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Optimizing Silver Nanoparticles for Pigment Identification in Art

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Optimizing Silver Nanoparticles for Pigment Identification in Art

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Neuroscience from The College of William and Mary

by

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Abstract

Surface-enhanced Raman spectroscopy (SERS) is a powerful tool for the identification of organic colorants within art samples. The SERS substrate that is widely used, a colloidal suspension of silver nanoparticles (AgNPs), does not always provide reproducible spectral results even when the same procedure is followed within the same laboratory conditions. An investigation to find a metric that can classify each new batch of AgNPs as optimal or suboptimal for application onto a precious art sample are discussed. Next, a quality assurance protocol for SERS-based identification of organic pigments in art is presented. Lastly, pretreatment extraction techniques for sample intervention prior to the application of AgNPs are illustrated.
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Chapter 1

Introduction

The crimson red colorant embedded within the paint layers of the model’s cheek fades to a pale pink as gallery lights illuminate the aging portrait. Organic colorants, made from extracted plant and animal sources, fade due to light exposure over time. The identification of the specific colorant that the artist carefully selected lends insight into the artist’s process. This precise identification can be used to date and authenticate the piece of art as well as drive conservation or restoration efforts tailored to the specific colorant. Colorants are identified by collecting the unique vibrational fingerprint of the molecule. The identification of organic molecules within paint samples requires the use of a technique with both high sensitivity and extreme selectivity. Typical analytical techniques like Raman spectroscopy, high-performance liquid chromatography (HPLC), and Ultraviolet-visible (UV-Vis) spectroscopy lack the combination of high sensitivity and selectivity required for the identification of organic molecules within paint samples. Raman spectroscopy suffers from insensitivity due to competing fluorescence from the organic colorant,\(^1\) HPLC’s limits of sensitivity requires a relatively large sample size that would be too damaging to the painting to remove,\(^2,3\) and UV-Vis does not have the selectivity for collecting an organic molecule’s vibrational fingerprint especially when presented in a complex mixture like a paint sample.\(^4\) One solution to these selectivity and sensitivity hindrances is to use surface-enhanced Raman spectroscopy (SERS).

SERS is a powerful analytical tool capable of enhancing signals even down to the single molecule level.\(^5-7\) SERS combines the selectivity of Raman spectroscopy with the use of a substrate that quenches the competing fluorescence from organic pigments and amplifies the molecular signal by \(10^6\) or more.\(^5\) The ubiquitous and powerful SERS substrate used to identify
organic colorants in this study was a colloidal suspension of silver nanoparticles (AgNPs). The colloidal suspension of AgNPs, or more simply called a batch of colloids, are directly applied to the small art sample for identification. Many studies show successful identification of organic colorants in cultural heritage objects like textiles and paintings. However, research suggests that variation in experimental conditions like the preparation of the SERS substrate leads to variation in spectra obtained. In fact, batch-to-batch variation of newly synthesized colloids was observed even when the protocol, laboratory conditions, and researcher remained the same.

The suspicions of batch inconsistency reached a peak when a routine synthesis resulted in a stark “white” batch which differed visually from the expected, “turbid green” color of previous batches. Before sending the waste container of these presumably non-active particles away, the peculiar white batch was tested using standard metrics. Surprisingly, these “white” particles passed every test with flying colors. Two important questions arose from this finding: what are the different chemical properties between “white” and “green” colloids? And are “white” colloids actually better than “green” colloids for SERS studies involving art as the preliminary test results would suggest?

In this thesis, the theory of SERS will be discussed followed by a proposal for a quality assurance protocol for AgNPs. Then a discussion of a red colorant sample extraction technique, that would precede the application of AgNPs on an art sample, will follow.

1.1 A Brief Background of Raman Spectroscopy

Light is composed of photons that either interact with, or directly pass through molecules in its path. If light interacts with a molecule, the incident photon is either absorbed or scattered. The photon is absorbed by the molecule if the energy of the incident photon matches the energy
difference between the electronic ground and excited states of the molecule.\textsuperscript{16} Scattering, on the other hand, is broken down into two different processes: Rayleigh and Raman scattering (\textbf{Figure 1}). Incident photons promote the molecule from its ground vibrational state to a “virtual state”, which is followed by scattering processes. Rayleigh scattering is an elastic scattering process where the energy of the incident photon is equal to the energy of the scattered photon. Raman scattering is an inelastic scattering process that can occur at anti-Stokes or Stokes frequencies.\textsuperscript{17} As shown in \textbf{Figure 1}, in Raman scattering, there is a difference in energy between the incident photon and the scattered photon. According to the Boltzmann distribution, Stokes Raman scattering is more likely to occur as compared to anti-Stokes scattering, and is therefore the focus of the work presented herein. For Stokes Raman scattering, the scattered photon has less energy than the incident photon. The energy difference between incident and scattered photons is termed the Raman shift. The Raman shift has units of wavenumbers (cm\textsuperscript{-1}),\textsuperscript{16} and corresponds to the energy of a specific vibrational mode of a molecule. Non-linear molecules possess \(3N - 6\) vibrational modes, where \(N\) represents the number of atoms in a molecule (or \(3N - 5\) for linear molecules). Each vibrational frequency is represented as a peak in a Raman spectrum, which is a plot of intensity versus Raman shift in units of cm\textsuperscript{-1}.\textsuperscript{1} Ultimately, the full Raman scattering spectrum is the unique vibrational fingerprint of a molecule that allows for its identification.

Though the selectivity of Raman spectroscopy is quite useful in analytical applications, it also has several limitations that impede its application to the identification of organic colorants. Raman is a relatively weak process and scattering is often overwhelmed by the fluorescence from organic chromophores.\textsuperscript{1} These limitations have so far prevented Raman spectroscopy from identifying extremely small samples of organic pigments in art. Although Raman spectroscopy has selectivity, the technique exhibits relatively poor sensitivity.
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**Figure 1.** An energy diagram for Rayleigh and Raman scattering processes.

1.2 Surface-enhanced Raman Spectroscopy (SERS) Theory

SERS substrate was born in 1974 from the discovery by Fleischmann et al. that a Raman signal could be amplified by placing an analyte close to an electrochemically roughened silver electrode. Three years later, two more independent publications surfaced which furthered the work of Fleischmann et al. by recognizing that a Raman signal could be amplified by $\sim 10^6$ through surface enhancement. As SERS grew in popularity, the $10^6$ signal enhancement reported in early studies increased to amplifications as high as $10^{10}$ when factoring in normal Raman enhancement. Notably, even single molecules can be studied using SERS. SERS amplification is owed mostly to the electromagnetic interaction of light with metals.

The electromagnetic (EM) field enhancement is due to localized surface plasmon resonance (LSPR) which is shown in **Figure 2.** LSPR is determined by the dielectric constant of the material. Also called the relative permittivity, the dielectric constant helps predict the

**Figure 2.** LSPR. Adapted from Kosuda et al..
electrical behavior of the material in a certain environment. Ag and Au along with other materials have either a small positive imaginary, or negative real, dielectric constant. Equation 1 models that a metal nanoparticle with a negative dielectric constant, $\varepsilon_{in}$, derives high enhancement of the EM filed at the surface of the nanoparticle, $|E_{out}|^2$. Equation 1 where $\varepsilon_{out}$ is the dielectric constant of the environment, $|E_0|^2$ is the incident field intensity is:

$$|E_{out}|^2 = 2E_0^2 |(\varepsilon_{in} - \varepsilon_{out})/(\varepsilon_{in} + 2\varepsilon_{out})|^2$$  \(1\)

When designing SERS experiments, it is important to consider three different factors concerning the EM enhancement mechanism which include: the structure of the SERS substrate,\(^5\) the excitation wavelength, and the distance between the analyte and the metal nanoparticle.\(^4\) Materials and structures of SERS substrates influence the strength of the signal amplification. Metals like Ag and Au are common substrates because of their ability to support strong plasmon resonances. A solution of Ag or Au nanoparticles that exhibit a range in enhancing strengths is favorable to this study compared to uniform metal substrates because there is a chance of forming “hot spots”. These “hot spots” exhibit the highest enhancement factors which are required for the detection of single molecules as previously mentioned.\(^5\) Hot spots, or areas of high enhancement, yield the best chance for signal acquisition form an organic pigment that historically can be difficult to identify. Distance plays a role in the overall enhancement as well. Due to the range of LSPR, analytes do not have to actually adsorb to the surface of the nanoparticle which is advantageous, especially for analytes with low affinity for the substrate since they can still be detected at a distance from the surface of the nanoparticle. The consideration that the attachment of the analyte to the particle is not necessary emphasizes that SERS enhancement is largely dependent on EM enhancement rather than chemisorption.\(^4\) The last EM enhancement factor to consider is the excitation wavelength. The largest enhancement
occurs when the incident and scattered photons are enhanced by the LSPR, and when the LSPR 
\( \lambda_{max} \) sits in between the peak excitation frequency and the vibrational frequency. In other words, 
maximum enhancement is dependent on the frequency of the laser as well as the plasmon 
frequency of the metallic nanoparticle.\(^4\)

The contribution of the chemical enhancement mechanism should still be considered 
though it is not as significant of a contributor to overall enhancement as the EM mechanism. The 
chemical enhancement is due to the interaction or chemisorption of the analyte and the metal 
surface. Chemisorption involves a charge transfer between the analyte and metal which generates 
a resonance Raman effect.\(^4\) Combining all of these contributions, SERS enhancement is 
promising for identifying organic pigments in art.
Chapter 2
Initial Observations and Revision of Synthesis Protocols

2.1 Lee and Meisel Method

Lee and Meisel\textsuperscript{21} colloids have been used by > 3000 papers. The particles synthesized by this method are used for many applications including single molecule studies, glucose sensing, and pH sensing just to name a few. The procedure published by Lee and Meisel details a procedure specifically for colloidal suspensions of citrate-reduced AgNPs used in this project. Though gold is another common substrate, silver is preferable to gold and the other possible metals for SERS sensing, because it is less expensive, more stable, simpler to prepare, and absorbs in the plasmon resonance region of interest.\textsuperscript{14} It is also previously discussed why a solution, containing concentrated particles with high likelihood of forming many hot spots, is preferable to other possible structures of SERS substrates. For these reasons, colloidal suspensions of citrate-reduced AgNPs were used for all past and are currently used in all ongoing experiments conducted by the Wustholz group.

The Lee and Meisel method states: “AgNO\textsubscript{3} (90 mg) was dissolved in 500ml of H\textsubscript{2}O and brought to boiling. A solution of 1% sodium citrate (10mL) was added. The solution was kept on boiling for ca. 1 h.”\textsuperscript{21} While the procedure is straight forward and easy to perform in various lab settings, the simplicity leads to variation between batches. After lots of variation was noted between batches (which will be discussed in depth later on) using this simple procedure, measures were added to make the procedure more detailed to yield a more precise product from each synthesis.
2.2 The Call for Investigation: The Curious White Batch

Figure 3 showing two different colored batches of colloidal suspensions of AgNPs in clear vials sparked curiosity. From now on, the “white” batch will be referred to as batch J and the “green batch” will be called batch A. These two batches were synthesized on the same day using the same protocol for AgNP synthesis outlined by Lee and Meisel as discussed in the previous section. On the day of synthesis, the adsorbed citrate intensity, or the characteristic peak for AgNPs at ~1400 cm\(^{-1}\), acquired while the colloid spots were still wet, showed a stark difference as seen in Figure 4. The adsorbed citrate intensity is nearly 15,000 counts mW\(^{-1}\)s\(^{-1}\) higher for batch J compared to batch A (emphasized by the vertical black bars in Figure 4).

Though batch J exhibited a higher adsorbed citrate intensity, batch J appeared less different than batch A when other metrics were analyzed. Batches A and J both produced a SERS spectrum of a common analytical red dye, alizarin. The spectra had relatively the same intensity of peaks and were the same in all other respects as seen in Figure 5. Since the SERS spectra of alizarin dye did not lend information as to which batch is optimal for application on art, the investigation of other test metrics was conducted to find a new set of diagnostics for...
SERS activity that would distinguish optimal AgNP batches from those that are suboptimal for application on art.

![Figure 5. Comparison of SERS spectra of alizarin collected using batch A and J.](image)

2.3 Modified Synthesis of AgNPs Proposal

The modified method for AgNP synthesis takes into account heating, stirring, and timing in more detail than the Lee and Meisel synthesis stated above. Relevant to this study, Munro et al. also noted the need to make the procedure more reproducible by supplementing Lee and Meisel’s procedure with more detailed parameters. This current study’s first step towards a modified Lee and Meisel’s procedure ensured the hot plate was reaching the target temperature and remained at that temperature throughout the duration of the synthesis. Heat is essential for the reaction of the AgNO₃ and sodium citrate. Next the of the size of stir bar and stir speed were considered to achieve a steady and vigorous vortex throughout the reaction vessel that is also essential to the reaction. Finally, timing of when to add the reagents and when to stop the synthesis was explored to optimize the final product. The new protocol targets a more precise
final volume and color of solution as indication of less batch-to-batch variation. Here is the new AgNP synthesis protocol:

1. **Preparation**

   a. Inspect glassware for cleanliness (all glassware should have been cleaned with aqua regia and ultrapure H₂O immediately following last synthesis). Any residual AgNPs on the bottom of the 1000 mL flask are especially bad in that they contaminate future syntheses.
      
      i. 1000 mL Erlenmeyer flask
      ii. amber storage bottle and cap
      iii. 10 mL Graduated cylinder
      iv. Glass funnel
      v. 2” Stir bar
      vi. 50 mL Volumetric flask w/stopper

   b. Place all glassware in base bath for at least 24 hours

   c. Rinse all glassware at least 5 times with ultrapure H₂O before use.

   d. Obtain the gray Corning Hot Plate (5”x7” approx. dimension, S/N 153517206156).

2. **Sodium Citrate Preparation**

   a. In the 50 mL volumetric flask add 0.5 g Na₃C₆H₅O₇ (Alfa Aesar, ACS, 99.0% min) with a little ultrapure H₂O

   b. Swirl flask until all solid is completely dissolved

   c. Add more ultrapure H₂O to fill to the 50 mL line on the flask (use glass pipette to fill to the line precisely)

   d. Invert the flask several times to mix, sonicate for 30 seconds if necessary.
3. Synthesis

a. Plug in hotplate. Set temperature to 500°C (high heat setting) and wait until the number stops flashing, indicating it has reached the target temperature.

b. Add 500 mL ultrapure H₂O to the 1000 mL flask.

c. Drop 2” stir bar in the flask.

d. Unfurl the hood curtains and center the flask on the hotplate with the heat setting at 500°C, and stir speed set to 1150 rpm (so that there is a vortex extending from the surface of the solution to the bottom of the flask).

e. Turn off the lights. Obtain AgNO₃ (99.9995%-Ag, STREM Chemicals Inc.) from the desiccator, and using the designated spatula, weigh 91 mg on weigh paper.

f. After the flask has heated for 10 min, add AgNO₃ to the 1000 mL flask while on a slow stir, being careful not to let the AgNO₃ hit the sides of the flask. Flask can be removed and carefully swirled to be sure that all of the crystals are off of the walls of the flask at this stage.

g. Keeping the heat setting and stir speed at 500°C and 1150 rpm respectively. The level of the solution should exceed ~600 mL when vigorously stirring (as fast a stir as possible while keeping the solution contained within the flask).

h. Keep heating and stirring for 20 minutes until a vigorous boil is achieved with certainty--with reflux visible at the neck of the flask (check for vigorous boil by turning the stir down to 0, and then back up to 1150 rpm if a vigorous boil has not yet been reached).

i. While waiting for a vigorous boil, measure 10 mL of 1% sodium citrate solution in a 10 mL graduated cylinder using a glass pipette.
j. When a vigorous boil is reached (20-25 min), add sodium citrate solution to the flask while on a slow stir, then turn stir down completely to remove flask and swirl by hand being careful to dissolve any sodium citrate that may be on the sides of the flask. Return flask to the center of the hot plate and return stir speed back up to 1150 rpm.

k. Let solution heat and stir for 30 minutes. With these settings, you should note a color change from clear to turbid green occurs within the first 5 min, then eventually to a more milky-white hue by the end of the 30 min. Note that the color change from turbid green to white may not be the most SERS active.

l. Turn off the heat and stir. Note the final volume of the solution (which should be ~300 mL including stir bar).

m. Allow flask to cool in the dark hood

n. Transfer solution to amber glass storage bottle using the glass funnel. Cap the bottle and wrap Parafilm around the top. Label bottle with Silver Nanoparticles followed by “[initials]-[Roman numeral lab notebook volume]-[page number]”.

o. Store in refrigerator.

4. Cleaning

All glassware involved in the synthesis is washed using aqua regia. Aqua regia is a 3:1 volume ratio solution of HCl/HNO₃ that is a strong enough acid solution to remove the AgNPs that coat the bottom of the reaction vessel after synthesis. It is essential to remove all residual AgNPs because contamination of the glassware ruins the next synthesis, and new glassware for each new batch is not an economical requirement.
Then all glassware is rinsed with ultrapure water 5 times. Lastly, all glassware is emerged in base bath for at least 24 hours. The base bath removes any residual aqua regia that would be detrimental to the next synthesis.

Cleaning Protocol:

a. Rinse the reaction vessel, stir bar, funnel with ultrapure H$_2$O. Dispose in AgNP waste.

b. Immediately after synthesis, dress in PPE (splash goggles and lab coat) and take all glassware used in synthesis, a 100 mL beaker, a recrystallization dish, sodium bicarbonate, and pH paper test strips to the hood for aqua regia.

c. Make aqua regia by adding 60 mL hydrochloric acid (HCl) to 100 mL beaker, then slowly add 20 mL of nitric acid (HNO$_3$).

d. Carefully swirl aqua regia around the inside of the glassware starting with the 1000 mL flask. Pour the aqua regia from the 100 mL beaker into the funnel containing the sir bar that is resting on top of the 1000 mL flask. Transfer aqua regia back to the 100 mL beaker slowly, while making sure to contact the entire inside surface. Go back and forth between the 1000 mL flask and the 100 mL beaker at least twice to ensure thorough cleaning of the 1000 mL flask, stir bar, and funnel. Reuse the aqua regia while you continue to clean the rest of the glassware.

e. After all items in step 4d are clean, transfer aqua regia to recrystallization dish for neutralization. Carefully add small amounts of sodium bicarbonate (NaHCO$_3$). This addition will cause the solution to bubble-up and fizz; make sure it does not bubble-over the sides of the dish. Once the reaction subsides,
swirl the dish to increase exposure to any unreacted NaHCO₃. Test with pH paper. Add more NaHCO₃ until the tested solution is neutralized (pH ~7). Pour contents down sink drain while flushing the drain with tap water (this is the only time tap water is used.)

f. Rinse each item with ultrapure H₂O. Fill glass vessels completely at least five times. Place items on a WypAll® on the benchtop to dry. Label benchtop surface with tape including “Clean,” date, and initial. Do not write on the drying pad surface or surface of glassware.

2.3.1 Importance of Proper AgNP Storage

Overtime, the colloidal suspension of AgNPs can aggregate or fall out of solution completely. AgNPs are also susceptible to oxidation both independent and dependent of light. In order to maintain stability for an appropriate amount of time, colloids are refrigerated in an amber bottle wrapped in Parafilm. A more comprehensive list of factors to consider for increased duration of particle stability include: maintaining high AgNP concentration, keeping the suspensions in the dark, increasing the citrate:silver molar ratios, and purging with nitrogen gas as opposed to air. This study suggests that particle stability is variable from batch-to-batch. It was observed that some batches are still able to collect SERS spectra for more than a year after synthesis while others fall out of solution in as short as about a month. Since synthesis requires time and material expenses, it is of interest to properly store each batch to increase batch longevity.
Chapter 3

Investigation of Test Metrics for Quality Assurance of AgNP Batches

3.1 Introduction

Moving forward with a more detailed protocol, the persisting variation noted between different batches of colloids, considering a variety of metrics, was further motivation to continue the search for metrics, or characteristics, that make a batch of colloids optimal for application on precious art samples. Paint samples taken from precious pieces of art deserve conscientious efforts to extract all of the information possible. Each sample is unique and precious. Although SERS is powerful enough to detect a target analyte within extremely small samples that minimally alter the piece of art, the small sample (i.e. ~nanograms)\textsuperscript{24} is still a manipulation of the artwork that deserves conscientious consideration. Also, some paintings that are sampled reside in private collections where sampling from a piece can only happen once; thus, if the AgNPs are suboptimal and fail to identify which pigment is in the sample, then there is not a always a second opportunity for more sampling from the same piece of art in order to gain that information. It is crucial to assure the AgNPs that are set on the art sample are above the threshold for an optimal batch of colloids.

Expansion of the investigation from just the two comparison batches, A and J discussed in section 2.2, to 10 representative batches was the next step towards designing a new quality assurance protocol. From the 149 batches previously synthesized that were prepared by 10 different researchers in the same laboratory conditions, 10 of these batches were carefully chosen as representatives for quality assurance testing. Except for batches A and J, all other batches were synthesized using the amended protocol detailed above. The 10 colloid batches were
synthesized within a two-year period but the various degrees of time elapsed since synthesis, or age of the batch, differed significantly. Some measurements were taken just about a month after synthesis, while other measurements were taken over a year after synthesis. The differing batch ages provided interesting characteristics that were hypothesized to affect the quality, or SERS activity, of the particles. Several characteristics of a batch of colloids were investigated as candidates for a test metric that would set the threshold for what makes a batch suitable for pigment identification. These characteristics include: average enhancement factor (AEF), adsorbed citrate intensity, identification of a dye, maximum absorbance, and particle size. None of the metrics listed set a clear threshold, thus these metrics failed to define what an optimal batch of AgNPs is for application onto an actual art sample.

3.2 Enhancement Factor Discussion

Wanting to quantify the enhancing capabilities of different colloid batches, the possibility of calculating the enhancement factor (EF) of each batch was explored. The electromagnetic enhancement factor can be calculated either theoretically or analytically. This study was more interested in an analytical approach for calculating the EF so that each batch of colloid can be evaluated directly without prior assumptions. The equation for EF taken from Kosuda et al.\textsuperscript{4} is:

\[ EF = \frac{I_{\text{SERS}}/N_{\text{SERS}}}{I_{\text{NRS}}/N_{\text{NRS}}} \]  

(2)

\( I_{\text{SERS}} \) is the intrinsic SERS intensity of the sample. This value can be measured by collecting a SERS spectrum of colloid and then plotting the spectrum as intensity (counts mW\(^{-1}\) s\(^{-1}\)) versus wavelength (rel. cm\(^{-1}\)). The \( I_{\text{SERS}} \) would be the maximum intensity of the most intense characteristic peak which is adsorbed citrate at \(~1400\) cm\(^{-1}\). \( N_{\text{SERS}} \) is the number of molecules adsorbed to the surface of the nanoparticle. \( N_{\text{NRS}} \) is the number of molecules in the volume of
solution being analyzed. Lastly, $I_{\text{NRS}}$ is the intensity of signal gathered via normal Raman spectroscopy which is where an issue arises. Normal Raman spectroscopy does not work for organic molecules. The fluorescence the molecules produce impedes the collection of a vibrational fingerprint which renders this equation useless for studies involving organic molecules. A different way to calculate EF without the need for a normal Raman measurement was needed.

A possible solution sprung from the idea that $I_{\text{SERS}}$ is proportional to the average EF (AEF) multiplied by concentration of AgNPs as portrayed by Equation 3:

$$I_{\text{SERS}} \propto [\text{AgNP}] \cdot \text{AEF} \quad (3)$$

$I_{\text{SERS}}$ can be measured directly from the sample as described above. $C$ is the concentration of the colloidal solution which can be derived from Beers Law. Beers Law is:

$$A = C \cdot \varepsilon \cdot \lambda_{\text{max}} \quad (4)$$

$A$ is the UV-Vis absorbance in arbitrary units. The wavelength at which the sample exhibits maximum absorbance is $\lambda_{\text{max}}$. Lastly, $\varepsilon$ is the extinction coefficient that can be taken from a published table of values in the supplementary publication of Paramelle et al.\textsuperscript{25} Paramelle et al. calculated the extinction coefficients experimentally by quantifying silver ion concentration of AgNPs, and reported that the experimental values matched literature values. The concentration of the solution ($C$) can be solved for once the other three values are input into Equation 4. Using this value for concentration and the known $I_{\text{SERS}}$ value for the respective variables in Equation 3, AEF can be estimated. AEFs of different batches of AgNPs were estimated using this method, but there were several challenges. The first is that $I_{\text{SERS}}$ of the adsorbed citrate peaks of AgNP SERS spectra proved to be an inconsistent and unreliable metric (which will be described in section 3.3). The second is that the reliance on literature values for $\varepsilon$ is not ideal when compared
to analytical values measured in the lab. The values for ε assigned using the table published in Paramelle et al.\textsuperscript{25} are based on $\lambda_{max}$ and particle size. As is discussed in sections 3.6 and 3.5 respectively, particle size and $\lambda_{max}$ may not be reliable metrics for these estimations either. The search for a technique beyond the use of equations requiring the input of unreliable variables was the next step in the search for a quality assurance test metric.

3.3 Adsorbed Citrate Intensity Lacks Trend

3.3.1 Sample Preparation The colloids were centrifuged (Eppendorf, 1 mL aliquots with ~0.93 mL of supernatant removed) for two cycles at 12,000 g for 15 min per cycle. Supernatant was removed after each round of centrifugation in order to increase the concentration of the particles in solution. Then 0.75-$\mu$L of the centrifuged colloidal paste was spotted onto a glass coverslip. The samples were all analyzed while still wet using an inverted microscope with a 632.8 nm laser detailed elsewhere.\textsuperscript{10,26,27} Accounting for possible variation, ≥3 tubes of colloid where prepared, and spectra from ≥3 different spots were gathered for each batch of colloid. Intensity was valued based off of the most prominent peak representative of adsorbed citrate within the colloid spectrum at ~1400 cm\textsuperscript{-1}.​
3.3.2 Results and Discussion

![Graph of adsorbed citrate intensity over time for four different colloids.]

**Figure 6.** Adsorbed citrate intensity (at ~1400 cm\(^{-1}\)) from SERS spectra of four different colloids tracked over time.

Intensity was thought to have a direct relationship to the SERS-activity of a colloid, with high intensity of the adsorbed citrate peak at ~1400 cm\(^{-1}\) indicating better enhancing capabilities. Intensities of peaks, acquired while the colloid sample spot was wet, were tracked over time to test this relationship. Batches that were tracked on benchmark days following synthesis exhibited average intensities of 9000 ± 5000 ADU mW\(^{-1}\) s\(^{-1}\). This is a considerable amount of variation with consequently no visible trend. Some of this information is presented in **Figure 6**. For instance, batch I had the highest intensity at ~day 1, batch H had the highest intensity at ~day 20, and batch J had the highest intensity at ~ day 35. Since the batch with the highest adsorbed citrate intensity varied day-to-day, adsorbed citrate intensity does not help determine which batch is best for art.
Figure 7. SERS spectra of (left) a) madder lake pigment b) colloid batch J, (right) a) 10^{-4} M alizarin dye in ethanol b) colloid batch B.

Another major indication that intensity is not a reliable metric is best exemplified by batches that have completely lost adsorbed citrate intensity due to aging, but are still able to identify target analytes. Looking at the spectrum for colloid batch B in Figure 7, there is no peak at ~1400 cm^{-1} which is the usual peak used as the I_{SERS} value. Thus, this colloid would be deemed as non-active and not trusted for analyte detection. Also seen in Figure 7 however, is a characteristic peak for alizarin dye obtained using that colloid. This means that the colloid was still functional for SERS-based analysis despite its classification of having zero intensity. Similarly, colloid batch J also appears to have zero intensity, considering again the ~1400 cm^{-1} peak, but amplified a signal from madder lake pigment. Examining both of these examples, adsorbed citrate intensity does not seem to be linked to a batch of colloid’s SERS capabilities as previously thought.

3.4 Alizarin is Identified by All Batches

3.4.1 Introduction Natural dyes like alizarin have been commonly used as analytes by the Wustholz group to test batches of AgNPs for suitability for application on art samples. In
these experiments, AgNPs are combined with a solution of $10^{-4}$M alizarin in ethanol either on a glass coverslip or in a glass vial. Batches of AgNPs positively identify alizarin by collecting a SERS spectra of $10^{-4}$M alizarin in ethanol that exhibit major peaks, consistent with previous studies, at 1599, 1413, 1327, 1295, 1160, and 342 cm$^{-1}$.27–29

3.4.2 Sample Preparation Colloids were centrifuged as described in 3.3.1. Alizarin was prepared for SERS testing either on a glass coverslip, or in a glass vial. For preparation on a glass coverslip, 1 µL of colloid was spotted onto the glass coverslip and let dry. Then 1 µL of $10^{-4}$M alizarin in ethanol solution was spotted on top of the dried colloid spot. If preparing a sample in a glass vial, colloid was centrifuged as previously stated then re-suspended up to 0.9 mL and then 0.1 mL $10^{-4}$M alizarin in ethanol solution was added to the same vial. Both samples on the glass coverslips and in the glass vials were analyzed using an inverted microscope with 632.8 nm wavelength laser.

3.4.3 Results and Discussion

Since adsorbed citrate intensity did not set a threshold for optimal batches, a colloid’s SERS-activity was then measured based off of its capability to collect a positive SERS spectrum of a common analytical molecule, alizarin dye. Referring back to Figure 5, the two visually distinct batches A and J provided two SERS spectra of alizarin dye (prepared in a glass vial) exhibited relatively the same intensity and clarity of peaks. Furthermore, although batch J sat unrefrigerated and

![Figure 8. SERS spectra of alizarin obtained using the same colloids (a) freshly synthesized and (b) ~1 year after synthesis.](image)
exposed to room lights for over a year, batch J (prepared on a glass coverslip) positively identified alizarin dye shown in Figure 8. In fact, all 10 batches positively identified alizarin dye. Taken together, these findings suggest that the ability of a batch of AgNPs to identify alizarin dye is not reflective of a batch’s greater ability to identify any organic pigment in art.

### 3.5 Range of Absorbance Spectra

Literature values for the maximum absorbance ($\lambda_{max}$) spectra of citrate-reduced silver nanoparticles range from 406 to 450 nm with a full width at half maximum (fwhm) that varies from 115 to 300 nm.$^{22}$ The 10 batches have maximum absorbance values that range from 399 to 420 nm and obvious differences in fwhm contributing to a diverse array of peak shape illustrated by Figure 9. Thus, the $\lambda_{max}$ values for batches A-J do not vary as much as reported values which may indicate that there is not a lot of variation in particle size distribution between batches.$^{22}$ Paramelle et al. suggest that spectra with smaller fwhm values and larger absorbance values (tall, thin shape) compared to a wider, lower absorbance values indicate that the batch of nanoparticles has smaller particle diameter sizes.$^{25}$ The next section will show that particle size distribution does seem to be variable between batches keeping in mind that data on

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**Figure 9.** UV-Vis absorbance spectra for colloid batches A-J.
particle size is limited to two batches, and that a more comprehensive data set may lead to
different conclusions. The differing peak shapes like those that exhibit a shoulder could be due to
varying degrees of aggregation. The differences in absorbance also relates to the age of the
colloid batch; generally, the older batches do not have as high of an absorbance as the batches
synthesized more recently. In conclusion, absorbance spectra provide information requiring
further future exploration of particle size and concentration that render $\lambda_{max}$ values as an
unsuitable metric for quality assurance of AgNPs at this time.

### 3.6 Discussion on Concentration and Particle Size

The first hypothesis (beyond adsorbed citrate intensity) for the difference between batch
A and batch J was thought to be a difference in concentration or particle size. Due to higher heat
settings and longer duration of synthesis, batch J was thought to be more concentrated than batch
A. While imaging techniques like transmitting electron microscopy (TEM) were not available,
particle size could be measured using a Dynamic Light Scattering (DLS) instrument.

The number-weighted distribution shows a mean diameter of 3.9 nm for Batch J and 1.2
nm for Batch A. The intensity-weighted distribution lists a mean diameter of 16.4 and 102.6 nm
for Batch J and 1.2, 3.8, and 30.2 nm for Batch A. Lastly, the volume-weighted distribution
shows a mean diameter of 15.4 and 95.9 nm for Batch J and 1.1, 3.4, and 28.3 nm. Possible
sources of error include the fit error of 1.11 and 1.24 for Batch J and A respectively as well as
the residual value of 0 and 49.82 for batch J and A respectively. The fit error and residual were
considered to assure the instrument was taking accurate measurements.

Though differences were found between batch A and batch J, the findings from particle
sizing were inconclusive overall. The colloidal suspension of silver nanoparticles is known to be
heterogeneous with spherical, rod-shaped, and complexes of multiple particles dispersed throughout the solution.\textsuperscript{31} This was not reflected in the data. The limited sample size and different fit error and residual values between scans resulted in particle sizing being classified as an ineffective metric for determining if batch J or batch A is optimal for application on art.

### 3.7 Conclusion

Though the 10 batches of AgNPs clearly differ according to the various investigations in this chapter, the investigations failed to find a metric that clearly defines which batches are optimal for application onto art. Adsorbed citrate intensity varied significantly, thus did not indicate clearly which batches are above threshold for an optimal colloid. Positive identification of alizarin dye is obtained by all colloids, so no threshold could be set based on this metric either. Finally, absorbance spectra characteristics and particle sizes require further research with a wider scope to perhaps find a trend in which characteristics of the absorbance spectra and size of particles are shared by optimal batches.

The search for a reliable metric for deciding whether a newly synthesized batch of colloid is suitable for application onto an art sample continued past the metrics elucidated in this chapter. A shift from quantitative and physical characteristics of batches, to the analytical capabilities, or strong SERS activity of the batch was the next goal to establish an optimal colloid batch from a suboptimal colloid batch. In other words, the next chapter will introduce testing colloids using reference samples of the target analytes. Testing with multiple analytes is a more direct approach towards knowing which colloids are optimal for identifying pigments in art without the need to classify each batch by chemical and physical characteristics.
Chapter 4


4.1 Introduction

The many test metrics of colloids measured in chapter 3 including, adsorbed citrate intensity, SERS-based identification of alizarin dye, absorbance spectra characteristics, and particle size, did not lend information as to what makes an optimal batch of colloid for organic pigment identification in art. In this chapter, investigations ensued to develop a new quality assurance procedure for AgNPs for increasing reliability of the SERS-based detection of organic colorants. The Wustholz lab have synthesized 149 batches of colloid, but 10 batches (A-J) were thoughtfully chosen to represent the group of 149 for discussion purposes throughout this thesis.

The goal for this part of the project was to create a quality assurance procedure that is based purely off of functionality rather than namable characteristics like adsorbed citrate intensity values and absorbance spectra. Since an easy to identify analyte like alizarin dye does not provide an indication of the scope of a colloid’s SERS-activity, a series of analytes of increasing difficulty to detect were assembled.

4.2 Discussion of Red Colorants as Analytes

The focus of this project is to detect specifically red organic colorants that are prone to fading in paintings. The red colorants selected as the test analytes for each new batch of colloid include: alizarin dye, carmine pigment, carmine paint, madder lake pigment, and madder lake paint. Water soluble dyes, considering their even dispersal and concentration throughout a
sample and higher probability of landing in close proximity to the metal nanoparticle, are easier to identify than colorants in insoluble pigments and paints. Paint samples from actual works of art are more intricate in that they may include oils, resins, binders, mordents, and even contaminants.\textsuperscript{2,10,29}

Alizarin dye was already discussed earlier as an easier analyte to detect. Alizarin is water-soluble, unlike the other pigments and paints. Solubility allows a sample to have a homogenous spot, in terms of even analyte dispersal, for the AgNPs to coat. This even dispersal of analyte is an advantage of the dye because it increases the probability that the AgNPs will be close enough to the metal nanoparticle to amplify the signal. Alizarin is also not impeded by water insoluble components like pigments and paints are which do not usually allow the analyte to get as close to the metal nanoparticle as a water-soluble dye.

Carmine is made from cochineal insects, \textit{Dactylopius coccus}, commonly found feeding on cacti in tropical and subtropical America. The insects are boiled in ammonia or sodium carbonate solution and alum is added. A precipitation reaction occurs between alum and the red aluminum salt in the carminic acid solution. The product is dried into a pigment that was purchased from a supplier in this case. Carmine pigment had been almost as easy as alizarin to identify in the past, but its insoluble nature deems it one level up in difficulty.\textsuperscript{32}

Carmine paint is another level up in difficulty to identify, because paints involve different mediums, binders, mordents, contaminants, etc. that can interfere with the pigment’s signal which were discussed above. The sample taken from the prepared reference paint slide is susceptible to variability because of these factors.

Identification was harder for Madder lake pigment than it was for carmine. Madder lake is taken from the roots of a plant family called the Rubiaceae. Madder lake is composed of two
major molecules, alizarin and purpurin, and sometimes has small amounts of quinizarin, xanthopurpurin, pseudopurpurin, rubiadin and/or mujistin. The alizarin dye extracted from the roots was used historically to color clothing dating as far back as the 5th century BCE. Another interesting use of this red dye was that bones remaining from animals who ate the madder plant were stained with alizarin which allowed researchers to study bone development.

Alizarin and purpurin make up madder lake, so different stocks of madder lake could have slightly different wavenumbers of major peaks depending on which component is contributing more to the spectra as well as if other molecules of lesser amounts (mentioned above) are contributing as well. Figure 10 shows a SERS spectrum of alizarin and purpurin, and illustrates how these components relate to the overall SERS spectrum of madder lake shown directly above. The contribution from competing components is perhaps a part of the reason that madder lake is more difficult to identify compared to carmine.

Madder lake paint is the last level of difficulty. Madder is the most difficult analyte to identify within the selected group of red dyes, and the complex nature of paint increases the difficulty to identify as well. Observations of the complex nature of madder lake paint is discussed further in later sections.

Figure 10. SERS spectra of (a) reference madder lake (b) reference purpurin (c) reference alizarin (d) blank colloids.
4.3 Experimental

Colloids were centrifuged (Eppendorf, 1 mL aliquots with ~0.93 mL of supernatant removed) for 2 cycles at 12,000 g for 15 min per cycle. For alizarin detection, 0.75 or 1 uL of blank colloid was placed onto glass coverslips and allowed to dry. Then 0.75-1 uL of a 10^{-2} or 10^{-4} M solution of alizarin (Acros Organics, 97%), ethanol (Fisher), and ultrapure water was placed on top of the dry colloid spot. For the detection of carmine naccarat and madder lake (Kremer Pigments), a small amount of pigment was gathered via the tip of surgical blades (Feather Safety Co.) and placed onto glass coverslips. Then 0.75-1 uL of colloid was placed on top. For the detection of carmine and madder reference paints (prepared using madder lake, carmine naccarat, flake white, and linseed oil obtained from Kremer Pigments), miniscule pieces of paint were cut from the reference slide using the tip of the surgical blade and placed onto glass coverslips. Then 0.75-1 uL of colloid was placed on top.

4.3.1 Relative Intensity Determination

Sometimes an acquired SERS spectrum of carmine or madder lake has characteristics distinct from fluorescence and the colloid spectrum, but is not quite distinguishable as a positive identification. First, a benchmark for what makes a spectrum an indisputably positive identification of the analyte had to be set. After gathering multiple reference spectra for both carmine and madder lake, essential peaks and the ratio between certain peaks were delineated. Not only the wavenumber of the peaks matter, but also the shape of the spectrum overall. If all of the characteristic peaks mentioned are accounted for, then the relative intensities of two or three of the peaks for carmine and madder respectively are measured. After evaluating the reference spectra for carmine, it was decided that the peaks at ~1300 and 459 cm^{-1} should be used to diagnose a spectrum as
identifiable or ambiguous. The ratio between the more intense 1300 peak and less intense 459 peak was calculated for all reference spectra. The mean ratio was 3:1, so any spectrum with a ratio $\geq 3:1$ was deemed a positive identification of carmine pigment. Madder lake has seven peaks that were classified as essential to a positive identification which are at 1610, 1543, 1418, 1326, 1296, 1162, 343 cm$^{-1}$. The peaks selected as reference peaks for madder lake are 1417, 1290, and 1610 cm$^{-1}$. If these three peaks have a ratio of $4 \pm 2 : 4 \pm 2 : 1$ respectively, then the spectrum is a positive identification for madder lake. Two standard deviations from the average are accepted based off of acceptable variation amongst the reference peaks used to set this benchmark. Figure 11 shows examples of spectra for both carmine and madder that are a positive identification, marked with a check, and those that are too ambiguous for identification, are marked with an “X”.

Figure 11. SERS spectra of reference red lake pigments obtained using various colloid batches. (c) carmine lake with batch J, (d) carmine lake with batch A, (e) madder lake with batch G, (f) madder lake with batch D, and (g) blank colloids.
4.4 Results and Discussion

Table 1 summarizes the results for 10 representative batches of colloid put through the quality assurance protocol. Each of these batches were synthesized using the same protocol outlined above, but have different characteristics. Batches showed variation using the previous testing measures discussed in chapter 3. These different batches were purposefully selected in hopes that a trend for a better AgNP batch would come to light.

The first thing of importance to note is that all 10 batches successfully amplified the signal for alizarin dye. This finding reaffirms the need to test each batch with an analyte that is not naturally as easily obtained. The second finding to note is that colloids that were successful in identifying carmine pigment were also successful in identifying carmine paint. This provokes two thoughts. The first being that one of those analytes can be omitted from testing in the future. The second is that carmine is easier to identify than madder as was hypothesized early on in this study. Another conclusion from Table 1 is that madder may be unreasonably difficult to detect without pretreatment of the sample before the addition of a colloid. In other words, perhaps a colloid should not be discarded if it is unable to gather a signal from madder paint. This conclusion provoked a shift from colloid optimization to sample pretreatment measures. The
flow chart in Figure 12 summarizes the steps that should be taken for quality assurance of each newly synthesized batch of colloids.

**Figure 12.** Schematic of QA protocol.
Chapter 5

Pretreatment Strategies for Madder Lake Extraction

5.1 Introduction

After AgNPs pass the proposed quality assurance protocol, the unique paint sample should also receive extra considerations. SERS has been successful in the positive identification of organic colorants using silver colloids,\textsuperscript{27,37} but the inconsistency that other studies have noted point towards a need for sample pretreatment measures.\textsuperscript{28,38} The unpredictable nature of working with madder lake and carmine, validates the motivation to develop an extraction procedure. The Wustholz group in the past recognized a need to decrease inconsistent sample cooperation in specifically yellow colorants.\textsuperscript{26} The published yellow organic pretreatment strategy will be discussed in relation to the more recent extraction of red colorants. A step accounting for extraction procedures is added to the quality assurance protocol of AgNPs which allows for more colloids to pass the test for SERS activity, owing difficulties in identification to the nature of the sample rather than a suboptimal batch of AgNPs.

5.2 Attempt to Identify Madder in Charles Wilson Peale Samples

The interest in optimizing identification of red colorants started with samples taken from paintings done by Charles Willson Peale. Charles Willson Peale was an American painter who lived from 1741 to 1827 and was well known for his portraits. He is of special interest to this project because he noted in his journal that he noticed fading of the flesh tones in his pieces. He then claimed to stop using carmine, the pigment he blamed for the fidelity of his flesh tones, and instead allegedly started using madder lake.
The samples were tested to see if they did in fact contain madder lake instead of carmine. A positive identification for madder lake would confirm Peale’s change in process. A positive identification for what appeared to be madder lake was not fully successful as shown in Figure 13. This motivated the creation of madder extraction techniques that would produce indisputable madder lake spectra from the art samples.

**Figure 13.** SERS spectra of (a) an art sample of Portrait of James Lewis by Charles Willson Peale. (B) madder lake reference (c) blank colloids.

The spectrum labeled (a) in Figure 13 is not a discernible spectrum. The high signal-to-noise ratio and lack of distinct peaks prevents a positive identification of the colorant. Unfortunately, this ambiguous signal was typical of the Peale art samples collected. The plateau from ~1300 cm\(^{-1}\) to ~1600 cm\(^{-1}\) could point towards this spectrum being a stubborn madder lake signal since its three most prominent peaks fall within this range. Through the exploration of reference samples (where a known pigment is analyzed), it was observed that carmine lake behaves differently than madder. When carmine lake is subjected to laser excitation the prominent peak at ~1300 cm\(^{-1}\) appears suddenly from the background noise. Madder on the other
hand seems to grow gradually from the background noise, eventually building up to distinct peaks. Behavior that more closely mimics that which is seen while analyzing reference madder lake is seen when looking at the Peale samples (like the one in the Figure 13).

5.3 Exploring the Extraction of Organic Colorants

5.3.1 Previous Extraction of Organic Colorants

While studies promote the use of hydrofluoric acid (HF) for the extraction of the colorant from binders within the paint matrix,\textsuperscript{29,38} this study focuses on finding a less hazardous approach. Studies with success in extracting colorants of interest from fibers and textiles highlight the use of hydrochloric acid (HCl) and methanol (MeOH).\textsuperscript{3,37} Moving on from textiles and fabrics, HCl and NaCl yielded success in pretreatment and subsequent identification of the blue colorant, indigo, but the same treatment applied to solid samples proved unsuccessful.\textsuperscript{39} A need for solid sample extraction, or what is necessary for real art samples, became apparent. Roh et al. successfully employed HCl and MeOH for identification of yellow colorants which results lend a foundation for this study of red colorants.\textsuperscript{26}

5.3.2 Protocol for Red Colorant Extraction

Microscopic samples retrieved from the Paintings Conservation Laboratory as part of the Colonial Williamsburg Foundation were spotted with 0.75 $\mu$L of the pretreatment extraction solution. The solution is a 1:2 mixture of 1M HCl in MeOH. The solution was allowed $\sim$1 hour to evaporate followed by the application of 0.75 $\mu$L of colloid. SERS studies were performed on an inverted microscope equipped with a 632.8-nm laser described in detail elsewhere.\textsuperscript{10,26,27}
5.3.3 Results and Discussion

The protocol outlined in the previous section was applied to samples of art hypothesized to include madder lake. The spectrum shown in Figure 14 was not as discernible as hoped. While more definition is seen in the ~1300-1500 cm\(^{-1}\) range, the lack of a peak at 1610 cm\(^{-1}\) means that these spectra would not be a confirmed positive identification for madder lake. The samples did contain a positive identification for carmine however as shown in Figure 15. Although carmine was not the colorant of interest, the positive identification confirms that the pretreatment protocol does not preselect for a certain red colorant. In the future, different solvents, concentrations of solvents, and application procedures will be explored to improve the red colorant extraction protocol in hopes of obtaining indistinguishable madder lake spectra from real works of art.

![Figure 14.](image1.png)  
**Figure 14.** (a) SERS spectrum from De Cool Dress #2 from a private collection. (b) madder lake extracted reference SERS spectrum. (c) SERS spectrum of blank colloid.

![Figure 15.](image2.png)  
**Figure 15.** SERS spectra of samples from *Portrait of Queen Elizabeth I (1533-1603)*, British, 1590-1600, oil on canvas transferred from wood, accession #1945-20. The Colonial Williamsburg Foundation, Gift of Mr. Preston Davie.
5.5 Concluding Remarks and Future Directions

SERS is a technique with high sensitivity and selectivity for identification of organic pigments in art, but this study was motivated by the need to optimize the SERS substrate, AgNPs. Variation between different colloid batches motivated a search for a threshold of which colloids are optimal for application on art. The quality assurance flowchart for assessing the quality of each new synthesis embraces the variation that each batch of colloid inevitably has, while ensuring that suboptimal batches are not used in experiments involving precious art. This quality assurance will hopefully provide the best chance for positive identification of red organic pigments in art in the future. Pretreatment of paint samples, motivated by the goal of obtaining a positive identification of madder lake within Charles Willson Peale art samples, shows promise but will require further experimentation to optimize the procedure. Eventually the combination of quality assurance of the SERS substrate and pretreatment extraction of art samples, will hopefully lead to consistent future success with organic red colorant identification.
References


