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A SERS and SEM-EDX Study of the Antiviral Mechanism of Creighton Silver Nanoparticles against Vaccinia Virus

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science

By

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Abstract

Anders, Catherine Binns. M.S., Department of Chemistry, Wright State University, 2012. A SERS and SEM-EDX Study of the Antiviral Mechanism of Creighton Silver Nanoparticles against Vaccinia Virus

Silver nanoparticles (AgNPs) are well-recognized as antiviral agents but little is known about their mechanism of action. In this study, it was hypothesized that unfunctionalized, Creighton AgNPs of an average diameter of 11 nm will form covalent bonds with vaccinia virus (VV) mainly through the external, entry fusion complex (EFC) proteins. The EFC is housed on the external membrane of VV and contains 9-12 proteins having numerous cysteine groups, intramolecular disulfide bonds, aromatic moieties and myristic acids bound to the N-terminus of glycine residues. VV \( (10^{12} \text{ PFUs}) \) was incubated at 37\(^{\circ}\)C for one hour with Creighton AgNPs that were size selected (1-25 nm in diameter) and concentrated (1,000 ppm silver) using tangential flow ultrafiltration. After incubation, the sample was rinsed three times to remove any unbound AgNPs from the VV. The VV-AgNP sample was then deactivated with formaldehyde and fixed onto glass slides and stubs for surface-enhanced Raman spectroscopy (SERS) and scanning electron microscopy-energy dispersive X-Ray (SEM-EDX) analysis, respectively. SERS maps containing over 2,600 spectra were collected and processed using in-house written MatLab codes. Six endmember spectra were extracted from the hyperspectral data set using a multivariate statistical analysis method, namely vector component analysis (VCA). The SERS analysis
of the six endmember spectra indicated an interaction trend similar to that reported in literature by other SERS studies on proteins exposed to AgNPs: carboxylic groups > peptide bond interactions (amide peaks) > aromatic AAs > thiol groups > small side chains. Additionally, the SEM electron backscatter images and the EDX spectrum of the VV-AgNP sample revealed the presence of silver and further supported the direct interaction between AgNPs and VV. These interactions confirmed the proposed hypothesis and suggested that the covalent bonding interactions might disrupt the VV ability to complete the entry/fusion steps of the viral replication cycle.
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Introduction

Smallpox and the Vaccinia Virus

Smallpox related diseases date back as many as 10,000 years to the ancient civilizations of Mesopotamia and regions near Egypt, and eventually spread to other civilizations\(^1\). During the latter two centuries, smallpox had caused epidemics and globally killed over 500 million people\(^1\). There are two primary infectious forms of smallpox: \textit{variola major} and \textit{variola minor}. \textit{Variola major}, the most dangerous form of the disease, is estimated to be fatal in 30-35\% of infections\(^2\). The quest to eradicate smallpox as a disease began in 1958 and continued tirelessly until smallpox was declared eradicated worldwide on October 26, 1979. This was two years from the last reported smallpox case, a day known as smallpox zero day\(^2\).

After its eradication, the smallpox virus was contained to two secure locations within the world: the first being the Centers for Disease Control and Prevention, in Atlanta, GA, and the second known as the State Research Institute of Virology and Biotechnology (also known as Vector) in Novosibirsk, Siberia. However, it is believed that since the containment of the virus, viral stocks have landed in the hands of many nations linked to terrorist regimes and hostile leadership including Iraq and North Korea\(^3\).
Other more prevalent orthopoxviruses, such as monkey-pox virus (MPV) also present challenges and potential threats as bioterrorist agents. MPV, which causes smallpox-like infections in primates, could be especially problematic considering the evolutionary similarities between primates and humans\(^3\)-\(^4\). Furthermore, remaining supplies of smallpox vaccine are limited and would only immunize approximately 10% of the world’s population. The implementation of a large scale vaccination program in the event of a bioterrorist threat or large scale cross-species orthopoxvirus infections would be ineffective in preventing potential pandemic scenarios\(^1\).

Antiviral drugs offer the best options to mitigate the effects of widespread viral infections. However, most antivirals are agent specific and no antiviral drugs have been tested for use with poxvirus infections\(^5\). There is a great need for the development of a broad-spectrum antiviral treatment that can be used against a variety of viral agents. Silver nanoparticles (AgNPs) have a proven history of antimicrobial properties against a variety of biological agents\(^6\)-\(^9\) including LIVP strain of smallpox (SPV)\(^10\) and vaccinia virus\(^5\). Experimentation with the actual smallpox virus is not possible today given the protected status of \textit{variola major} and \textit{variola minor}. Vaccinia virus (VV), the gold standard for the development of the smallpox vaccine, is readily available and is an effective laboratory model for the study of smallpox and related orthopoxviruses\(^11\)-\(^12\).
Silver nanoparticles (AgNPs)

The preparation and characterization of nanostructures (1-100 nm in at least one dimension) has been a very active sector for the past decades primarily due to their unique physical, chemical and antimicrobial properties\textsuperscript{13,6-7}. In general, colloidal AgNPs exist as zerovalent metal particles, and have low coordination numbers and highly polarized surfaces\textsuperscript{14-15}. Seeking a more stable environment, colloidal AgNPs will preferentially bind to organic and biological molecules through covalent interactions with common electron donors such as oxygen, nitrogen, thiol and phosphate groups\textsuperscript{14}. The small size of nanomaterials offers many advantages in the commercial sector due to large surface area to volume ratios yet their toxicity is often much lower to their bulk or ionic counterparts\textsuperscript{16}. This has led to exponential growth in the use of AgNPs in consumer products and biomedical applications in the past decade. Nanomaterials have also received considerable attention in many research areas including environmental pollutants\textsuperscript{17}, biomedical devices\textsuperscript{6}, electronics\textsuperscript{18}, pharmaceutical products\textsuperscript{7} and sensors\textsuperscript{19-21}.

The antimicrobial properties of AgNPs have been extensively studied against a variety of biological agents including bacteria\textsuperscript{6-7}, fungi\textsuperscript{7} and numerous viruses (\textit{e.g.}, HIV-1\textsuperscript{8,22}, hepatitis B\textsuperscript{23}, herpes simplex virus (HSV)\textsuperscript{24,9}, monkey-pox virus\textsuperscript{4}, tacaribe virus\textsuperscript{25}, LIVP strain of smallpox (SPV)\textsuperscript{10} and vaccinia virus (VV)\textsuperscript{5}). Much of the virus-related research has focused on the size-dependent relationship of the NPs and their level of antiviral activity.
Elechiguerra et al. found that AgNPs (1-10 nm in diameter) synthesized using three different methods demonstrated antiviral capabilities against the HIV-1 virus. The first formulation consisted of non-spherical AgNPs obtained from Nanotechnologies, Inc. that were embedded in a foamy carbon matrix. Ultrasonication and a TEM electron beam were employed to disrupt the bonds between the NPs and carbon, and to release the majority of the AgNPs from the matrix. The other two formulations were poly (N-vinyl-2-pyrrolidone) (PVP) coated AgNPs and bovine serum albumin (BSA) conjugated AgNPs. High angle annular dark field (HAADF) scanning transmission electron microscopy demonstrated that the AgNPs bound to the GP-120 glycoprotein knobs protruding from the external membrane of the HIV-1 virus. Furthermore, the HAADF images revealed an ordered distribution of the AgNPs suggesting that the NPs bound preferentially to the GP-120 glycoprotein knobs, which were spaced approximately 22 nm apart. All three formulations showed antiviral activity at concentrations above 25 μg mL⁻¹. However, the AgNPs that had been released from the foamy carbon matrix (i.e., unfunctionalized AgNPs) exhibited the greatest antiviral activity. This increase in inhibition was attributed to the ability of the AgNPs to bind directly to the GP-120 glycoprotein knobs without bonding interferences from the functional groups (i.e., PVP and BSA) present in the other formulations.

Rogers et al. examined the antiviral effects of different AgNP formulations (polysaccharide-coated and non-coated) and sizes (10-80 nm) against monkey-
pox virus (MPV) in conjunction with African green monkey (Vero) kidney cells using a plaque reduction assay. Both AgNP types demonstrated antiviral activity against MPV at concentrations as low as 12.5 μg mL⁻¹. However, only the 10 nm polysaccharide-coated AgNPs exhibited consistent plaque reduction capabilities, and suggested a size-dependent antiviral activity⁴.

Bogdanchikova et al. investigated the size-dependent antiviral effects of two different medicinal preparations of unfunctionalized AgNPs (i.e., collargol and protargol) against SPV. The different formulations had AgNPs of average diameters of 10 nm and 2 nm, respectively¹⁰. The 10 nm collargol AgNPs were found to have a larger antiviral activity against SPV than the 2 nm protargol AgNPs¹⁰. The author concluded that the large surface area to volume ratios of the smaller AgNPs resulted in the rapid oxidation of the zerovalent NPs to ionic silver (Ag⁺) in the aqueous solution¹⁰. The resulting change in the antiviral activity of the oxidized AgNPs led to the conclusion that AgNPs exert a greater inhibitory effect against SPV than ionic silver¹⁰.

Despite the growing amount of research with noble metal NPs and various virus strains, a specific viral inhibition mechanism has not been determined yet. Most literature studies strongly suggested that the primary inhibition is a result of the NP disruption of the binding, fusion and/or entry steps of the viral replication cycle⁴,⁸-⁹,²²-²⁵. The HIV-1 study showed that the AgNPs bound to the GP-120 glycoprotein knobs of the virus through Ag-S interactions.
This is achieved through the reduction of disulfide bonds of the GP-120 proteins that permanently alters the virus and its ability to bind with and infect the host cell. Similar inhibition mechanisms were proposed for the disruption of the replication process of MPV, tacaribe virus and HSV. However, observation of magnesium NPs within cultured cells suggested that the interruption of intracellular biochemical pathways could also play a role in the inhibition mechanisms of such NPs.

Trefry (2011) examined the binding and fusion steps of the viral replication cycle of VV that was exposed to NovaCentrix AgNPs (25 nm ± 10 nm in diameter). The viral adsorption to host cells (i.e., binding) was found to be unaffected by AgNP concentrations ranging from 2 – 128 µg mL\(^{-1}\). This demonstrated that AgNPs did not alter the VV’s ability to bind to the host cell at such concentrations. Antiviral mechanisms associated with the entry/fusion complex (EFC) were explored by examining the cytoprotective inhibition (cells incubated with AgNPs pre-infection), the virucidal protection (VV incubated with AgNPs prior to infection) and the post-infection inhibition (AgNPs applied as treatment post-infection). The minimum inhibitory concentrations 50% (IC\(_{50}\)) for the three treatment conditions were found to be 91 µg mL\(^{-1}\), 48 µg mL\(^{-1}\) and 88 µg mL\(^{-1}\), respectively. This confirmed that VV’s fusion with the cell or entry into the cell was disrupted by the AgNPs. In addition, the virucidal condition of VV inhibition was also investigated using Creighton AgNPs that had been filtered using tangential flow ultrafiltration (TFU). These AgNPs were synthesized in our
group, and had a narrow size distribution of 1-20 nm with an average diameter of 11 nm\textsuperscript{27}. The IC\textsubscript{50} of these NPs was 128 \( \mu \text{g mL}^{-1} \). While a specific mechanism was not proposed for the viral inhibition, the short incubation times employed in this study (1 hr) and the observed antiviral activity of the two different types of AgNPs strongly suggested that the inhibition occurred at the early stages (entry and fusion) of the viral replication cycle\textsuperscript{5}. Furthermore, the entry/fusion inhibition scenario was supported with western blot testing using antibodies specific for the G9 protein, a member of the EFC of VV. This analysis revealed a dose-dependent viral inhibition and suggested some form of covalent interaction between the AgNPs and the G9 proteins of VV\textsuperscript{5}.

Several trends emerge from the above studies on various viral pathogens exposed to various AgNPs. (1) The viral inhibition mechanism involves strong interactions between the AgNPs and the virus that disrupt the early stages of the viral replication cycle. Given the attraction of AgNPs to electron rich, organic constituents, covalent bonding between the AgNPs and the amino acids (AAs) of the viral proteins should be investigated. Additionally, the previous research on VV supports the idea that the most probable interactions are occurring through the protein moieties responsible for the entry and fusion steps (EFC proteins) of the replication cycles. (2) Considering the enhanced antiviral activity that was reported for unfunctionalized and functionalized AgNPs of an average diameter of about 10 nm, and the high toxicity of AgNP capping agents or enclosing matrices in host cells (\textit{e.g.}, foamy carbon matrix and PVP), unfunctionalized
AgNPs of a similar average diameter should be considered and tested as effective antiviral agents.

The Creighton synthesis method is an inexpensive and simple procedure, which results in the formation of unfunctionalized, round AgNPs of 1-100 nm in diameter.\textsuperscript{28-29} The polydispersity and concentration of the Creighton colloidal AgNPs can be further controlled through TFU. This thesis will show that TFU may be implemented for the size-selection and extreme concentration of large volumes of Creighton AgNPs. Afterward, these AgNPs of an average diameter of 11 nm will be used in antiviral mechanism studies.\textsuperscript{30-31}

**Surface-enhanced Raman spectroscopy (SERS) and scanning electron microscopy – energy dispersive X-ray (SEM-EDX)**

In this work, SERS and SEM-EDX measurements will be performed to investigate the antiviral mechanism of unfunctionalized, Creighton AgNPs with sizes in the 1-25 nm range and an average diameter of 11 nm.

Surface-enhanced Raman spectroscopy (SERS) is an effective method for the detection of covalent bonding interactions between AgNPs and electron-rich functional groups of proteins such as carboxylic, amide, thiol and aromatic AA groups.\textsuperscript{32-33} Previous research indicated that AgNPs have preferential binding to EFC proteins. The following interaction trend with AgNPs emerged from these
studies: carboxylic groups > peptide bond interactions (amide peaks) > aromatic AAs > thiol groups > small side chains\textsuperscript{32-34}. Given the complexity of the protein structure EFC of the VV\textsuperscript{11,35}, numerous interactions with the unfunctionalized, Creighton AgNPs would be expected. In this work, hyperspectral collection methods that combine movable microscope stages with Raman instrumentation will be employed to obtain a large, representative set of SERS spectra. Furthermore, multivariate analysis techniques, specifically principal component analysis (PCA) and vector component analysis (VCA), will be utilized to mathematically identify the representative spectral patterns within the complex hyperspectral dataset and to provide a statistical correlation of the representative spectra to the compiled dataset\textsuperscript{36-39}.

Finally, scanning electron microscopy – energy dispersive X-ray (SEM-EDX) will be employed to confirm the direct, strong interactions between the AgNPs and VV. SEM electron backscattering will be exploited to differentiate between the organic constituents of the VV and any covalently attached AgNPs. The contrast of backscattered electron images is directly related to the atomic number of the elements in the sample\textsuperscript{8}. More intense, brighter areas of the image will correspond to heavier atoms (i.e., silver), whereas lighter elements (i.e., organic constituents) will appear as darker areas\textsuperscript{40}. Furthermore, EDX will facilitate the elemental composition analysis of the VV samples treated with AgNPs.
Hypothesis

Unfunctionalized, Creighton AgNPs of an average diameter of 11 nm will covalently bind to VV mainly through the external, entry fusion complex (EFC) proteins.

Specific aims

1) To fabricate, characterize and manipulate Creighton AgNPs of an average diameter of 11 nm through UV-Vis absorption spectrophotometry, inductively coupled plasma optical emission spectroscopy (ICP-OES), micro-Raman spectroscopy, transmission electron microscopy (TEM), scanning electron microscopy – energy dispersive X-Ray (SEM-EDX), and tangential flow ultrafiltration.

2) To demonstrate the proposed antiviral mechanism through micro-Raman spectroscopy, surface-enhanced Raman spectroscopy (SERS) and scanning electron microscopy – energy dispersive X-ray (SEM-EDX).
Technical Approach

Specific Aim 1:

Reagent solutions and glassware preparation

Silver nitrate (AgNO₃) and sodium borohydride (NaBH₄) were obtained through Fisher Scientific Inc. All solutions were prepared in highly purified water (>17 MΩ) that had been autoclaved at 121°C for 20 min using a liquid sterilization cycle. All glassware used for synthesis was cleaned by submersion in an acid bath (10 % HNO₃ bath for 12-24 hours) that was followed by a base bath (1.25 M NaOH in a 40% ethanol base bath for 4-12 hours). Glassware was rinsed three times with deionized water and one time with highly purified water after each cleaning step. Afterward, the glassware was sterilized in an autoclave at 121°C on a 20 minute gravity cycle.

Creighton synthesis of silver nanoparticles

A modified Creighton method²⁸,³¹ was utilized to synthesize the AgNP colloid through the reduction of AgNO₃ with NaBH₄. A 2 mM solution of NaBH₄ was prepared by dissolving 0.0227 ± 0.0012 g of NaBH₄ in 300 mL of autoclaved water. A 1 mM solution of AgNO₃ was prepared by dissolving 0.0085 ± 0.0009 g AgNO₃ in 50 mL autoclaved water. The reaction was carried out in an ice bath at
2 ± 1°C by adding AgNO₃ drop wise to the NaBH₄ at a rate of 1-2 drops/sec while stirring at a rate of 325 rpm. To ensure the complete reduction of AgNO₃, the colloidal solution was stirred for additional 50 minutes within the ice bath. The final colloid was refrigerated for a minimum of four days prior to characterization by UV-visible absorption spectrophotometry and micro-Raman spectroscopy. Colloidal batches of AgNPs, which passed the above nanomaterial characterization tests, were combined. Figure 1 illustrates the synthesis setup and the final color of the colloidal suspension of AgNPs. This Creighton colloid was found to be stable for up to six months when stored at 4°C.

Figure 1: A) Creighton colloid synthesis setup and B) Characteristic golden-yellow color of the Creighton colloidal AgNPs.³₀
Characterization of silver nanoparticles

UV-Vis Absorption Spectrophotometry

*Working Principle:* When subjected to electromagnetic excitation, some organic and inorganic molecules will absorb radiation characteristic to the ultra-violet (UV) or visible (VIS) range of the electromagnetic spectrum (approximately 200-800 nm). Molecules that absorb UV-VIS radiation, commonly known as *chromophores*, must contain electrons that are capable of undergoing the allowed electronic transitions corresponding to this absorption\(^{41}\). Briefly, the molecules must possess an electron in an \( \pi \)-orbital or a non-bonded electron contained in a \( n \)-energy level that can be excited to an antibonding \( \pi^* \)-orbital and an antibonding \( \sigma^* \) or \( \pi^* \)-orbital, respectively (Figure 2)\(^{41}\).

Molecules that absorb radiation from the VIS portion of the electromagnetic spectrum such as many transition metals will transmit a color visible to the human eye that is complementary to the color of the radiation actually absorbed by the molecular species\(^{41}\). Creighton colloidal AgNPs, which emit a characteristic golden yellow color, actually absorb radiation in the violet portion of the spectrum at approximately 400 nm. The intensity of the color that is observed by the naked eye often reflects the intensity of the characteristic radiation that is absorbed by the molecule\(^{41}\).
Figure 2: Allowed electronic transitions for UV-VIS absorption: A) $\pi$ to $\pi^*$, B) $n$ to $\pi^*$ and C) $n$ to $\sigma^*$.

The absorbed radiation or absorbance ($A$) is expressed mathematically as the logarithmic ratio of the incident radiation power ($P_0$) to the power of the radiation transmitted from the sample ($P$). This is equal to the negative log of the transmittance ($T$)\(^4\) (eq 1).

$$A = \log \frac{P_0}{P} = -\log T \quad (1)$$

Spectra are normally plotted as a function of the absorbed radiation (in arbitrary units) versus the wavelength (in nm). Typical spectra contain one or more broad absorption bands that span a range of wavelengths and exhibit a maximum
absorption wavelength ($\lambda_{\text{max}}$). The broad appearance of the absorption bands are a result of the simultaneous absorption of closely spaced vibrational and rotational energy transitions that are not resolved into discrete peaks\textsuperscript{38}.

The shape, symmetry and intensity of the absorption bands can be used to qualitatively define the molecular species or quantitatively estimate the concentration of the analyte\textsuperscript{38}. The approximate concentration can be obtained through the use of Beer's law relating the absorbance (A) to the analyte concentration in moles per liter (c), the path length (b) and the extinction coefficient of the analyte ($\epsilon$) by the following equation\textsuperscript{42}

$$A = \epsilon bc$$

where in this study, $\epsilon = 1.9 \times 10^4$ dm$^3$ mol$^{-1}$ cm$^{-1}$ (for Creighton AgNP clusters with $\lambda_{\text{max}} = 400$ nm)\textsuperscript{43} and $b = 1$ cm (for standard cuvettes).

**Sample preparation:**

A 1:10 by volume dilution was prepared by pipetting 0.25 mL of AgNP colloid into a cuvette of 1 cm path length (Fischer Scientific, 4.5 mL capacity) containing 2.5 mL of highly purified water. A separate cuvette was prepared with highly purified water for a blank baseline correction. The resulting solution was mixed thoroughly. The outside walls of both cuvettes were cleaned with a dry Kimwipe prior to analysis.
Instrumental Analysis:

A Cary 50 UV-VIS-NIR spectrophotometer from Varian Inc. (Figure 3) set to absorbance mode was utilized to obtain the baseline corrected absorption spectrum of the AgNP samples. Sample scans were collected within the 200-800 nm spectral range using 1 nm scanning intervals and a fast scan rate of 4800 nm min\(^{-1}\). Prior to sample analysis, a baseline correction was obtained from the absorption spectrum of the high quality water. The resulting spectra were plotted and analyzed using Origin 8 software.

![Image of Cary 50 UV-VIS-NIR spectrophotometer](image)

*Figure 3: Cary 50 UV-VIS-NIR spectrophotometer from Varian Inc. used to measure the SPR peak of the AgNP colloid.*

Micro-Raman Spectroscopy

Micro-Raman spectroscopy was used to verify the purity of the Creighton AgNP batches before tangential flow ultrafiltration (TFU). The working principal,
sample preparation and instrumental analysis parameters are outlined under Specific Aim #2.

**Tangential Flow Ultrafiltration (TFU)**

**Working Principle:**

Tangential flow ultrafiltration (TFU) is a recirculating filtration technique that is commonly employed for the weight-based isolation of biological materials such as proteins, cells and viruses. In this work, it has been adapted for the effective size selection and concentration of AgNPs$^{30-31}$. Traditional dead-end filtration techniques such as centrifugation result in nanoparticle aggregation. To overcome this limitation, synthesis methods often employ chemically aggressive capping agents or solvent systems to achieve stability upon nanomaterial concentration or specific size-selection$^{44}$. The results of this study will show that TFU (Figure 18) results in stable, homogenous concentrates and eliminate the need of capping agents or solvent systems.

Briefly, the liquid sample (e.g., colloidal suspensions of nanoparticles) is passed through a series of hollow fiber membranes of various pore sizes (from 1,000-kD down to 10-kD) and surfaces areas (from 370 cm$^2$ down to 5 cm$^2$) to achieve the desired size selection and level of concentration, respectively. In TFU, the retentate feed is parallel to the membrane filter in comparison to most filtration methods that utilize a perpendicular flow. Larger particles are retained by the
specific size lumen of the filter (retentate), whereas particles smaller than the filter pore size will pass through (filtrate) (Figure 4).

Figure 4: TFU illustration of the selection process through the hollow fiber membrane. (Used with permission from Joshua D. Baker)

This parallel circulation helps prevent membrane clogging and aggregation events that are common in traditional methods. As the retentate continually feeds through the hollow fiber membrane, increasing amounts of excess water and synthesis byproducts are removed through the passing filtrate, while the AgNPs are retained with the recirculating retentate resulting in increased concentrations of AgNPs in the final colloidal suspension (Figure 5)
Figure 5: Schematic illustrating the TFU working principle: a-c represent successive recirculating passes through the TFU system.

**Sample preparation:**

Each large batch of Creighton colloid (4.0 to 5.0 L) was processed through the TFU system without the need of additional sample preparation.

**Instrumental Analysis:**

A KrosFlo II Research filtering system (Spectrum Laboratories, Rancho Dominguez, CA) was used to size select and concentrate 4.0-5.0 L of AgNP colloid (Figure 6).
Figure 6: KrosFlo II TFU system used for size selection and concentration of the AgNP colloid.

The TFU process was completed in a series of three steps. In the first step, AgNPs and AgNP-aggregates of 50-nm in diameter and larger were eliminated using a 50-nm MidiKros® polysulfone module (460 cm²). Secondly, a 100-kD MidiKros® polysulfone filter (200 cm²) was employed to further size select and concentrate the AgNPs contained in the filtrate collected from the 50-nm filter. Size 17 masterflex tubing was used with a pump rate of 700 mL min⁻¹ for steps one and two. Finally, the concentrate retained from step 2 underwent a further volume reduction using a 100-kD MicroKros® polysulfone filter (20 cm²) connected with size 14 masterflex tubing and a pump rate of 90 mL min⁻¹. Both steps 2 and 3 eliminate AgNPs less than 1 nm in diameter and result in approximate volume reductions of 100 fold. An original colloid batch of 5 L would be reduced to approximately 50 mL in step 2 and then to approximately 4 mL in step 3.
Inductively Coupled Plasma - Optical Emission Spectroscopy (ICP-OES)

**Working principle:**

When at room temperature, the electrons in an atom will occupy the ground state. Upon exposure to flame or ionized plasma, these electrons will be excited to an energetic state. The lifetime of these excitations are normally very short ($\sim 10^{-7} - 10^{-9}$ s) and result in the emission of a photon of a wavelength ($\lambda$) and an energy corresponding to the transition between the two energy levels. The characteristic excitation and emission process is depicted in Figure 7.

Figure 7: Excitation and emission process. A - D correspond various levels of excitation energy: A and B) Atomic excitation; C) Ionization and D) Ionization and excitation. $\lambda_1 - \lambda_4$ represent characteristic emission patterns: $\lambda_1, \lambda_2$ and $\lambda_3$) Atomic emissions and $\lambda_4$) Ionic emission.
In ICP-OES, the excitation source is a plasma created by the inductively-coupled ionization of a flowing argon stream\textsuperscript{42}. The temperature range of the resulting ionized plasma spans from 6,500 to 10,000 K, and creates an environment capable of exciting most elements on multiple atomization or ionization levels\textsuperscript{41}. As a result, a complex emission spectrum is obtained for each element present. Within the emission spectrum, intense spectral lines will be present and will occur even for very low analyte concentration\textsuperscript{41}. The wavelengths of these emission lines are normally chosen for the sample analysis and usually correspond to the electronic transition from an excited state to the ground state ($\lambda_1$ or $\lambda_2$) according to the Boltzmann Distribution Law\textsuperscript{41}. ICP-OES offers many advantages over other atomic emission techniques such flame atomic absorption spectroscopy. A few examples include the simultaneous multiple element analysis, the low detection limits (down to the $\mu$g L$^{-1}$ level) and the elimination of many spectral and chemical interferences\textsuperscript{42}.

**Sample preparation:**

Aliquot samples collected from the original Creighton colloid and the two 100-kD colloidal retentates were chemically digested using HNO$_3$. Each colloidal sample was volumetrically pipetted into a 50 mL beaker containing approximately 5 mL of highly purified water. One milliliter of trace metal grade HNO$_3$ (Optima grade, Fisher Scientific) was then added. The solution was gently heated at 225°C on a hotplate and allowed to gradually evaporate until nearly dry. The
samples were then volumetrically diluted in a 2% HNO$_3$ matrix. The dilution factors used in this study were 1:1,000 for the original colloid, 1:25,000 for the first 100-kD MidiKros® retentate (200 cm$^2$ module), and 1:250,000 for the second 100-kD MicroKros® retentate (20 cm$^2$ module).

The following standards were prepared from a 10,000 µg mL$^{-1}$ (ppm) silver standard solution for trace metal grade analysis (Ultra Scientific) in a 2% HNO$_3$ matrix: 3, 7, 10, 15, 25, 50, and 100 µg L$^{-1}$.

**Instrumental analysis:**

The amount of silver present in each colloidal sample was quantified using a 710E spectrometer (Varian Inc.) (Figure 8). The ICP-OES instrument parameters were as follows: wavelength for Ag (328.068 nm), radio frequency power (1.20 kW), plasma flow (15.0 L min$^{-1}$), auxiliary flow (1.50 L min$^{-1}$), and nebulizer pressure (200 kPa). Each sample was measured in triplicate with a replicate read time of 10 s, a between-measurement stabilization time of 15 s and a sample uptake delay of 30 s. Method blanks were introduced between every sample to reduce potential contamination and a standard curve was run every 10 samples to verify the stability of the emission intensity. The resulting spectra were plotted using Origin 8 software and data was analyzed using Microsoft Excel.

**Working Principle:**

Electron probe characterization methods (e.g., SEM-EDX or TEM) are important tools for imaging, chemical composition determination and surface analysis of nanomaterials. In these microscopy techniques, an electron beam referred to as the primary (1⁰) probe projects electrons onto the surface of the sample and elicits a variety of scattering effects. The scattering is highly dependent on the mean free path of the electron, the scattering cross-section and the composition and thickness of the sample.
SEM-EDX is typically used for thick sample materials when compared to TEM. SEM-EDX measures the electron-sample interactions that occur in an interaction volume typically of 1 – 5 µm in depth, and is dependent upon the density of the sample and the electron energy. While several electron interactions are possible, SEM primarily measures secondary \((2^0)\) and backscattered electrons. When coupled with EDX, characteristic X-rays that are emitted from electron cascade events can also be measured.

Low energy \(2^0\) electrons, excited from the K-shell of the atom, are produced when \(1^0\) electrons release sufficient energy at the atom’s core to overcome the work function of the material. The SEM image formed from the collection of the \(2^0\) electrons can be used to elucidate the morphology or topology of the sample surface or the shape of particles present in the sample. Additionally, the vacancy left behind by the excited \(2^0\) K-shell electron is subsequently filled when an electron from a higher energy level cascades down to fill the vacancy. The X-rays emitted by the cascading electrons may be differentiated using an EDX detector to provide a unique spectral fingerprint for the atoms present. The backscattered electrons are produced by \(1^0\) electrons in a lower yield than the \(2^0\) electrons. These backscattered electrons are deflected backwards from the surface and depend on the atomic species and the volume of excitation. The elemental composition of the sample can be determined through topographical images generated from backscattered electrons with brighter areas corresponding to elements of high atomic numbers.
Comparatively, TEM is an effective technique for the analysis of thin samples (≈100 nm). TEM is commonly used for estimating the size, shape and size distribution of nanomaterials. In TEM, there is no appreciable electron-sample interaction as the electrons transmit through the sample. The amount of electrons transmitted is dependent upon the atomic or molecular weight of the sample\(^{40}\). Lighter weight atoms typically transmit more electrons than heavier atoms, which exert more influence on the transmitting electrons\(^{40}\). As a result, light areas correspond to lower weight species and darker, more electron-dense areas are occupied by heavier atoms in the resulting bright field image on the phosphor screen\(^{40}\).

**SEM-EDX Sample Preparation:**

Three samples were prepared for SEM-EDX analysis: 1) the final 100-kD retentate of AgNPs (8538.9 mg mL\(^{-1}\)), 2) vaccinia virus (10\(^{12}\) PFU) and 3) vaccinia virus that was incubated with 1000 mg mL\(^{-1}\) of AgNPs for 1 hour at 37\(^{0}\)C, washed and filtered through a 100 nm filter to remove any unbound AgNPs. Both virus samples were deactivated with formaldehyde prior to application. A small volume of each sample was applied to a stub and allowed to dry.
**SEM-EDX Instrumental Analysis:**

A Jeol JIB 4500 SEM (Figure 9) equipped with an EDX detector was utilized for data collection.

![Jeol JIB 4500 SEM](image)

Figure 9: Jeol JIB 4500 scanning electron microscope used for SEM-EDX measurements.

**TEM Sample Preparation:**

The original Creighton AgNPs and the final 100-kD retentate of AgNPs were diluted in highly purified water as described in reference 30. Twenty microliters of each sample were deposited onto 300-mesh formvar-coated gold grids (Electron Microscopy Sciences) and allowed to dry in a desiccator before viewing within one day.
TEM Instrumental Analysis:

A Phillips EM 208S transmission electron microscope operating at an accelerating potential of 70 kV was used to visualize the nanoparticle samples as described in reference 30. A high resolution Gatan Bioscan camera was used to capture the electron micrographs. ImageJ software was used to analyze the tagged image files (TIF). One AgNP was defined by a complete and enclosed perimeter. Two hundred particles per sample were analyzed. Origin 8 software was employed to construct a TEM size histogram for the diameters of the selected AgNPs.

Specific Aim 2:

Virus Sample Preparation

A virus stock was prepared and donated by Dr. John Trefry in Dr. Dawn Wooley’s laboratory.

Micro-Raman and Surface-enhanced Raman Spectroscopy (SERS)

Working Principle:

Theory shows that the Raman effect is an inelastic backscattering process that results when molecules in a sample are excited by a monochromatic laser
beam$^{46}$. When an incident photon from the laser beam collides with the molecule, the electron cloud becomes polarized and the electrons are excited to a virtual energy state raising the potential energy of the molecule ($h\nu_0$) above the ground state (Figure 10).

![Diagram of energy levels](image)

**Figure 10**: Virtual energy state attained by excited electrons

The majority of the excited molecules will relax immediately back down to the initial ground state by emitting a photon of energy identical to the incident photon. This phenomena is commonly known as Rayleigh scattering or elastic scattering (Figure 11b)$^{47}$. Approximately 1 in $10^8$ molecules experience inelastic scattering or Raman scattering, and emit a photon of energy that is either greater than or less than the energy of the incident photon. Stokes scattering (Figure 10a) occurs when the final vibrational state is more energetic than the initial state and the photon is shifted to a lower frequency. This Stokes radiation has less energy than the incident photon ($h\nu_S = h\nu_0 - h\nu_f$)$^{47}$. The anti-stokes scattering
(Figure 11c) occurs when the final vibrational state is less energetic than the initial state. The anti-Stokes radiation has a higher frequency and more energy than the incident photon \((h\nu_{AS} = h\nu_0 + h\nu_f)^{47}\).

![Diagram of Raman spectroscopy](image)

Figure 11: Fundamental principle of Raman spectroscopy: a) Stokes scattering, b) Rayleigh scattering and c) Anti-Stokes scattering.\(^{48}\)

The inelastic backscattering modes are symmetric relative to the Rayleigh peak with Stokes peaks and anti-Stokes peaks occurring at lower and higher wavenumbers than the Rayleigh scattering, respectively. Stokes peaks are generally higher in intensity and most often chosen for analysis since the \(\nu = 0\) energy level is more populated than the higher energy levels of the anti-Stokes scattering according to the Boltzmann Distribution Law\(^{42}\).

Raman spectroscopy offers several advantages as an analytical tool. Samples in various physical states can be analyzed including liquids, solids and gases with minimal sample preparation. Raman spectroscopy is also a non-invasive technique which facilitates the sample recovery for further analysis by...
other methods. Additionally, Raman spectroscopy provides a unique molecular fingerprint and in general sharp Raman bands that enable multiplex detection. Its selection rules make this technique complimentary to infrared (IR) spectroscopy. Finally, a large range of wavenumbers (100 – 4000 cm\textsuperscript{-1}) can be analyzed to obtain the entire spectral region of interest without changing filters, gratings or detectors.

One inherent disadvantage is that low signal intensity may occur for dilute or lowly concentrated samples due to the extremely low number of photons that experience inelastic backscattering (approximately one in a million). Fluorescence interferences may also result especially with higher energy excitation sources. Furthermore, sensitive biological samples may experience undesired photodecomposition depending on the acquisition parameters chosen for analysis. While inconvenient, most of these limitations can be managed by employing surface-enhanced Raman spectroscopy (SERS).

The SERS effect was discovered in 1974, when Fleischmann and coworkers noticed an enhancement in the signal of the Raman spectra of pyridine located in the close proximity to a silver electrode\textsuperscript{49}. This enhancement, initially thought to be due to presence of a larger number of pyridine molecules adsorbed onto the nano-roughened silver electrode\textsuperscript{49}. Later, it was shown that this enhancement (up to 10\textsuperscript{6}) was much too large to be accounted for by the increase in surface area of the silver electrode alone. It was attributed to a
combination of electromagnetic and charge transfer enhancement mechanisms\textsuperscript{50}.

The electromagnetic enhancement mechanism is a result of the resonance surface plasmons of noble metal nanomaterials such silver, gold and copper nanoparticles and nanofilms. Silver nanoparticles have unique electromagnetic properties that emerge from their lone s electrons (\textit{i.e.}, the so-called surface plasmons). When the noble nanoparticles are exposed to an electrical field at the frequency of the laser beam ($E_{\text{incident}}$), the particles become polarized in the direction of the electrical field and subsequently produce an electrical field ($E_{\text{inside}}$) that contributes additively to the incident electrical field ($E_{\text{total}}$) (Figure 12).

<table>
<thead>
<tr>
<th>Before Interaction</th>
<th>After Interaction with laser light $\vec{E}_{\text{incident}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Free electron metal particle</strong></td>
<td></td>
</tr>
<tr>
<td>$\vec{E}_{\text{incident}}$</td>
<td>$\vec{E}<em>{\text{incident}}$ $\vec{E}</em>{\text{inside}}$ $\vec{E}<em>{\text{total}} = \vec{E}</em>{\text{incident}} + \vec{E}_{\text{inside}}$</td>
</tr>
</tbody>
</table>

Figure 12: Schematic of the electromagnetic enhancement mechanism in SERS.
The presence of a molecule in close proximity to the nanomaterial surface will likewise create a resonance effect\textsuperscript{51}. This electromagnetic mechanism accounts for a total enhancement of the Raman signal of up to $10^5$-$10^6$, whereas the chemical adsorption mechanism or charge transfer theory will contribute a maximum of $10^3$ to the signal enhancement\textsuperscript{51}. In 1999, Xu and coworkers demonstrated that the total SERS enhancement factor may reach values as high as $10^9$-$10^{11}$ for analyte molecules located in the interstitial space between two nanoparticles. This SERS enhancement is highly dependent on the distance between the two nanoparticles and the laser polarization with respect to the nano-dimer axis\textsuperscript{52}. The highest SERS enhancements were later observed experimentally for small junctions of linked AgNPs (< 5 nm) with the analyte molecule located in this hot spot and a polarization along the AgNP-dimer axis\textsuperscript{51}-\textsuperscript{53}.

\textit{Sample preparation:}

\textbf{AgNP Colloid:} AgNP colloid was transferred into a clean 2 mL quartz cuvette with plastic plug. A Kim wipe was used to clean off finger prints, smudges or colloid from the surface of the cuvette prior to placing the cuvette on the microscope stage.
**Viral samples and controls:** Three sample spots were applied to an autoclaved glass microscope slide for micro-Raman analysis: 1) 100-kD colloidal AgNP suspension (1000 mg mL\(^{-1}\); AgNP alone), 2) vaccinia virus (10\(^{12}\) PFU; VV alone) and 3) vaccinia virus that was incubated with 1000 mg mL\(^{-1}\) of AgNP for 1 hour at 37\(^\circ\)C, washed and filtered through a 100 nm filter to remove any unbound AgNP (AgNPs & VV). Both virus samples were deactivated with formaldehyde prior to application. Figure 13 illustrates the sample slid configuration.

![Figure 13: Microscope slide preparation for virus samples.](image)

**Instrumental analysis:**

A LabRamHR 800 Raman system (Horiba Jobin Yvon, Inc.) (Figure 14) equipped an Olympus BX41 confocal Raman microscope) and a holographic grating of 600 grooves/mm was employed for the both the AgNP colloid purity analysis and virus sample analysis.
**Parameters for AgNP colloid purity:** Laser focusing of the AgNP colloid was conducted in video mode using the 50x objective lens. Directly observation of the focal point and confocal cone of the laser in the colloidal suspension facilitated the focusing procedure. A He-Ne laser (632.8 nm) with approximately 17 mW of laser power at the sample was employed as the excitation source. The confocal hole was set at 300 μm. Micro-Raman spectra were collected over a 100 – 4000 cm$^{-1}$ acquisition window using the 50x long working distance air objective lens. An exposure time of 30 s with 5 accumulation cycles was utilized. A CCD detector (1024 x 526 pixels) with a spectral resolution of 1 cm$^{-1}$ was employed for spectral data collection. The micro-Raman spectra were plotted and analyzed using Origin 8 software.

**Parameters for Virus Sample Analysis:** Laser focusing of the virus samples and controls were conducted in video mode using the 100x objective lens. A He-Ne laser (632.8 nm) was employed as the excitation source with a confocal hole set at 300 μm. A D1 filter was utilized to attenuate the laser power by a factor of 10 resulting approximately 1.7 mW of laser power at the sample. Micro-Raman maps using a 1 μm step size were collected over a 100 – 2000 cm$^{-1}$ acquisition window using the 100x objective lens. An exposure time of 4 s with 3 accumulation cycles was utilized. All multivariate analysis techniques were executed using MatLab v. 7.11.0.584 (R2010b) with in-house written codes. Origin 8 software was used to plot all represented spectra.
Hyperspectroscopy and Multivariate Analysis Methods

**Working principle:**

Given the complex and heterogeneous morphology of biological samples, advanced spectroscopic techniques may be needed to accurately access the molecular fingerprint of the sample. For example, hyperspectroscopy combines vibrational spectroscopy techniques such as Raman spectroscopy with movable microscope stages to facilitate the evaluation of the molecular composition over a selected, 2D space of the sample. Traditional vibrational spectroscopy typically allows for the fast acquisition of point spectra from a specific location on the sample and requires a minimal analysis time. The data obtained in the form of a 2D plot is easily evaluated with the help of univariate analysis techniques and provides information about molecular composition, structure and interactions.
within a sample. Given the simplicity of the analysis, vibrational spectroscopy is a preferred analysis method for homogeneous samples in many scientific disciplines such as chemistry and biology. In contrast, hyperspectroscopy allows for the collection of multiple sample measurements using mapping techniques with a movable microscope stage on which the sample is placed. The resulting data is compiled into a 3D hyperspectral dataset that can be examined using both univariate or multivariate analysis techniques. This provides information about the molecular composition and morphology of the sample. The wealth of information that can be gleaned from this type of analysis makes hyperspectroscopy very suitable for heterogeneous samples. However, extensive acquisition and analysis time may be required.

Hyperspectral datasets are 3D matrices consisting of a 2D spatial mapping surface, which is typically represented with x and y coordinates, and a series of wavelengths constituting the third dimension called spectral axis (Figure 15).
Figure 15: Hyperspectral dataset representations. The images in the left and right columns represent the spatial orientation of the data selection and the corresponding spectral image taken from hyperspectral data obtained from micro-Raman analysis of the cross section of chick embryo bones. A) Spatial selection is equivalent to point spectrum measurements; B) Several point spectrum measurements corresponding spatially to a data obtained from a linear section across the sample surface; C) An RGB image obtained from the analysis of the signal response obtained at that specific wavenumber from the spectral axis for all 2D points on the sample surface.  

The representative 2D data image or spectra obtained is determined by the location of the selected data within the 3D hyperspectral array. If a single
spectrum was desired (Figure 15a), the spatial selection would be equivalent to a point measurement in vibrational spectroscopy. Evaluating a series of points scanned on the surface (a line in the 2D plane) across the entire spectral axis would yield multiple point spectra within the same plot (Figure 15b). Lastly, a color map (Figure 15c) can be created using RGB imaging techniques to indicate “hot spots” or points of high intensity for a specific vibrational mode\textsuperscript{38}. This can be achieved by analyzing the signal response obtained at that specific wavenumber from the spectral axis for all 2D points on the sample surface.

Given the complex nature of a hyperspectral dataset, well-developed data analysis protocols are recommended. The first step in the process is the elimination of any anomalous cosmic ray features or spikes followed by the application of a baseline correction or normalization algorithm for the reduction or elimination of noise related spectral components and fluorescence\textsuperscript{38}. After preprocessing, a reduction of the data set may be needed to eliminate spectra attributed to noise related features or non-sample spectra. Principal component analysis (PCA) is a data reduction algorithm that is commonly employed in such situations (to be discussed later)\textsuperscript{38}. The actual analysis of the hyperspectral dataset depends on the desired result. Univariate analysis techniques such as single peak area integration offer relatively rapid analysis of the complex dataset\textsuperscript{55}. However, more complex algorithms termed multivariate analysis methods are needed if a comprehensive look at the morphological characteristics of the sample is desired\textsuperscript{54,38}. 
The underlying premise of multivariate analysis techniques is that the hyperspectral dataset will contain a fixed number of spectral features called “endmembers”. These endmembers will account for the majority of the spectral variance within the data set\(^{36,56}\). The multivariate algorithms determine the fraction of the data set that each endmember accounts for, the so called fractional abundances\(^{36}\). When these fractional abundances account for most of the variance of the spectral data set (usually 95\%) then the entire spectral data set can be represented by these endmember spectra or a linear combination of these spectra and noise related features\(^{54,36}\). The noise contribution to the cumulative variance of the dataset is in general smaller than 1\%\(^{36,54}\). A good example of endmember representation is provided by De Juan \textit{et al} (2004)\(^{37}\). Briefly, they performed an analysis of five pharmaceutical blend samples with known, varying amounts of an excipient ingredient and an active pharmaceutical ingredient for proof of concept (API). The first sample was prepared with 100\% excipient and no API, the next four samples contained 80\% excipient, 20\% API; 60\% excipient, 40\% API; 40\% excipient, 60\% API and finally 20\% excipient, 80\% API\(^{37}\). The PCA multivariate analysis method was used to extract the representative endmembers, termed principal components (PC), from the hyperspectral datasets of each sample (Table 1).
Table 1: Percent cumulative variance determined by increasing PCA model size\textsuperscript{37}.

<table>
<thead>
<tr>
<th>Number of Principal Components</th>
<th>% of active pharmaceutical ingredient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>96.33</td>
</tr>
<tr>
<td>2</td>
<td>96.9</td>
</tr>
<tr>
<td>3</td>
<td>97.08</td>
</tr>
<tr>
<td>4</td>
<td>97.21</td>
</tr>
<tr>
<td>5</td>
<td>97.25</td>
</tr>
</tbody>
</table>

For the first sample, 96.33% of the variance of the dataset was accounted for with one PC or the excipient. As previously stated, all the remaining PCs added less than 1% to the cumulative variance and therefore are considered noise-related features\textsuperscript{37}. Two PCs were necessary for the second sample analysis, namely excipient and API and accounted for 95.26% of the variance. With samples three through five, a third PC was needed to account for the majority of the variance, which the authors attributed to an impurity component\textsuperscript{37}. The identified PC(s) correlated very well with the composition of the five samples. Overall, this study demonstrates that PCA could be successfully used for the identification of spectral features of individual components of a complex sample.

The PCA technique is a relatively popular multivariate method because of its mathematical simplicity. PCA is an unsupervised method that identifies spectral patterns or similarities within a dataset called PCs\textsuperscript{37}. PCA works well in differentiating noise related spectra and can help reduce the dimensionality of the
dataset if needed even when a different multivariate technique is ultimately desired for the analysis\textsuperscript{54}. As a principal analysis technique, PCA works best with data that has some degree of reproducibility. Mathematically, the PCs are eigenvectors calculated from the covariance of the data matrix. The first PC is the eigenvector with the highest eigenvalue and will explain the highest percentage of variance in the dataset\textsuperscript{37}. The second PC accounts for the next highest percentage of the residual variance (the eigenvector with the second highest eigenvalue) and is orthogonal to the first eigenvector and so forth (Figure 16)\textsuperscript{37}.

![Figure 16: Geometric representation of PCs\textsuperscript{54}.](image)

Finally, all spectra in the original data set are projected onto the PC basis set and expressed as linear combinations of the PCs. The individual spectra are then
given a “score” indicating how well the given spectrum correlates with the PC\textsuperscript{54}. These score projections are useful for grouping spectra according to their correlation to the PCs and provide a useful tool for differentiating samples based on their spectral similarities\textsuperscript{57}.

Another common multivariate technique used for unsupervised datasets is vector component analysis (VCA). Like PCA, VCA reduces the data set to a finite number of endmembers that are a linear representation of the dataset\textsuperscript{56}. Unlike PCA, VCA selects endmembers that are “pure component spectra” and are considered outliers of the hyperspectral dataset\textsuperscript{56}. Each pure component spectrum is assigned a different vector\textsuperscript{56}. The combination of these vectors forms a simplex with the most extreme spectra defining the edges of the simplex boundaries\textsuperscript{56}. Each spectrum within the dataset is then defined as an orthogonal projection onto the set of endmembers\textsuperscript{56}. Since the mathematical basis of VCA focuses on spectral differences, the representative set of VCA endmembers should define the most significant spectral features of the hyperspectral dataset\textsuperscript{39}. This makes VCA a useful multivariate technique for complex biological samples, which in general exhibit a large number of unique spectral differences within the spectral dataset.

Miljkovic \textit{et.al} (2010) applied the VCA algorithm to several HeLa cell hyperspectral Raman datasets and imaged the cells using a finite number of endmembers to obtain an RGB image of the sampled cells. The algorithm was able to identify unique biological components of the cells including mitochondrial
tubules, nuclei, nucleoli, phospholipid inclusions, and the cytoplasm. These label-free Raman images (see reference) demonstrated a high degree of correlation when compared to the images produced by traditional fluorescent imaging techniques. Given that the VCA images displayed several cellular components at the same time, a huge advantage was achieved over the fluorescence techniques, which are normally constrained to one biological component. Considering the biochemically diverse nature of different cellular components, this multivariate method proved successful in isolating unique spectral characteristics of the cellular matrix.

**Instrumental Analysis:**

**Pre-processing**

Combined representative hyperspectral data sets were created in MatLab v. 7.11.0.584 (R2010b) for the VV-AgNP and control samples (AgNP alone and VV alone). Prior to the application of any multivariate algorithms, all spectra within the dataset were normalized according to the minimum and maximum intensity values present in the individual spectra. First, the lowest negative intensity value of the spectrum was subtracted from all points within the spectrum to normalize the baseline to zero. If no negative values were observed within the spectrum, the step was not performed. Next, all points within the spectrum were divided by the maximum intensity value within the spectrum to create a spectral
dataset where all spectral points lied between zero and one. This process corrected for baseline anomalies or spectral artifacts.

For the VV-AgNP sample, a signal-to-noise threshold algorithm was used to select representative spectra from the dataset that exhibited 30% or greater of the maximum baseline corrected relative peak intensities. The following selected bands of interest were used for this screening process: 450-550 cm\(^{-1}\), 550-650 cm\(^{-1}\), 650-750 cm\(^{-1}\), 750-850 cm\(^{-1}\), 850-950 cm\(^{-1}\), 950-1050 cm\(^{-1}\), 1000-1100 cm\(^{-1}\), 1100-1200 cm\(^{-1}\), 1200-1300 cm\(^{-1}\), 1300-1400 cm\(^{-1}\), 1400-1500 cm\(^{-1}\), 1600-1700 cm\(^{-1}\) and 1800-1900 cm\(^{-1}\). Most of the spectral features characteristic to the VV-AgNP interaction fell within the above spectral ranges. After selection, each spectrum was eliminated from the larger hyperspectral data set to avoid the selection of duplicate spectra. Finally, the compiled selected spectra were examined individually to eliminate any anomalous spectra that were not previously discarded in the preprocessing protocol.

**Multivariate Data Analysis:**

**Vaccinia virus control:** No multivariate data analysis techniques were applied to the vaccinia virus control dataset.

**AgNP control:** PCA was applied to the normalized hyperspectral dataset. Any PC that contributed more than 1% to the cumulative variance of the dataset was
retained, while any PC that added less than 1% was considered a noise-related component and was discarded.

**Vaccinia virus-AgNP sample:** VCA was utilized to select 50 representative endmember (EM) SERS spectra. This number of EMs \( N = 50 \) was chosen to provide a large enough set of representative SERS spectra that would comprise all unique spectral features. At the same time, this number of EMs would not contain an overwhelming amount of repetitive SERS spectra. Origin 8 software was used to plot all EM spectra and to assign the SERS peaks. A pairwise correlation coefficient algorithm was then employed to quantify the spectral profile and the degree of similarity in each of the 50 EMs with all other representative endmembers. The correlation threshold values were 95%, 90%, 85%, 80% and 75%. After correlation, six representative SERS spectra were selected by examining individually each of the 50 EMs. These six EMs accounted for the most unique spectral features and corresponded to a threshold value of 85% \( (i.e., \text{they demonstrated the lowest correlation in the pairwise comparison}) \). At higher correlation values \( (i.e., 90-95\%) \), too few representative SERS spectra were selected and some unique vibrational modes were lost, whereas at lower correlation values \( (i.e., 75 – 80\%) \), too many repetitive SERS spectra were included. The correlation algorithm was then employed a second time to determine the spectral similarity of these representative EMs to the entire compiled hyperspectral dataset using the same correlation thresholds.
Results and Discussion

Specific Aim 1:

Synthesis of silver nanoparticles

Five liters of AgNP colloid were successfully synthesized using a modified Creighton method\(^\text{29}\). For the reaction, 50 mL of AgNO\(_3\) (0.0516 g of AgNO\(_3\) in 300 mL of autoclaved water) was reacted with 300 mL of NaBH\(_4\) (0.0463 g of NaBH\(_4\) in 600 mL autoclaved water). The resulting colloidal suspension contained 5.06 x 10\(^{-5}\) moles of Ag\(^+\) equating to 5.46x10\(^{-3}\) g of Ag\(^+\) in a total reaction volume of 350 mL. The theoretical yield was 15.6 µg mL\(^{-1}\) of silver.

Characterization of silver nanoparticles

UV-Vis absorption spectrophotometry

UV-Vis absorption spectrophotometry was used to estimate the approximate shape, average size, size distribution, aggregation state, and concentration of the colloidal AgNPs. The estimates can be made using the surface plasmon resonance peak of the colloidal AgNPs. AgNPs have special optical properties that are associated with the lone s electrons (surface plasmons) present in silver metal atoms\(^\text{29}\) of a [Kr]5s\(^1\)4d\(^{10}\) electron configuration. When exposed to incident laser light, the surface plasmons are polarized by the electromagnetic field of the laser light (i.e., the s electrons will move away from
the nucleus in the direction of the electrical field). Inevitably, the surface plasmons will experience an electrostatic attractive force back towards the nucleus of the silver atom. It is these collective oscillations of the electrons (surface plasmon resonance) that gives rise to the characteristic yellow color of the colloid and the surface plasmon resonance (SPR) maximum at approximately 400 nm.

The approximate shape, average size and aggregation state of the Creighton AgNPs can be verified by the number of absorption bands present and the \( \lambda_{\text{max}} \) of the SPR peaks. The typical UV-Vis absorption spectrum for single element spherical nanostructures (\( i.e., \) having an aspect ratio of 1:1) exhibits one SPR peak. Nanorods, which are anisotropic in shape have differing dimensions for the transverse and longitudinal axes, will absorb UV-Vis radiation at two separate \( \lambda_{\text{max}} \) values. Mohapatra et.al (2010) demonstrated this principal with the UV-Vis absorption spectrum of silver nanorods that contained two distinct peaks at 420 and 582 nm corresponding to the transverse and longitudinal SPR bands, respectively. Furthermore, if the Creighton AgNPs form larger aggregates, the SPR peak broadens and red-shifts to higher wavelengths. Likewise, a blue-shift would be indicative of a NP suspension with particles of smaller sizes. Mie theory (eq. 3) was used to quantify this wavelength/particle size-dependence and the average diameter of the Creighton AgNPs.
\[ D = \frac{\left(\lambda_{\text{max}}^2 V_f\right)}{\pi c \omega} \]  

where \( \lambda_{\text{max}} \) is the maximum wavelength value (\( \lambda_{\text{max}} \)), \( \omega \) represents the full width at half maximum (FWHM) of the SPR peak, \( c \) refers to the velocity of light (2.998 \( \times 10^8 \) m s\(^{-1} \)), and \( V_f \) denotes the Fermi level electron velocity of silver (1.4 \( \times 10^6 \) m s\(^{-1} \))\(^{60} \). The estimated average particle size for the Creighton AgNP suspension depicted above (\( \lambda_{\text{max}} = 394 \) nm and a FWHM of 52.4 nm) was determined to be 4.4 nm. TEM analysis (discussed later) revealed the actual average particle size for the AgNP colloid was 28.1 nm.

The SPR peak obtained for the Creighton AgNPs through UV-Vis spectrophotometry is pictured in Figure 17. The sharp, symmetrical nature of this peak with a \( \lambda_{\text{max}} \) at 394 nm is indicative of small (average diameter of 10-20 nm), spherical and moderately aggregated AgNPs.

![Figure 17: UV-Vis absorption spectrum of the colloidal AgNPs\(^{30} \).](image)
Using Lambert Beer’s law (eq. 2)

\[ A = \varepsilon bc \]  

the average silver concentration of the colloid was estimated at 9.47 \( \mu g \) mL\(^{-1}\) where \( \varepsilon = 1.9 \times 10^4 \) dm\(^3\) mol\(^{-1}\) cm\(^{-1}\) (for AgNP clusters with a \( \lambda_{max} = 400 \) nm)\(^{43}\), \( A = 0.18 \) (Figure 17) and \( b = 1 \) cm. The actual silver yield obtained through ICP-OES (discussed below) was 14.3 and 15.2 \( \mu g \) mL\(^{-1}\) for two representative batches. These values are more accurate (i.e., close to the theoretical yield of 15.6 \( \mu g \) mL\(^{-1}\)).

In general, the shape, average size, size distribution, aggregation state and concentration of AgNPs obtained from the SPR absorption peak are estimations at best and more sophisticated techniques such as TEM and ICP-OES are necessary for the accurate determination of these parameters. However, UV-VIS absorption spectrophotometry demonstrated to be a valuable, inexpensive and time efficient screening tool that facilitated the selection of “acceptable” colloidal batches of AgNPs for further quantification and use in biological applications.
Tangential flow ultrafiltration (TFU)

The size selection and concentration of Creighton AgNPs was accomplished in three steps. The schematic depicted in Figure 18 demonstrates the steps involved in the TFU process, the filter modules employed and the colloidal suspensions retained in each step of the process. In the first step, five liters of colloid was filtered through a 50 nm MidiKros® polysulfone (200 cm²) module resulting in a volume reduction of approximately 100 mL. In the second step, the remaining 4.9 L of 50 nm filtrate was then processed using the 100-kD MidiKros® filter (200 cm²). The retentate volume of 50 mL was saved and resulted in a 98-fold volume reduction. In the last step, the retentate from step two (100-kD retentate) was filtered through a 100-kD MicroKros® polysulfone module (20 cm²). The final 100-kD retentate volume was 4 mL, which corresponded to an additional 12.5-fold volume reduction.

Overall, a 1,225-fold volume reduction was achieved through the three-step TFU process. Since the volume reductions are dependent upon the initial volume of Creighton colloid and the surface area of the filtration modules, the TFU process can be modified to obtain the desired concentration and volume of final retentate of AgNPs. In this study, the two volume reduction steps resulted in retentate volumes of approximately 50 mL (100-kD module of 200 cm²) and 4 mL (100-kD module of 20 cm²), respectively. If a smaller volume of colloid is processed, the initial volume reduction would be smaller (i.e., the initial volume reduction would be approximately 60-fold for 3 L of colloid). Likewise, if filtration
modules with smaller surface areas are chosen, the retentate volumes would decrease (i.e., larger volume reductions) and the final concentration of AgNPs would increase.

Figure 18: Schematic illustrating the three-step TFU process. The green-shaded boxes indicate the AgNP colloidal samples retained for analysis. Vial photographs show A) Original colloid, B) 50 nm filtrate collected after processing the original colloid through the through the 50 nm filter, C) 100-kD retentate obtained after the first volume reduction using the 100-kD MidiKros filter (200 cm$^2$) and D) 100-kD final retentate resulting from the second volume reduction using the 100-kD MicroKros filter (20 cm$^2$).
The drastic change in color from light yellow for the original Creighton colloid to a very dark brown for the final 100-kD retentate (Figure 18) is a clear indication of the large degree of concentration associated with the extreme volume reduction (from 5 L down to 4 mL of colloidal suspension) of the three-step TFU process.

**Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES)**

ICP-OES was used to quantify the silver amount in the original Creighton AgNP colloid and the 100-kD final retentate. A linear calibration curve was constructed from eight silver standards (0, 3, 7, 10, 15, 25, 50, and 100 μg L\(^{-1}\)) prepared in a 2% nitric acid (Figure 19). The correlation coefficient of the calibration curve (R\(^2\) = 0.9999) validated the curve linearity over a wide range of silver concentrations.

![ICP-OES calibration curve](image)

*Figure 19: ICP-OES calibration curve prepared from eight Ag standards (0, 3, 7, 10, 15, 25, 50, and 100 μg L\(^{-1}\)).*
The silver amount in each of the digested colloidal suspensions (i.e., samples A, C and D from Figure 18) was extrapolated from the linear ICP-OES calibration curve. Table 2 lists the silver amounts that were obtained for two independent Creighton colloid batches.

Table 2: Silver amounts determined by ICP-OES for the representative TFU samples of two independent batches: A) Original colloid, B) Initial 100-kD retentate, and C) Final 100-kD retentate.

<table>
<thead>
<tr>
<th>Batch 1</th>
<th>Volume of Suspension (mL)</th>
<th>Silver amount (µg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original Colloid (A)</td>
<td>5,000</td>
<td>14.3</td>
</tr>
<tr>
<td>100-kD concentrate (B)</td>
<td>50</td>
<td>1,020</td>
</tr>
<tr>
<td>100-kD concentrate (C)</td>
<td>4</td>
<td>10,400</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Batch 2²⁰</th>
<th>Volume of Suspension (mL)</th>
<th>Silver amount (µg mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Original Colloid (A)</td>
<td>4,000</td>
<td>15.2</td>
</tr>
<tr>
<td>100-kD concentrate (B)</td>
<td>50</td>
<td>683.1</td>
</tr>
<tr>
<td>100-kD concentrate (C)</td>
<td>4</td>
<td>8,540</td>
</tr>
</tbody>
</table>

The percent yield for the Creighton reaction was estimated to be 91.6% and 97.4% for the two colloid batches. The percent reaction yield was estimated as the ratio of the actual yield to theoretical yield as indicated below:
\[ \% \text{Reaction yield} = \frac{14.3 \text{ } \mu g \text{ } mL^{-1}}{15.6 \text{ } \mu g \text{ } mL^{-1}} \times 100\% = 91.6\% \] (4)

In the first step of the TFU process, an original Creighton volume of 5 L (14.3 \text{ } \mu g \text{ } mL^{-1}) was concentrated into 50.0 mL of colloidal suspension (1,020 \text{ } \mu g \text{ } mL^{-1}). This corresponds to a concentration factor and a percent recovery of approximately 71-fold and 71.0\% recovery of AgNPs, respectively.

\[ \text{Concentration factor} = \frac{10,020 \text{ } \mu g \text{ } mL^{-1}}{14.3 \text{ } \mu g \text{ } mL^{-1}} = 71.3 \] (5)

\[ \% \text{Recovery} = \frac{10,020 \text{ } \mu g \text{ } mL^{-1} \times 50 \text{ } mL}{14.3 \text{ } \mu g \text{ } mL^{-1} \times 5000 \text{ } mL} \times 100 = 71.3 \% \] (6)

During the second step of the TFU process, 82.2\% of the AgNPs were retained with a concentration factor of approximately 10-fold.

\[ \text{Concentration factor} = \frac{10,400 \text{ } \mu g \text{ } mL^{-1}}{1020 \text{ } \mu g \text{ } mL^{-1}} = 10.2 \] (7)

\[ \% \text{Recovery} = \frac{10,437.3 \text{ } \mu g \text{ } mL^{-1} \times 4 \text{ } mL}{1015.4 \text{ } \mu g \text{ } mL^{-1} \times 50 \text{ } mL} \times 100 = 81.6 \% \] (8)

For the entire TFU process, an approximate 730-fold concentration resulted in a 58.2\% recovery.

\[ \text{Concentration factor} = \frac{10,400 \text{ } \mu g \text{ } mL^{-1}}{14.3 \text{ } \mu g \text{ } mL^{-1}} = 727.3 \] (9)

\[ \% \text{Recovery} = \frac{10,400 \text{ } \mu g \text{ } mL^{-1} \times 4 \text{ } mL}{14.3 \text{ } \mu g \text{ } mL^{-1} \times 5000 \text{ } mL} \times 100 = 58.2 \% \] (10)
Similar results were observed for the total TFU process for batch two: an approximate 560-fold concentration and a 56.2% recovery.

\[
\text{Concentration factor} = \frac{8.540 \, \mu g \, mL^{-1}}{15.2 \, \mu g \, mL^{-1}} = 561.8
\]  

\[
\% \, \text{Recovery} = \frac{8.540 \, \mu g \, mL^{-1} \times 4 \, mL}{15.2 \, \mu g \, mL^{-1} \times 4000 \, mL} \times 100 = 56.2 \%
\]

The remaining 40% of AgNPs that were not retained through the TFU process could be attributed to larger AgNPs and AgNP-aggregates removed through the 50 nm filtration step. AgNPs of smaller diameters that were not recovered through the successive steps of the TFU process might also contribute to this percent loss.

Overall, TFU proved to be a relatively rapid (6 hours) and inexpensive concentration method for AgNPs (from 15.2 \( \mu g \, mL^{-1} \) of silver in the original colloid to 10,400 \( \mu g \, mL^{-1} \) in the final 100-kD retentate). In this process, most of the excess reagents and synthesis byproducts were eliminated together with the water without the use of additional solvent. Therefore, TFU may be successfully utilized for the “green” manipulation of nanoparticles in preparation for nanotoxicity or SERS-based sensing studies.
Transmission electron microscopy (TEM)

The TEM images, size histograms and data presented in this section are part of a study that has been accepted for publication in the Journal of Visualized Experiments (JoVE)\textsuperscript{30}. TEM micrographs and size histograms of the original Creighton colloid and the final 100-kD retentate of AgNPs are depicted in Figure 20.

Figure 20: TEM micrographs and TEM size distribution histograms of original Creighton AgNP colloid (A and B) and final 100 kD AgNP colloidal suspension (C and D). The inset on the original colloid histogram (B) represents an expanded view of the 41-75 nm size range for comparison. The size bar is 100 nm for the TEM micrographs\textsuperscript{30}.
Visual inspection of the TEM micrographs and TEM size histogram of the original Creighton colloid revealed a polydispersed colloidal suspension containing AgNPs and AgNP-aggregates larger than 50 nm in diameter (approximately 1.0% of the total number of AgNPs) and a size distribution of 1-75 nm. In comparison, the final 100-kD retentate contained no AgNPs and AgNP-aggregates of 50 nm and larger, and had a much narrower size distribution. It consisted mostly of AgNPs of 1-20 nm in diameter having only 12.4% of the AgNPs present in the 21-40 nm size range. The decrease in the number of AgNPs in the 1-5 nm size bins was also significant, namely from 33.2% for the original Creighton colloid down to 21.3% for the final 100-kD suspension. Consequently, the average AgNP diameter increased from 9.3 nm for the original Creighton colloid to 11.1 nm for the final 100-kD colloidal suspension.

Overall, the TEM results demonstrated that the three-step TFU process was extremely effective in the size selection of AgNPs of 1-25 nm in diameter. This isolation procedure can be tuned to further size select or narrow the size distribution range of AgNPs by using different combinations of filtration modules (pore sizes from 1,000-kD down to 10-kD and surfaces areas from 370 cm² down to 5 cm²). TFU eliminates the need of using cappingfunctionalizing agents to better control the size and aggregation state of nanoparticles during their synthesis.
Elemental analysis characterization using SEM-EDX

Figure 21 shows three SEM images that were collected from the final 100-kD retentate of colloidal AgNPs deposited onto a carbon stub.

![SEM Images](Image)

**Figure 21**: SEM-EDX results for 100-kD retentate of AgNPs. A, B, and C represent the SEM images with an acceleration voltage of 10 kV for A and B and 15kV for C. D depicts the EDX spectrum corresponding to image C.

It is evident from the SEM images that nanoparticles in the 0-50 nm size range are not readily distinguishable at either magnification level. The images most likely reflect the presence of large aggregates formed during the sample preparation process. Four distinct peaks were observed in the EDX spectrum (Figure 21D) between 2.5 and 3.5 keV. These peaks are characteristic of the
binding energies associated with the Ag$_{L\alpha}$, Ag$_{La}$, Ag$_{Lb}$ and Ag$_{Lg}^{61-62}$. Two other significant peaks were also present in the spectrum, namely C$_{Ka}$ (0.25 keV), S$_{Ka}$ (2.3 keV) and O$_{Ka}$ (0.57 keV). The C$_{Ka}$ peak was attributed to the carbon mounting stub, whereas the sulfur and the trace amount of oxygen detected may be residual contamination from the polysulfone filtration module used in the TFU process. Table 3 showed that the 100 kD retentate sample of AgNPs contained mostly silver (60.1%) and smaller amounts of carbon and sulfur (29.6% and 8.7%, respectively). The weight ratio of silver to sulfur was found to be 6.9 : 1.0.

Table 3: Percentage weight of elements present in the final 100-kD retentate of AgNPs as revealed by EDX analysis.

<table>
<thead>
<tr>
<th>Element</th>
<th>Wt.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C$_{Ka}$</td>
<td>29.6</td>
</tr>
<tr>
<td>O$_{Ka}$</td>
<td>3.0</td>
</tr>
<tr>
<td>S$_{Ka}$</td>
<td>8.7</td>
</tr>
<tr>
<td>Ag$_{L}$</td>
<td>60.1</td>
</tr>
</tbody>
</table>

The SEM-EDX results confirmed that the TFU process produced a final 100-kD retentate comprising of primarily silver with a minimal amount of sulfur contamination resulting from the TFU process.
**Micro-Raman Spectroscopy**

**Original Creighton colloid**

The purity of the Creighton colloid was verified through micro-Raman spectroscopy (Figure 22).

![Raman spectrum of AgNP colloid](image)

**Figure 22: Raman spectrum of AgNP colloid.**

Only two Raman peaks characteristic to the bending (1640 cm\(^{-1}\)) and stretching (3240-3420 cm\(^{-1}\)) vibrational modes of H\(_2\)O were observed in the Raman spectra, which shows that the AgNP colloid is free of organic contaminants.
**100-kD final retentate (1000 µg mL⁻¹)**

One hundred micro-Raman point spectra were collected on the 100-kD retentate of AgNPs. Visual inspection of the individual spectra revealed a high degree of spectral reproducibility and similarity. Because of the similarities in these Raman spectra, PCA was conducted to determine the most representative spectra. Three PCs were identified and accounted for 99.6% of the cumulative variance of the hyperspectral dataset (Figure 23).

![Micro-Raman spectrum](image)

**Figure 23:** Micro-Raman spectrum containing the three principal components accounting for 99.6% of the variance in the hyperspectral dataset (N = 100 spectra).

The largest portion of the variance was attributed to PC1 (90.6%). The PC2 and PC3 contributions to the cumulative variance were much smaller (4.8%...
and 4.2%, respectively). All other PCs contributed less than 1% to the cumulative variance of the dataset and were discarded as noise-related components. All PC Raman spectra contain a weak, broad band at \( \approx 220 \text{ cm}^{-1} \) probably due to an Ag-S or Ag-O stretching mode\(^{63,64}\). This result is consistent with the SEM-EDX finding of a minimal amount of sulfur present in the 100-kD final retentate. The broad peak in between 1200 and 1700 cm\(^{-1}\) is characteristic to amorphous carbon\(^{53}\) that is associated with the decomposition of organic matter under the laser light (e.g., polysulfone membrane residues or organic impurities from air). The micro-Raman spectra of PC1 and PC3 exhibited an additional weak feature at \( \approx 993 \text{ cm}^{-1} \), which may be attributed to a ring breathing mode\(^{33}\) of the ultrafiltration membrane residues.

Micro-Raman spectra confirmed the purity of the original Creighton colloid. A single contaminant band was observed in the control micro-Raman spectra of the predominant PC of the final 100-kD retentate probably coming from TFU residues. This is extremely encouraging because the SERS spectra collected on the VV incubated with AgNPs from 100-kD concentrate will exhibit minimum interferences from the AgNPs alone.

Overall, the modified Creighton method for the synthesis of AgNP proved to be an effective technique for the fabrication of non-functionalized small, spherical, polydispersed AgNPs. Furthermore, large volumes can be synthesized that will remain stable for up to six months with refrigeration. The TFU process provides an efficient means by which to limit the polydispersity, size select to a
narrow distribution and concentrate AgNP suspensions without the addition of aggressive solvent systems or capping agents. The final concentration ranges can be tailored somewhat by varying the volume of the original Creighton AgNP processed through the first step of the TFU process. In general however extremely concentrated 100-kD suspensions from the final TFU step have a relatively short shelf-life (<2 weeks) due to the large number of particles present in a very small volume. This requires diligent preparation and careful project planning in order to maximize the effective use of the AgNP suspension before aggregation occurs.

**Specific Aim 2:**

**Vaccinia Virus Control**

**Background information about Vaccinia Virus (VV)**

VV is an enveloped virus that contains a dsDNA genome approximately 200 kb long. The general shape of a mature virion is described as ellipsoidal with dimensions of 360 nm x 270 nm x 250 nm.

Mature virions (MV), the simplest form of VV, consist of a lipid bilayer external membrane that surrounds the lateral bodies and border the biconcave core containing the double-stranded genomic DNA. Figure 24 is a pictorial representation of a mature VV.
Figure 24: Pictorial representation of the vaccinia virus.

The external membrane of the VV houses approximately 25-30 different proteins\textsuperscript{11}. Nine of these proteins, namely A21, A28, G3, H2, L5, A16, G9, J5 and O3 have been identified as being a part of a well-organized and stable formation of proteins termed the entry-fusion complex (EFC)\textsuperscript{35,66}. Three other EFC associated proteins have been identified, specifically F9, I2 and L1, but they do not appear to be integral to the formation or stabilization of the EFC\textsuperscript{67}. F9 and I2, in particular, assume many of the key functions of the EFC but efforts to verify the proteins’ integral part of the complex remain inconclusive\textsuperscript{68-69}. Three of the EFC proteins (A16, G9 and J5) have similar amino acid (AA) sequences.
including a C-terminus trans-membrane domain\textsuperscript{35}, numerous invariant cysteine groups\textsuperscript{11} and disulfide bonds (4-10)\textsuperscript{35}. The A16 and the G9 protein in this group are also myristoylated proteins, \textit{i.e.}, a myristic acid is bound to the glycine terminus of the protein (Figure 25)\textsuperscript{11}.

![Myristic Acid](image)

**Figure 25:** Myristoylated terminal moiety of a protein.

In contrast, the other six proteins (A21, A28, G3, H2, L5 and O3) are heterologous with N-terminus trans-membrane domains, contain fewer invariant cysteine groups or disulfide bonds (0-2)\textsuperscript{35,66} and do not present myristoylated moieties\textsuperscript{11}. Table 4 summarizes several of the most significant structural differences of the nine EFC proteins in terms of the presence of myristoylated glycine AA N-terminus residues, trans-membrane terminus AAs (AAs), the number of cysteine groups, the intramolecular disulfide bonds and the number of aromatic AAs. These structural differences were emphasized in this study because of their high abundance of electronegative atoms (\textit{e.g.}, O, N, and S) and the possible interaction with electron withdrawing AgNPs.
Table 4: Structural differences in the nine EFC proteins relevant to the interaction mechanism with AgNPs. The total number of AAs in the sequence of each EFC protein is given in parenthesis.

<table>
<thead>
<tr>
<th>Protein (# AAs)</th>
<th>Terminus Atom</th>
<th># of Cysteine Groups</th>
<th># of Disulfide Bonds</th>
<th># of Aromatic Amino acids</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A21 (117)</td>
<td>N</td>
<td>5</td>
<td>2</td>
<td>20</td>
<td>70</td>
</tr>
<tr>
<td>A28 (146)</td>
<td>N</td>
<td>5</td>
<td>2</td>
<td>25</td>
<td>71</td>
</tr>
<tr>
<td>G3 (111)</td>
<td>N</td>
<td>1</td>
<td>0</td>
<td>18</td>
<td>72</td>
</tr>
<tr>
<td>H2 (378)</td>
<td>N</td>
<td>5</td>
<td>2</td>
<td>28</td>
<td>73</td>
</tr>
<tr>
<td>L5 (133)</td>
<td>N</td>
<td>2</td>
<td>1</td>
<td>27</td>
<td>74</td>
</tr>
<tr>
<td>O3 (35)</td>
<td>N</td>
<td>1</td>
<td>0</td>
<td>9</td>
<td>66</td>
</tr>
<tr>
<td>A16 * (378)</td>
<td>C</td>
<td>20</td>
<td>8-10</td>
<td>77</td>
<td>75-76</td>
</tr>
<tr>
<td>G9 * (340)</td>
<td>C</td>
<td>14</td>
<td>4-7</td>
<td>54</td>
<td>35, 76-77</td>
</tr>
<tr>
<td>J5 (133)</td>
<td>C</td>
<td>8</td>
<td>4</td>
<td>18</td>
<td>35</td>
</tr>
</tbody>
</table>

* denotes the presence of a myristoylated glycine residue.

All of the EFC proteins are integral membrane proteins, which may transverse the external membrane of the virus at least once if not multiple times.

The AA constituents of the proteins that are located externally to the membrane

---

67
will be the first to encounter a host cell and likewise the first to interact with AgNPs. A comprehensive look at these AAs is summarized in Figure 26.

![Graph showing the AA composition of EFC proteins](image)

Figure 26: A summary of the AA composition of the nine EFC proteins residing outside of the external membrane of VV.

The individual proteins are responsible for the formation of the EFC, its fusion to the host cell membrane and the entry of the viral DNA into the host cell. All of the EFC proteins participate in the fusion and entry functions of the complex while A28, H2 and O3 are integral to the EFC formation\(^{35,66,73}\). Although the complex structure of the EFC remains unknown, three pair-wise interactions have been identified: A28 and H2\(^73\), A16 and G9, and G3 and L5\(^67\). The role of
one EFC protein J5 remains unknown; however, attempts to isolate a mutant without the J5 protein have been unsuccessful\textsuperscript{35}.

**Estimation of the VV external surface coverage with AgNPs**

The VV stock contained $10^{12}$ plaque forming units (PFUs) or virions per 1 mL of virus stock. By calculating the surface area of a mature VV, the number of AgNPs needed to form a monolayer of coverage may be estimated. The dimensions of a mature VV reported above are characteristic of a scalene spheroid of three non-equal spatial dimensions. Making the assumption that some degree of variability will be present in the proportions of the individual virions, the dimensions describing a prolate spheroid or $360$ nm x $260$ nm x $260$ nm were used to greatly simplify the surface area (SA) calculations (eq 13).

$$SA = 2\pi \left( a^2 + \frac{ac}{\sin a} \right) \text{ where } a = \arccos \frac{a}{c}$$

(13)

The SA of a VV was determined using two equal spatial dimensions, $a = b = 260$ nm, and $c = 360$ nm as follows:

$$SA = 2\pi \left( 260^2 + \frac{260 \times 360 + 0.7638}{\sin(0.7638)} \right) = 1.075 \times 10^6 \frac{nm^2}{virion}$$

(14)

The average AgNP diameter in the 100-kD colloidal concentrate was determined to be $11.1$ nm ($r = 5.55$ nm) from the TEM size histogram. Assuming a spherical
shape, the average volume and surface area for a single AgNP was calculated as follows (eq 15 and 16).

\[
V_{AgNP} = \frac{4}{3} \pi r^3 = \frac{4}{3} \pi (5.55)^3 = 716.1 \text{ nm}^3
\]  
(15)

\[
SA_{AgNP} = 4\pi r^2 = 4\pi (5.55^2) = 387.1 \text{ nm}^2
\]  
(16)

For a 1 mL stock containing \(10^{12}\) PFU, the number of AgNPs needed for monolayer coverage was estimated as follows:

\[
\frac{SA \text{ of a single VV}}{SA \text{ of a single AgNP}} = \frac{1.075 \times 10^6 \text{ nm}^2\text{virion}}{387.1 \text{ nm}^2\text{AgNP}} = 2777.2 \frac{\text{AgNPs needed for monolayer coverage}}{\text{virion}}
\]  
(17)

\[
2777.3 \frac{\text{AgNPs}}{\text{virion}} \times \left( \frac{10^{12} \text{ virion}}{1 \text{ mL of virus stock}} \right) = 2.78 \times 10^{15} \frac{\text{AgNPs needed for monolayer coverage}}{\text{mL of virus stock}}
\]  
(18)

Using the silver density \(78, D_{Ag} = 10.49 \frac{g}{cm^3} \times \left( \frac{cm^3}{(10^7)^3 \text{ nm}^3} \right) = 1.049 \times 10^{-20} \frac{g}{nm^3}\), and the \(V_{AgNP}\), the silver mass of a single AgNP was determined (eq 19):

\[
\text{mass of a single AgNP} = D_{Ag} \times V_{AgNP}
\]

\[
= 1.049 \times 10^{-20} \frac{g}{nm^3} \times 716.1 \text{ nm}^3 \times \left( \frac{1 \mu g}{10^{-6} g} \right) = 7.512 \times 10^{-18} \mu g \text{ of Ag}
\]  
(19)

For the AgNP treatment condition of 1,000 \(\mu g\) mL\(^{-1}\), the number of AgNPs present in 1 mL was found (eq 20):
This represents approximately 48,000 times the number of AgNPs needed to achieve monolayer coverage in 1 mL of the viral stock solution (eq. 21).

\[
\frac{1000 \mu g}{mL} \frac{\mu g \text{ of } Ag}{\text{a single } AgNP} = 1.33 \times 10^{20} \frac{AgNPs}{mL}
\]

(20)

The concentration of 1000 μg mL\(^{-1}\) employed in this study was significantly higher than the lowest inhibitory concentration (IC\(_{50}\) = 48 μg mL\(^{-1}\)) reported for AgNPs with VV\(^{5}\). However, monolayer coverage of AgNPs will greatly enable the SERS detection of the silver covalent bonding interactions believed to responsible for the viral inhibition mechanism.

**Elemental analysis characterization using SEM-EDX**

Figure 27 displays three SEM images of the VV control that was deposited onto a copper stub for microscopy measurements. The first SEM image (Figure 27a) depicts a large cluster of virions. A closer examination of the image revealed the presence of several ellipsoidal particles with at least one dimension between 200 – 300 nm. This size is consistent with the approximate dimensions of VV\(^{65}\) of 360 nm x 270 nm x 250 nm. However, attempts to focus upon and completely resolve the individual virions proved very difficult. This was probably due to a thin biofilm covering the sample, which was also visible in the
first image. The SEM image in Figure 27b illustrates an individual virion with measured dimensions of 352 X 288 nm.

Figure 27: SEM-EDX results for VV control. A, B, and C represent SEM images collected with acceleration voltages of 15 kV, 20 kV, and 20 kV, respectively. D depicts the EDX spectrum corresponding to image C.

The EDX spectrum of the VV control which corresponds to the SEM image in Figure 27c is shown in Figure 27d. The major chemical components of the sample are carbon and oxygen as indicated by the large $C_{K\alpha}$ peak at 0.26 keV and the smaller $O_{K\alpha}$ band at 0.52 keV, respectively. A trace amount of calcium was also detected, which is mostly likely a contaminant from the PBS buffer. The large copper binding energies are from the copper mounting stub. The relative
weight percent (Wt%) for each element was included in Table 5. The Wt% findings confirmed the predominance of carbon (55.8%) and oxygen (22.2%). The a carbon to oxygen weight ratio was approximately 2.5 : 1.0. However, the most significant result of this set of SEM-EDX measurements was the absence of any silver in the VV control.

Table 5: Percentage weight of elements present in the VV control sample as revealed by the EDX analysis.

<table>
<thead>
<tr>
<th>Element</th>
<th>Wt.%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C_{Ka}</td>
<td>55.8</td>
</tr>
<tr>
<td>O_{Ka}</td>
<td>22.2</td>
</tr>
<tr>
<td>Ca_{Ka}</td>
<td>2.1</td>
</tr>
<tr>
<td>Cu_{K}</td>
<td>19.7</td>
</tr>
</tbody>
</table>

**Micro-Raman Spectroscopy**

Over 100 point spectra were collected from various locations on the VV control sample to ascertain whether any appreciable signal could be detected without the enhancement benefits of SERS. Figure 28 depicts 20 point spectra for the VV control compared to a point spectrum taken from a control glass slide.
Figure 28: Micro-Raman point spectra \((N = 20)\) of virus control samples in comparison with a point spectrum collected from the glass microscope slide control.

All VV control micro-Raman spectra were similar to the point spectrum collected from the glass microscope slide. This confirmed the low sensitivity of the Raman spectroscopy method at these analyte concentrations and further encouraged the planned SERS measurements with AgNPs.

**Vaccinia Virus treated with 1,000 \(\mu\)g \(\text{mL}^{-1}\) of AgNPs**

**Elemental analysis**

Three SEM images of the VV-AgNP sample deposited onto an aluminum stub are shown in Figure 29.
Figure 29: SEM-EDX results for VV- AgNP sample. A depicts the electron backscatter SEM image using an acceleration voltage of 15 kV. B, C and D represent SEM images collected with an acceleration voltage of 15 kV and the corresponding EDX spectrum for the VV-AgNP control, respectively.

The first frame (Figure 29a) depicts an SEM image collected from backscattered electrons. With electron backscattering, heavier elements such as silver will produce a larger angular deflection and will appear brighter in the corresponding images. In the above image, the presence of AgNP in the rinsed VV-AgNP sample strongly suggests that the AgNPs are attached to the VV sample. The presence of the silver also improved the conductivity of the sample resulting in the ability to obtain a greater degree of resolution for the 20 electron detector image (Figure 29b). Consequently, several spheroidal particles (red...
circles) were observed and appeared to have relatively similar sizes. ImageJ software was used to measure the largest dimension of each spheroid. The size distribution for these spheroids ranged from 278 – 392 nm. The random distribution of particles having dimensions consistent with that of VV suggests that the observed particles correspond to individual virions.

Figure 29d depicts the EDX spectrum obtained for the VV-AgNP sample in Figure 29c. The predominant peaks in the spectrum correspond to the Ag_{La} and Ag_{Lb} binding energies of silver (between 2.5 and 3.5 keV), and the Al_{Ka} peak associated with the aluminum mounting stub (1.5 keV). Silver and Al were represented 31.4 % and 34.1%, respectively, by weight (Table 6).

Other significant peaks were C_{Ka} (16.6%), O_{Ka} (10.9%), Na_{Ka} (2.3%), Mg_{Ka} (2.4%), S_{Ka} (1.6%) and Cl_{K} (0.7%). The carbon and oxygen moieties may be due to AA residues present in the VV component. Trace amounts of PBS solution remaining after the rinsing step may result in the presence of Na, Mg and Cl peaks.
Table 6: Percentage weight of elements present in the VV-AgNP sample as revealed by EDX analysis.

<table>
<thead>
<tr>
<th>Element</th>
<th>Wt.% for elements</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{Ka}$</td>
<td>16.6</td>
</tr>
<tr>
<td>$O_{Ka}$</td>
<td>10.9</td>
</tr>
<tr>
<td>$Na_{Ka}$</td>
<td>2.3</td>
</tr>
<tr>
<td>$Mg_{Ka}$</td>
<td>2.4</td>
</tr>
<tr>
<td>$Al_{Ka}$</td>
<td>34.1</td>
</tr>
<tr>
<td>$S_{Ka}$</td>
<td>1.6</td>
</tr>
<tr>
<td>$Cl_{K}$</td>
<td>0.7</td>
</tr>
<tr>
<td>$Ag_{L}$</td>
<td>31.4</td>
</tr>
</tbody>
</table>

The electron backscatter images and the EDX spectrum of the VV-AgNP sample revealed the presence of silver and further supported the direct interaction between AgNPs and VV.

**Background Information on Surface-enhanced Raman spectroscopy on Viruses**

SERS has become an increasingly popular analytical tool for the detection and study of biological molecules. Pavel *et al.* (2008) used SERS to investigate two FynSH3 proteins modified with double-cysteine groups designed to act as bi-
functional linkers to colloidal AgNPs. The two respective mutant proteins were treated with a reducing agent to break the disulfide bonds and then incubated with Creighton colloidal AgNPs. The resulting SERS spectra revealed a Ag-S peak at 167 cm\(^{-1}\) and a very strong C-S peak at 677 cm\(^{-1}\) indicating the successful cleavage of the S-S bonds and the subsequent Ag-S covalent bond formation. Numerous interactions in between AgNPs and aromatic AAs were also observed specifically for tryptophan (707, 1084, 1132, and 1243 cm\(^{-1}\)), phenylalanine (1183 and 1591 cm\(^{-1}\)) and histidine (1399 and 1591 cm\(^{-1}\)).

Stewart and Fredericks (1999) employed SERS to interrogate the interaction mechanism between various di- and tri-peptides and an electrochemically nanoroughened silver surface. The SERS spectra showed that the primary mode of interaction between the peptides and the silver substrate was through the carboxylate terminus of the AA chain. This interaction was confirmed through the presence of bands of high relative intensity, which were associated with the carboxylate moieties, the peptide bond (amide peaks) and the AA functional groups located in the close proximity of the carboxyl terminus. Furthermore, the vibrational modes that were attributed to the functional groups closest to the amine terminus of the polypeptide were weaker or nonexistent. This suggested that the amine group was further away from the silver substrate. Two AA samples containing thiol groups were also investigated to determine if any Ag-S interactions may occur. In both cases the carboxyl group bands had higher relative intensity than the thiol group suggesting a preferred
nanosubstrate interaction with the carboxylic acids\textsuperscript{32}. When the carboxyl group was not present, the interactions involved mostly aromatic AAs as opposed to smaller side chains\textsuperscript{32}. It seems that an interaction trend with the silver nanosubstrate emerges here: carboxylic groups > peptide bond interactions (amide peaks) > aromatic AAs > thiol groups > small side chains. No explanation was provided in this regard.

Taking into account that well-established, viral detection methods such as polymerase chain reaction (PCR) assays\textsuperscript{79} are time consuming and expensive, recent studies have evaluated SERS as a potential alternative method. In order to obtain highly reproducible spectra, most of the studies used solid-state nanomaterial substrates. Colloidal nanomaterials have also been utilized but to a smaller extent. Table 7 lists the six SERS studies that were performed on viruses in the last two decades. Four of these studies utilized silver nanosubstrates (silver nanorods and silver hydrosols)\textsuperscript{57,79-81}, while the other two studies utilized gold substrates (Kharite SERS active substrate and focused ion beam (FIB)-fabricated nanosubstate)\textsuperscript{82-83}.

Given the AA rich environment of the external membranes of viruses, interactions were observed with all SERS substrates. The primary interactions present were with carboxylate, amide, aromatic AA especially tryptophan, tyrosine and phenylalanine and thiol groups. The SERS vibrational modes characteristic to these interactions are summarized in Table 8.
Table 7: Viruses investigated by SERS and the used nanosubstrate.

<table>
<thead>
<tr>
<th>Virus</th>
<th>Abbreviation</th>
<th>Virus type</th>
<th>SERS substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Norovirus strain MNV-4</td>
<td>MNV4(^{82})</td>
<td>Non-enveloped</td>
<td>Kharite SERS active substrate: a silicon wafer coated with gold. A 6 x 10 mm chip was attached to a glass slide. The chip included a 4 x 4 mm patterned SERS active area and an unpatterned gold reference area.</td>
</tr>
<tr>
<td>Adenovirus strain Mad-1</td>
<td>MAD(^{82})</td>
<td>Non-enveloped</td>
<td></td>
</tr>
<tr>
<td>Parvovirus strain MVMp</td>
<td>MVM(^{82})</td>
<td>Non-enveloped</td>
<td></td>
</tr>
<tr>
<td>Simian rotavirus strain SA-11</td>
<td>SA11(^{82})</td>
<td>Non-enveloped</td>
<td></td>
</tr>
<tr>
<td>Coronavirus strain MHV-A59</td>
<td>MHV(^{82})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Sendai virus strain</td>
<td>Sendai(^{82})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Herpesvirus strain Smith MSGV</td>
<td>MCMV(^{82})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Influenza A WSN33 H1N1</td>
<td>H1N1(^{83, 79})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Yaba monkey tumor virus</td>
<td>YMTV(^{90})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Pseudocowpox</td>
<td>PCPV(^{80})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Bovine papular stomatitis</td>
<td>BVSV(^{80})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Purified Respiratory syncytial virus (RSV)</td>
<td>RSV(^{79})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>RSV strain A2</td>
<td>A2(^{79, 57})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>RSV strain A/Long</td>
<td>A/Long(^{79, 57})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>RSV strain B1</td>
<td>B1(^{79, 57})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>RSV strain A2 with the deletion of the G gene</td>
<td>ΔG(^{79, 57})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Adenovirus type 6/tonsil strain 99</td>
<td>AD(^{79})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Rhinovirus type 4/strain 16/60</td>
<td>Rhino(^{79})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>HIV CXCR4-tropic strain</td>
<td>HIV(^{79})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Influenza A HKx31</td>
<td>HKx31(^{79})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Influenza A PR/8/34</td>
<td>PR8(^{79})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Trabala vishnou NPV</td>
<td>TVP(^{81})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Dendrolimus punctatus NPV</td>
<td>DPV(^{81})</td>
<td>Enveloped</td>
<td></td>
</tr>
<tr>
<td>Tobacco mosaic virus</td>
<td>TMV(^{84})</td>
<td>Non-enveloped</td>
<td>TERS: 20 nm silver coated tip</td>
</tr>
</tbody>
</table>

Silver nanorods: Rod length of 868 nm ± 95 nm; Diameter of 99 nm ± 29 nm; Average tilt angle of 71.3° ± 4.0 °C

Silver hydrosols: Synthesized using the Creighton method.
Table 8: Literature assignment of the SERS peaks observed during the interaction of various viruses with silver and gold nanosubstrates.

<table>
<thead>
<tr>
<th>Raman Shift (cm$^{-1}$)</th>
<th>Assignment</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td>238, 244</td>
<td>$\nu$(Ag-N)</td>
<td>DPV(s), TPV(vs)</td>
</tr>
<tr>
<td>276</td>
<td>aliphatic chain C-C bending $[\delta$(C-C)]</td>
<td>YMTV(m), PCPV(w), BVSV(s)</td>
</tr>
<tr>
<td>441, 456</td>
<td>rocking of COO$^-$</td>
<td>TPV(vw), DPV(vw)</td>
</tr>
<tr>
<td>455</td>
<td>$\nu$(S-S)</td>
<td>YMTV(vw), PCPV(vw), BVSV(vw)</td>
</tr>
<tr>
<td>521</td>
<td>skeletal stretching</td>
<td>BPMV(vw)</td>
</tr>
<tr>
<td>526$^{79}$, 540, 546$^{79}$</td>
<td>$\nu$(S-S)</td>
<td>MAD(w), MVM(m), SA11(w), BVSV(vw), RSV(w, w-sh)</td>
</tr>
<tr>
<td>577</td>
<td>$\gamma$(COO$^-$)</td>
<td>TPV(m)</td>
</tr>
<tr>
<td>592</td>
<td>glycine</td>
<td>HKx31(vw), PR8(vw), H1N1(vw)</td>
</tr>
<tr>
<td>612</td>
<td>$\delta$(COO$^-$)</td>
<td>DPV(vw)</td>
</tr>
<tr>
<td>633$^{79}$, 635$^{79}$, 640$^{82}$</td>
<td>tyrosine (skeletal)</td>
<td>A/Long(w-b), HKx31(vw), PR8(vw), H1N1(vw), MAD(w), MVM(vw), SA11(vw)</td>
</tr>
<tr>
<td>761$^{84}$</td>
<td>cysteine</td>
<td>TMV(w)</td>
</tr>
<tr>
<td>837$^{79}$, 844$^{82}$, 848$^{79}$</td>
<td>tyrosine</td>
<td>MAD(vs), MVM4, SA11(w), Sendai(m), MCMV(vs-b), MVM(s), MVH(vs), HIV(vw), RSV(m),</td>
</tr>
<tr>
<td>853</td>
<td>$\delta$(CCH), tyrosine</td>
<td>YMTV(w), PCPV(vw-b), BVSV(m), Rhino(w)</td>
</tr>
<tr>
<td>877</td>
<td>tryptophan</td>
<td>A/Long(vw), BPMV(vw), TMV(vw)</td>
</tr>
<tr>
<td>916$^{81}$, 921$^{82}$, 927$^{81}$, 932$^{84}$, 937$^{82}$, 943$^{92}$</td>
<td>$\nu$(C-COO$^-$), alanine</td>
<td>DPV(vw), MNV4(s), MVH(w), Sendai(vw), MCMV(vw), TP(vw), TMV(vw)</td>
</tr>
<tr>
<td>1001, 1002, 1003, 1005</td>
<td>phenylalanine (symmetric ring breathing mode)</td>
<td>MVH(vw), Sendai(m), MCMV(vw), YMTV(m), PCPV(w), BVSV(s), AD(vw), Rhino(vw), HIV(vw), HKx31(w), PR8(w), H1N1(w), TMV(ms)</td>
</tr>
<tr>
<td>1018$^{82}$, 1022$^{82}$, 1030$^{79}$, 1033$^{79}$</td>
<td>in-plane $\delta$(C-H), phenylalanine</td>
<td>MVM(vw), MAD(vw), MCMV(m), MVH(w), Sendai(m), AD(vw), Rhino(vw)</td>
</tr>
<tr>
<td>1031$^{81}$, 1037$^{84}$</td>
<td>$\nu$(C-N)</td>
<td>DPV(vw), TMV(vw)</td>
</tr>
<tr>
<td>1042$^{79}$, 1044$^{79}$, 1045$^{79}$, 1047$^{82}$, 1055$^{79}$</td>
<td>$\nu$(C-N)</td>
<td>MCMV(m), MVH(w), MAD(vw), SA11(vs), MVH(w), Sendai(w), RSV(vs), HKx31(w), PR8(w), H1N1(w), A/Long(vw), B1(vw), $\Delta$G(vw), A2(vw)</td>
</tr>
<tr>
<td>1085</td>
<td>$\nu$(C-N)</td>
<td>TPV(m)</td>
</tr>
<tr>
<td>Frequency (cm⁻¹)</td>
<td>Assignment</td>
<td>Species</td>
</tr>
<tr>
<td>-----------------</td>
<td>------------</td>
<td>------------------</td>
</tr>
<tr>
<td>1098⁸⁴</td>
<td>PO₂⁻ stretch, alanine</td>
<td>TMV(vw)</td>
</tr>
<tr>
<td>1122</td>
<td>T(NH₂)</td>
<td>DPV(vw)</td>
</tr>
<tr>
<td>1129</td>
<td>ν(C-N) and ν(C-C)</td>
<td>MVM(vw), MAD(s), SA11(m), MVH(m), Sendai(m), MCMV(vw), HKx31(ms), PR8(s), H1N1(s), TMV(w)</td>
</tr>
<tr>
<td>1156⁸⁴, 1158⁸²</td>
<td>ν(C-C)</td>
<td>TMV(vw), MAD(m), MCMV(vw)</td>
</tr>
<tr>
<td>1165⁸³, 1168⁹⁰, 117³⁸², 117⁹⁴</td>
<td>tyrosine, ν(CN)</td>
<td>H1N1(vw), YMTV(m-b), PCPV(vw-b), BVSV(m), MVH(vw), Sendai(m), TMV(vw)</td>
</tr>
<tr>
<td>1190</td>
<td>tyrosine, phenylalanine</td>
<td>A/Long(w-b)</td>
</tr>
<tr>
<td>1246⁸⁴</td>
<td>amide III</td>
<td>TMV(vw)</td>
</tr>
<tr>
<td>1248</td>
<td>δ(NH₂)</td>
<td>AD(m), DPV(vw)</td>
</tr>
<tr>
<td>1260</td>
<td>CH₂ in-plane deformation, tyrosine</td>
<td>H1N1(w), HKx31(w), PR8(w), H1N1(w)</td>
</tr>
<tr>
<td>1278</td>
<td>amide III</td>
<td>TMV(vw-s)</td>
</tr>
<tr>
<td>1296</td>
<td>CH₂ deformation</td>
<td>MAD(vw), MVM(vw), SA11(vw)</td>
</tr>
<tr>
<td>1332⁸⁴</td>
<td>T(CH₂), tryptophan, TMV(m)</td>
<td></td>
</tr>
<tr>
<td>1358⁸¹, 137¹⁹⁹, 139⁶¹</td>
<td>ν₅(COO'), tryptophan</td>
<td>DPV(vw), HIV(vw), TPV(w)</td>
</tr>
<tr>
<td>1401</td>
<td>ν₅(COO'), AAs</td>
<td>H1N1(w)</td>
</tr>
<tr>
<td>1443²⁸², 144⁸⁷⁹, 145⁴⁷⁹, 145⁵⁸⁴, 145⁷²⁸⁴</td>
<td>δ(CH₂)</td>
<td>MNV4(s), MAD(m), MVM(vs), SA11(w), YMTV(w-b), PCPV(w-b), BVSV(w-b), AD(w), Rhino(w), HIV(w), RSV(m), BPMV(m), TMV(s)</td>
</tr>
<tr>
<td>1480</td>
<td>amide II (coupling of C-N stretch and in-plane bending of N-H)</td>
<td>H1N1(vw-s)</td>
</tr>
<tr>
<td>1515</td>
<td>δ(N-H)</td>
<td>H1N1(w), YMTV(m-b), BVSV(vw)</td>
</tr>
<tr>
<td>1520</td>
<td>ν₅(sym(COO'))</td>
<td>DPV(vw)</td>
</tr>
<tr>
<td>1523</td>
<td>Tryptophan ν₅(sym(COO'))</td>
<td>HIV(vw)</td>
</tr>
<tr>
<td>1555⁸⁴, 1558⁸³</td>
<td>tryptophan</td>
<td>TMV(w), H1N1(w)</td>
</tr>
<tr>
<td>157⁴⁷¹, 157⁶⁷⁹, 158²⁸¹</td>
<td>ν₅(sym(COO'), tryptophan, tyrosine</td>
<td>DPV(vw), AD(m), TPV(w)</td>
</tr>
<tr>
<td>1597</td>
<td>tyrosine</td>
<td>MAD(vw), Rhino(vs)</td>
</tr>
<tr>
<td>161⁵³</td>
<td>tyrosine</td>
<td>H1N1(vs)</td>
</tr>
<tr>
<td>165⁷³, 165⁶⁸⁴</td>
<td>amide I</td>
<td>AD(w), TMV(vs)</td>
</tr>
<tr>
<td>167⁹²</td>
<td>amide I, ν(C=O)</td>
<td>MAD(vw)</td>
</tr>
</tbody>
</table>
Abbreviations key for band intensitites is as follows: vw - very weak, w - weak, m - medium, ms - medium strong, s - strong, vs - very strong, b - broad, and sh - shoulder.

Surface-enhanced Raman spectroscopy on Creighton silver nanoparticles interacting with vaccinia virus

In this study, 12 SERS-maps (10x10 µm to 40x40 µm with 1 µm step size) were collected on the Creighton AgNPs incubated with VV. These SERS maps contained a total of 2600 spectra. After the application of the signal to noise algorithm, 392 spectra were retained and compiled into a single hyperspectral dataset. VCA was then used to select 50 representative EMs from the compiled dataset. Pairwise correlations between each EM using a threshold of 85% further reduced the dataset to 10 representative spectra that contained the most unique spectral components. Of the ten remaining spectra, three were nearly identical to other spectra and were discarded. One spectrum contained little spectral information above the signal to noise threshold and was also eliminated leaving six spectra in the final representative set (Figure 30). The peak assignments of each of the six representative SERS spectra are provided in Table 9 (100 – 1000 cm\(^{-1}\)) and Table 10 (1000 – 2000 cm\(^{-1}\)).
Figure 30: Six representative normalized SERS spectra (A-F) as selected by VCA and correlation algorithms.
Table 9: Tentative assignment of the SERS peaks in the 100-1000 cm\(^{-1}\) spectral range of the six, representative EMs (denoted with A-F in Figure 30).

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>E</th>
<th>F</th>
<th>Band Assignment</th>
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</thead>
<tbody>
<tr>
<td>154</td>
<td>194</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\nu(\text{Ag-S})^{53})</td>
</tr>
<tr>
<td>229</td>
<td>238</td>
<td>238</td>
<td>234</td>
<td>230</td>
<td>232</td>
<td>(\nu(\text{Ag-N}), \nu(\text{Ag-O})^{86-87})</td>
</tr>
<tr>
<td>334</td>
<td>326</td>
<td>336</td>
<td></td>
<td></td>
<td></td>
<td>(\nu(\text{Ag-N})^{88-89})</td>
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<tr>
<td>395</td>
<td>392</td>
<td>394</td>
<td></td>
<td></td>
<td></td>
<td>Chain vibrations of myristic acid(^{34})</td>
</tr>
<tr>
<td>445</td>
<td>458</td>
<td>473</td>
<td>500</td>
<td>466</td>
<td></td>
<td>(\nu(\text{S-S})^{80,33,27})</td>
</tr>
<tr>
<td>482</td>
<td></td>
<td>488</td>
<td></td>
<td></td>
<td></td>
<td>(\delta(\text{C-C}=\text{O})^{33})</td>
</tr>
<tr>
<td>504</td>
<td></td>
<td></td>
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<td>(\nu(\text{S-S})^{33})</td>
</tr>
<tr>
<td>540</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\delta(\text{CC}_3)) deformation(^{34})</td>
</tr>
<tr>
<td>569</td>
<td></td>
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<td></td>
<td></td>
<td>(\gamma(\text{COO})^{81})</td>
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<tr>
<td>615</td>
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<td></td>
<td>(\delta(\text{COO})^{81})</td>
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<tr>
<td>622</td>
<td>625</td>
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<td>In plane (\delta(\text{C}=\text{C})) ring of tryptophan(^{87,34})</td>
</tr>
<tr>
<td>640</td>
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<td></td>
<td></td>
<td></td>
<td>Ring deformation of tyrosine(^{34})</td>
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<td>662</td>
<td>647</td>
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<td></td>
<td></td>
<td>(\nu(\text{C-S})^{32})</td>
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<tr>
<td>674</td>
<td></td>
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<td>(\nu(\text{C-C})^{32})</td>
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<tr>
<td>676</td>
<td>680</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\delta(\text{O}=\text{C-O})^{33})</td>
</tr>
<tr>
<td>727</td>
<td>727</td>
<td>694</td>
<td></td>
<td></td>
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<td>(\delta(\text{O}=\text{C-N})) of amide IV(^{33})</td>
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<tr>
<td>746</td>
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<td></td>
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<td>Tryptophan(^{32})</td>
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<tr>
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<td></td>
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<td>Glutamine(^{32})</td>
</tr>
<tr>
<td>819</td>
<td>828</td>
<td>831</td>
<td>828</td>
<td>816</td>
<td></td>
<td>(\delta(\text{C-SH})^{90})</td>
</tr>
<tr>
<td>886</td>
<td>841</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\nu_3(\text{C-N-C})^{33})</td>
</tr>
<tr>
<td>909</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>937</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\nu(\text{C-COO})^{32})</td>
</tr>
<tr>
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<td></td>
<td>Valine(^{34})</td>
</tr>
<tr>
<td>970</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(\nu(\text{C-C})^{90})</td>
</tr>
<tr>
<td>994</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Symmetric ring breathing mode of phenylalanine(^{90})</td>
</tr>
</tbody>
</table>

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Table 10: Tentative assignment of the SERS peaks in the 1000-2000 cm\(^{-1}\) spectral range of the six representative EMs (denoted with A-F in Figure 30).

<table>
<thead>
<tr>
<th>Representative Endmembers (EMs)</th>
<th>Band Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>A 1009</td>
<td>Tryptophan(^{32})</td>
</tr>
<tr>
<td>B 1023</td>
<td>Alanine (^{34})</td>
</tr>
<tr>
<td>C 1035 1030</td>
<td>In plane (\delta(CH)) of phenylalanine(^{32})</td>
</tr>
<tr>
<td>D 1043</td>
<td></td>
</tr>
<tr>
<td>E 1063</td>
<td>Histidine (^{34})</td>
</tr>
<tr>
<td>F 1100 1095 1088 1116 1139 1182</td>
<td>Tryptophan (^{34}), (\delta(NH_3)^{32}), Out of phase (\nu(CCC)^{90})</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>1190</td>
<td>Valine (^{34})</td>
</tr>
<tr>
<td>1210</td>
<td></td>
</tr>
<tr>
<td>1228 1229 1222</td>
<td>Pyrrole ring stretch(^{91})</td>
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<tr>
<td>1245</td>
<td>(\delta(NH_2)^{32})</td>
</tr>
<tr>
<td>1254 1253 1267</td>
<td>Tyrosine (^{34})</td>
</tr>
<tr>
<td>1294</td>
<td>Amide III (^{33})</td>
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<tr>
<td>1298</td>
<td>(\nu(C-C)) of myristic acid (^{34})</td>
</tr>
<tr>
<td>1302 1306 1326 1322 1348</td>
<td>Tyrosine (^{34}), Histidine (^{34})</td>
</tr>
<tr>
<td>1336</td>
<td>Tryptophan (^{33})</td>
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<tr>
<td>1387 1384</td>
<td>Serine (^{34})</td>
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<tr>
<td>1411 1404</td>
<td>(\nu_3(COO)^{32})</td>
</tr>
<tr>
<td>1430</td>
<td>Histidine (^{34})</td>
</tr>
<tr>
<td>1438</td>
<td>Myristic Acid (^{34})</td>
</tr>
<tr>
<td>1465 1451 1476 1477 1502</td>
<td>(\delta(H-C-H))</td>
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<tr>
<td>1531 1539 1539 (C-C(=O)-NH(_2)) of amide II (^{33})</td>
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</tr>
<tr>
<td>1563</td>
<td>Tryptophan (^{91})</td>
</tr>
<tr>
<td>1583 1572</td>
<td>Ring (\nu(C-C)) of phenylalanine (^{32})</td>
</tr>
<tr>
<td>1592</td>
<td>Tyrosine (^{32})</td>
</tr>
</tbody>
</table>
Covalent bonding interactions of silver with vaccinia virus

*Ag-N, Ag-O, and Ag-S interactions*

Silver has a preferential attraction to electronegative atoms and tends to covalently bond with oxygen, sulfur and nitrogen. Most of the representative spectra (B, D, E, and F) exhibited a well-defined peak consistent with $\nu$(Ag-N) or $\nu$(Ag-O)$^{86-87}$ between 229 – 238 cm$^{-1}$. Spectra A and C had a broader less pronounced peak but the interaction was still present. Spectra B contained two additional shoulder peaks at 154 cm$^{-1}$ and 194 cm$^{-1}$. The vibrational mode at 154 cm$^{-1}$ is consistent with $\nu$(Ag-S) interactions associated with large protein structures,$^{53}$ whereas the band at 194 cm$^{-1}$ may be attributed to $\nu$(Ag-Ag)$^{85}$ that occurs in general at 192 cm$^{-1}$.

**Carboxylic acid, myristic acid, amine group and peptide bond interactions**

The EFC proteins of VV are rich in carboxylic acids groups pertaining to the AA constituents and the two myristoylated proteins A16 and G9$^{11}$. As previously discussed, silver readily bonds with available carboxylic acid groups$^{32}$. 

\[\begin{array}{cc}
1598 & \nu_{\text{as}}(\text{COO})^{32} \\
1614 & \nu(\text{COO})^{32} \\
1631 & \text{Tyrosine}^{32} \\
1631 & \text{Histidine}^{34} \\
1638 & \text{Amide I}^{32} \\
1639 & \text{Amide II} \\
1643 & \text{Amide III} \\
1655 & \text{Amide IV} \\
\end{array}\]
This was evidenced by the presence of multiple vibrational modes characteristic to carboxylic acids in the representative SERS spectra. The most prominent carboxylic acid peaks appeared in the SERS spectra A and E at 1411 cm\(^{-1}\) and 1598 cm\(^{-1}\). These \(\nu_s(COO^-)\) and \(\nu_{as}(COO^-)\) stretchings dominated the spectrum A. Only the \(\nu_s(COO^-)\) was observed at 1404 cm\(^{-1}\) for spectrum E. Carboxylic signals of lower relative intensity were detected at 569 cm\(^{-1}\) (medium strong in spectrum B), 615 cm\(^{-1}\) (weak in spectrum C), 676 and 680 cm\(^{-1}\) (weak in spectra A and E), and 937 cm\(^{-1}\) (weak in spectrum E). These peaks were attributed to vibrational modes characteristic to terminal carboxylic groups, namely \(\gamma(COO^-)\) (spectrum B), \(\delta(COO^-)\) (spectrum C), \(\delta(O=C-O)\) (spectrum A), and \(\nu(C-COO^-)\) (spectrum E).

Interactions were also observed for the carbonyl group moieties of the peptide bonds comprising the backbone of the protein chain. A few examples include the \(\delta(C-C=O)\) peak at 482 cm\(^{-1}\) in spectrum B and a weak band at 488 cm\(^{-1}\) in spectrum D. In addition, three other interactions with the myristic acid constituents were detected. The corresponding carbon chain vibrations \((N > 3)^{34}\) were visible at 395, 392 and 394 cm\(^{-1}\) for spectra B, D and F, respectively. Spectra D also exhibited a very strong \(\nu(C-C)^{34}\) peak at 1298 cm\(^{-1}\) and a strong \(\delta(H-C-H)^{33}\) band at 1438 cm\(^{-1}\).

In the SERS spectra of proteins, amide bond interactions are often observed from the peptide bond formed between the carboxylic acid group and
the amine group of adjacent AAs. The associated vibrational modes, often
named amide I – IV, are characterized by a certain degree of carbonyl \( \nu(C=O) \),
\( \nu(C-N) \) and \( \delta(N-H) \). Weak amide I bands, primarily associated with carbonyl
adsorption, were observed in the 1638 – 1655 cm\(^{-1}\) spectral range for SERS
spectra B, D, E and F. The spectra A, C and F presented amide II peaks mostly
represented by \( \delta(N-H) \). This bending appeared at 1531 (medium strong) 1539
cm\(^{-1}\) (medium strong), and 1531 cm\(^{-1}\) (weak) in spectra A, C, and F, respectively.
The amide III mode derives much of its character equally from the carbonyl
adsorption and the N-H bending. It was found at 1294 cm\(^{-1}\) as a medium
intensity band in spectrum C. Finally, the amide IV contributions were observed
as \( \delta(O=C-N) \) bands at 727 cm\(^{-1}\) (weak in spectra A and B) and 694 cm\(^{-1}\) (very
strong in spectrum F). Weak \( \nu_s(C-N-C) \) modes were present at 886 cm\(^{-1}\) and 841
cm\(^{-1}\) in spectrum B and C, respectively, but could not be associated with a
specific amide band interaction.

Amine group interactions were only present in two of the representative
spectra (B and F). Spectrum F exhibited weak bands for \( \delta(NH_3^+) \) and \( \delta(NH_2) \) at
1139 and 1245 cm\(^{-1}\), respectively, whereas spectrum B had a weak intensity
\( \delta(NH_3^+) \) peak at 1148 cm\(^{-1}\).
Aromatic amino acid interactions

Five AAs, histidine, phenylalanine, tyrosine, tryptophan and proline contain aromatic ring-structure side chains. Of those, phenylalanine and tyrosine have benzene rings, histidine has an imidazole ring, tryptophan contains an indole group and proline forms an aliphatic ring between the amine group and the \( \alpha \)-carbon of the AA (pyrrolidine ring)\(^{92-93}\). SERS studies confirm that AgNPs are bind readily with the electron rich side chains of these AAs\(^{32,91}\).

Several aromatic AA interactions were observed in the representative spectra. The strongest bands (medium strong in intensity) associated with tryptophan were found in SERS representative spectra D and F at 1116 cm\(^{-1}\) and in spectrum A at 1563 cm\(^{-1}\). Several weak tryptophan interactions were also detected in the SERS spectra, specifically 1009 cm\(^{-1}\) for A, 622 cm\(^{-1}\) for B, and 746 and 1336 cm\(^{-1}\) for C. Weak tyrosine vibrational modes were observed in SERS spectra A (1326 cm\(^{-1}\)), B (1322 cm\(^{-1}\)), C (640 cm\(^{-1}\) and 1614 cm\(^{-1}\)) and E (1267 and 1592 cm\(^{-1}\)). Three significant bands were found for histidine at 1063 cm\(^{-1}\) (medium strong in D), 955 cm\(^{-1}\) (medium in B), and 1430 cm\(^{-1}\) (medium in C). In addition, weak intensity peaks were exhibited in spectrum F (1348 and 1629 cm\(^{-1}\)) and spectrum A (1631 cm\(^{-1}\)). The characteristic symmetric ring breathing mode of phenylalanine\(^{90}\) at 994 cm\(^{-1}\) was present in SERS spectrum F at medium intensity. The in plane \( \delta(\text{CH}) \) modes\(^{32}\) was detected at 1035 cm\(^{-1}\) as a medium intensity peak in A and weakly 1030 cm\(^{-1}\) in B along with a weak ring.
\( \nu(C-C) \) peak in spectrum C at 1583 cm\(^{-1}\). Finally, only one vibrational mode was observed for proline. This peak associated with the pyrrole ring stretch\(^{91}\) appeared at 1228 cm\(^{-1}\) (medium in C), 1229 cm\(^{-1}\) (medium strong in D) and 1222 cm\(^{-1}\) (medium strong in E).

**Cysteine group interactions**

The EFC proteins in VV contain multiple, invariant cysteine AAs and disulfide bonds. Direct interactions of EFC proteins with AgNPs through these groups are also possible. All SERS spectra with the exception of spectrum C presented weak \( \nu(C-SH) \) modes in the 816 – 831 cm\(^{-1}\) spectral region consistent with the functional group of cysteine\(^{90}\). Furthermore, spectra B and D exhibited \( \nu(C-S) \) at 662 cm\(^{-1}\) and 647 cm\(^{-1}\), respectively. Weak overtones of the \( \delta(C-C-S) \) band\(^{33}\) were detected at 326 – 336 cm\(^{-1}\) for spectra A – C.

In the EFC, 23 – 28 disulfide bonds are formed among the nine proteins\(^{35}\). Evidence of the disulfide bonds was present in several of the representative spectra. A very strong \( \nu(S-S) \) mode was observed at 458 cm\(^{-1}\) in spectrum B, while relatively weak bands were found at 445 cm\(^{-1}\) and 466 cm\(^{-1}\) in spectra A and D respectively. Spectrum B also exhibits a second \( \nu(S-S) \) peak at 504 cm\(^{-1}\) however this band is much weaker in intensity than the other peak. Spectra C contained two very strong, distinct and nearly symmetrical \( \nu(S-S) \) bands at 473...
cm$^{-1}$ and 500 cm$^{-1}$. The nature and proximity of these bands in the spectrum strongly suggests that AgNPs are covalently bonded to both electron rich sulfur atoms. This unique NP interaction would result in peaks with similar enhancement due to the sulfur atoms proximity to the AgNPs. However, the bands would be observed at different wavenumbers due to the varying AA constituents on the two different invariant cysteines.

**SERS spectra correlation**

The SERS results obtained in this work are consistent with those reported in the few SERS literature studies on other viruses. Strong covalent interactions were observed in between the Creighton colloidal AgNPs of 1-20 nm in diameter and the same protein moieties of VV. Figure 31 illustrates that the number of observed interactions was dominated by AA with aromatic functional groups and similar to the other SERS studies, interactions with small side chains were negligible. More exactly, the number of aromatic AA modes was observed approximately 5 times more frequently (i.e., with a ratio of 26.0 : 5.0 or 5.2 : 1.0) than smaller aliphatic side chain AAs. The predominance of aromatic AA modes is not surprising as these AA side chains tend to be oriented towards the outside of the protein structures (e.g., helixes, sheets, turns and folds) to reduce the steric hindrance of the overall conformation$^{92}$. As a result, AgNPs may come in contact with these electron rich moieties more readily.
Figure 31: Six representative normalized SERS spectra (A-F) together with the primary interactions (carboxylic groups, amide groups, aromatic amino acids and thiol groups) ranked in order from most frequent to least often.
Aromatic AAs, carboxylic group and amide bond modes were far more prevalent numerically than thiol modes. AgNP interactions with carboxylic moieties were observed more frequently than amide and thiol group interactions indicating the preferential binding of AgNPs for the carboxylic terminus of the protein versus the amine group terminus or the sulfur groups.

The interaction trend revealed by the analysis of the six EM spectra seems to be slightly different than observed in the previous SERS studies on other viruses: aromatic AAs > carboxylic groups> amide groups> thiol groups > small side chains. However, the carboxylic and amide modes were consistently stronger in intensity suggesting a closer proximity and stronger bonding interaction with the AgNPs. Furthermore, there are far more vibrational modes possible for the side chains of the five aromatic AA acids than for the carboxylic moieties. Considering $3N-6$ vibrational degrees of freedom with $N$ representing the number of atoms present in the molecule, the simplest side chain of these amino acids, benzene ($N = 12$) of phenylalanine, would have 30 vibrational modes (i.e., $3N - 6 = 3(12) - 6 = 30$). Of those, 12 are Raman active$^{46}$. Comparatively, the carboxylic acid moiety of proteins exhibits six Raman vibrations$^{32}$. Therefore, it is not surprising that the aromatic AA moieties were more numerous than the carboxylic modes and peptide bond interactions. For these reasons, it is believed that the observed AgNP interaction trends in this study are consistent with those reported in literature: carboxylic groups > peptide
bond interactions (amide peaks) > aromatic AAs > thiol groups > small side chains.

After the SERS representative spectra were determined, the correlation algorithm was employed a second time to determine the spectral similarity of the six representative EMs to the entire compiled hyperspectral dataset (392 spectra) using the same correlation thresholds (Table 11).

Table 11: Spectral correlation of EMs A-F to the compiled, hyperspectral dataset (392 spectra). The values represent the number of spectra in the compiled dataset that contain similar spectral characteristics to the representative EMs at specific correlation thresholds.

<table>
<thead>
<tr>
<th>EM</th>
<th>Correlation Threshold (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>95</td>
</tr>
<tr>
<td>A</td>
<td>1</td>
</tr>
<tr>
<td>B</td>
<td>3</td>
</tr>
<tr>
<td>C</td>
<td>1</td>
</tr>
<tr>
<td>D</td>
<td>1</td>
</tr>
<tr>
<td>E</td>
<td>1</td>
</tr>
<tr>
<td>F</td>
<td>1</td>
</tr>
</tbody>
</table>

Since the correlation coefficient algorithm compares each pixel of the spectral pairs, two spectra that were identical would correlate at 100%. Therefore, most of the EMs were only correlated to one spectrum in the dataset (i.e., the actual EM) at very high thresholds of 90% or high. Since many the spectra contain similar vibrational features, any one member of the dataset may
correlate to more than one EM especially at lower threshold values. For this reason, this analysis cannot definitely describe the spectral correlation for all members of the dataset. If an EM has very unique spectral features it will correspond to fewer dataset members even at lower thresholds. EM C only correlated to itself even down to a 75% correlation threshold. This is most likely a result of the very strong, nearly symmetrical $\nu$(S-S) bands at 473 cm$^{-1}$ and 500 cm$^{-1}$. The most highly correlated spectra were EMs B, A and E. At a 75% threshold, 166, 150 and 144 spectra from the compiled dataset were correlated to the EMs, respectively. The remaining two EMs (D and F) are more unique spectrally and therefore correlated to 88 dataset members at 75%. Cumulatively, the total number of dataset members accounted for at 75% was 637, a number that is well above the actual 392 spectra in the dataset. This confirmed the prevalence of similar spectral features and consistent interaction patterns in spectral dataset as a whole. However the representative SERS EMs also contained many unique features. This suggested that a very large number of spectra in the compiled dataset (if not all) may be accounted for by the representative EMs.
Conclusions

There is an ever increasing need for the development of a broad-spectrum antiviral drug that can be effective against a wide variety of viruses. AgNPs have a demonstrated antiviral activity and offer a viable option as potential antiviral agents. However, a more definitive mechanism of viral inhibition needs to be defined. This study confirmed the proposed hypothesis and showed for the first time that unfunctionalized, Creighton AgNPs of an average diameter of ~11 nm bind covalently to the AA moieties present in the EFC proteins of VV. However, given the abundance of proteins comprising the external membrane of VV (i.e., 25-30), AgNP binding to other proteins on the external VV membrane cannot be excluded. In this context, TFU demonstrated to be a “green” method for the size-selection and extreme concentration of large volumes of colloidal AgNPs with minimal aggregation.

Further research should focus on determining if any other steps of the viral replication cycle of the VV are also disrupted by the AgNPs or if the AgNPs are taken into the core of the VV. Given that VV does not have an active metabolism, the later scenario is unlikely. Also different AgNP formulations and different size distributions should also be investigated in a similar manner.
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