unit is completely separated from the main power. It has small switch right next to the laser. So when the switch is on the power of the laser is going to be on.

- Step 2) The new laser path had to be created so, the mirror in front of the laser need to be flipped up. The arrow in the picture below shows the adjustable mirror in front of the laser head.

- Step 3) Next, the main computer screen has a program called “Coherent Connection”. This is the program that controls the 785 nm laser setting. Once open the program, click
on the “ON” button. Then, the two gauges on the program window will show the laser output and temperature of the laser. The output can simply be changed by putting desired output into the box, then click ok. The calibration procedure is exactly the same as other lasers. One thing has to be pointed out is that 785 nm laser has extremely low intensity and it is really hard to see. So, it is always recommended to use the maximum power of the laser and also use the curtain around to help see the laser better.
APPENDIX (C)

SPARTAN SIMULATIONS OF RAMAN VIBRATION

One of the computers in the lab has a simulation program installed. The name of the program is called “Spartan” (Wavefunction, INC). This program can offer many different things. However, in this chapter, how to achieve a calculated IR or Raman spectrum with vibrational peak assignment, and how to test to see the formation of hydrogen bond between molecules are going to be explained.

1. Results/Discussion

In chapter 4, employing surface modified AuNPs with 2MELM and AuNPs without modifying surface were studied to find out the effect of LM. The one with 2MELM showed higher enhancement in two of the TMs, cyclohexanol and 1,3-cyclohexanediol. However, when the benzoic acid was a TM, there was no difference. To further prove and understand the experimental results, Spartan software was used to simulate the formation of hydrogen bonding between TMs and LM. Figure A.5 shows the simulation results of formation of hydrogen bonding between benzoic acid and 2MELM. B is showing the simulation result between cyclohexanol and 2MELM. C is showing the simulation result between 1,3-cyclohexanediol and 2MELM.

Figure A.5. Hydrogen bonding formation simulation using Spartan software. A is showing the simulation result between benzoic acid and 2MELM. B is showing the simulation result between cyclohexanol and 2MELM. C is showing the simulation result between 1,3-cyclohexanediol and 2MELM.
bonding between the target molecules and the linking molecule. The dotted green lines are indicating the formation of hydrogen bonding. The initial idea was if the TMs have more hydrogen bonding sites, it will have stronger affinity toward the LM; therefore, TMs can get closer to the AuNP surface resulting in higher enhancement. Figure A.5A is showing the formation of one hydrogen bond between the benzoic acid and 2MELM. Since benzoic acid can offer two different hydrogen bonding sites, it was expected to have two different hydrogen bonds at once. However, when two of the 2MELMs were added into the simulation, no hydrogen bond was formed. Steric hindrance plays an important role in this case because the locations of two possible hydrogen bonding sites are located too close to each other. Figure A.5B is showing the formation of hydrogen bond between cyclohexanol and 2MELM. Since cyclohexanol can only have one hydrogen bonding site, there is one hydrogen bond is formed. Figure A.5C is showing the formation of two hydrogen bonds between 1,3-cyclohexanol and 2MELM. As expected, two hydrogen bonding sites are fully functional without any interferences resulting in higher enhancement than other two TMs. Theses simulation results strongly supports the experimental results.

2. Raman and IR Spectrum of Molecule

- Step 1) Click on the new sheet that is located on top left of the screen. Then it will give you small window (model kit) with drawing option on the right side of the screen.
- Step 2) It will let the user to draw a molecule of interest. All user has to do is to choose whatever atom he/she needs on the model kit window, and then click on the main screen to have the atom on the main screen.
- Step 3) Once the molecule is drawn, the energy of the molecule need to be minimized. This can be done by clicking on the minimization button located in on icon bars on top of the screen. The image below show what the button looks like on icon bar.

- Step 4) Once the energy of the molecule is minimized, click on Setup \( \rightarrow \) Calculations… Then small window with what kind of calculations this program is going to run to simulate the molecule. Calculation setting is usually at Equilibrium Geometry at Ground state with Hartree-Fock 3-21 G in Vacuum. This setting usually does not need to be changed. However, if error message appears, user needs to change the calculation setting however the error message wants. In Compute section, user has to check box what kind of compute user wants. There are other sections; however, they usually do not have to be touched. Once user is done setting, click on Submit. Then next window shows up will ask where and what kind of name the user wants to save the results. Once set, click ok then small window will appear saying that the software is running. When it is done, another window will appear saying it is completed.

- Step 5) Click Display \( \rightarrow \) spectra. Small window with options of IR, Raman, NMR and UV/vis will appear. If user chose, Raman, click on Raman. Then, it should give you frequencies between the set values. Click on the Draw Calculated, it will give you calculated spectrum of molecule. Once the spectrum appears on screen. Users can click on the red line that appears on top of the peak to see where the exact frequency is. Also, when the peak is chosen, the molecule will move to show what kind of vibrational mode that peak belongs to.
3. Hydrogen bonding formation test

- Step 1) Draw molecules of interests following the exactly same steps explained in previous section.

- Step 2) Once molecules are drawn, minimize the energy of molecules following previous step.

- Step 3) Once energy minimization is done, there are two options to see the hydrogen bonding formation. Option one is to click on Model ➔ Hydrogen bonding. If the molecules are in suitable position, there should be possible hydrogen bonding appear with dotted line. However, if the molecules are not in near or correct position, nothing will happen. Option two is preferred way. Users can manually move around the molecules by left click of the mouse then drag the molecules. Once the molecules are in position that user wants, click on Model ➔ Hydrogen bonding. This should show hydrogen bonding (dotted line) if there is any possibilities.

- Step 4) Once hydrogen bonding is formed, click on file and save it however user wants it.
1. Copyright for Chapter 3

Gold nanoparticles have been used as effective surface-enhanced Raman spectroscopy (SERS) substrates for decades. However, the origin of the enhancement and the effect of the size of nanoparticles still need clarification. Here, gold nanoparticles with different sizes from 17 to 80 nm were synthesized and characterized, and their SERS enhancement toward both 4-aminophenol and 4-nitrophenol was examined. For the same number of nanoparticles, the enhancement factor generated from the gold nanoparticles increases as the size of nanoparticles increases. Interestingly, when the concentration of gold or the total surface area of gold nanoparticles was kept the same, the optimal size of gold nanoparticles was found to be around 50 nm when the enhancement factor reached a maximum. The same size effect was observed for both 4-aminophenol and 4-nitrophenol, which suggests that the conclusions drawn in this study might also be applicable to other adorbs during SERS measurements.

1. Introduction

Surface-enhanced Raman spectroscopy (SERS) is a surface sensitive technique that provides high Raman scattering enhancement of molecules adsorbing on a rough metal surface, such as silver, gold, and copper [1, 2]. Presently, there are two well-accepted theories describing the mechanism of SERS amplification: electromagnetic enhancement and chemical enhancement. Electromagnetic enhancement (EME) is responsible for up to $10^{7}$ to $10^{8}$ increase of Raman scattering which occurs as the surface Plasmon gets excited by incident light and amplifies the electromagnetic field of the metal surface [1, 2]. Chemical enhancement, on the other hand, provides up to $10^{9}$ increase of Raman scattering, and it happens when the molecule adsorbs strongly on the surface of the metal, which leads to changes of its polarizability. Due to the high enhancement, SERS technique has thus been studied extensively for the last few decades and it provides many advantages including non-destructive nature, low detection limit, high sensitivity, and easy sample preparation. SERS has been widely applied in many different areas [5-10].

Metal nanoparticles (NPs) especially Ag and Au NPs have been widely employed in SERS because of their unique physical properties that depend on size and shape of the NPs [11, 12]. Among the metals mentioned above, silver usually exhibits the highest SERS activity. It was reported that the optimal Ag nanoparticles size is 15 nm which generates the strongest SERS activity in solution [11]. Interestingly, the optimal size of Ag NPs was observed as 50 nm when off-resonance SERS is adopted [12]. On the other hand, gold has captured the most interests among recent studies. Gold metal is known to be biocompatible [10] and shows a strong excitation close to the IR region of light, which has attracted considerable interests in its use in biomedical systems [13]. Various research groups have studied and reported the relationship between the enhancement and the shape and size of the immobilized gold NPs on a substrate using different analytes like 4-aminophenol (4-ATP) [14, 15] and 5,5′-disulfobis(2-nitrobenzoic acid) (DSBNA) [16]. The results from all laboratories indicate that the SERS enhancement is highly dependent on many factors including the size of gold NPs, however, with controversial conclusions. When 4-ATP adsorbed on immobilized gold NPs, the SERS intensity from gold NPs with the size of 30 nm was lower than that from gold NPs with the size of 15 nm [14, 15]. When 4-ATP was adsorbed between Au NPs and a smooth Au substrate, the SERS intensity was found out to increase as the size of the gold NPs increases [14, 15]. When labeled gold NPs were immobilized at
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