

PROTEIN TEMPLATED SILVER NANOCCLUSERS

by
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ABSTRACT

Greyson Keel: Protein Templated Silver Nanoclusters
(Under the direction of Dr. Saumen Chakraborty)

Nanoclusters are smaller than their nanoparticle counterparts and have unique properties. Most research has been done on gold nanoclusters, but silver nanoclusters are said to be more robust. During the process of trying to create nanoclusters, several methods in the procedure were changed when gathering data: determining a successful silver concentration, working with the correct buffer, mixing gold and silver metals together, observing the effects of pH, and finding a good reducing agent. After experimenting with those changes, a successful protocol was adapted and modified from literature (Journal of Materials Chemistry, 2011, 21, 11205-11212) to produce the protein-templated Ag clusters. By using both NaOH and NaBH₄ as reducing agents, silver nanoclusters were formed and a brown color change was observed. Different silver concentrations were created. The results from the MALDI and native gel suggest that the formation of silver nanoclusters can be created at different sizes.

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Chapter 1: Introduction

1.1 Nanoparticles

Nanoparticles are particles ranging in size from one to one hundred nanometers surrounded by an interfacial layer. These nanoparticles are studied because they have unique electronic and atomic structures when compared to larger bulk materials. Since no two nanoparticles are the same, the polydispersity of nanoparticles is a major issue in nanotechnology.¹ A major thrust in this field would be to create truly uniform nanoparticles at the atomic level.¹ This has partially been accomplished through the creation of ultrasmall gold nanoparticles with diameters ranging from subnanometer to ~2.2 nm; this allows the fundamental properties of colloids to be found at the atomic level.¹ While there is a lot to still discover in the field of nanotechnology, scientific research on nanoparticles has a long history. Beginning in the fourth century, the Roman Empire's artisans made use of gold nanoparticles in their pottery to create a glittering effect. In the mid 1800's, Faraday's reaction created "potable gold" which was the basis of colloid science.¹ In 2000, when the National Nanotechnology Initiative was established, the era of nanotechnology was launched. Since then, nanoscience and nanotechnology has greatly impacted virtually every branch of the natural sciences.¹

Although there have been great advancements, there are still many unanswered questions regarding nanoparticles. First, the surface layer which includes organic

stabilizers and the organic-inorganic interface is poorly understood due to the inability of transmission electron microscopy to image this layer. Although scanning probe microscopies can image this surface layer at the atomic level, the buried interface cannot be accessed directly.¹ Nuclear Magnetic Resonance, Infrared Absorption, and Raman scattering techniques are beneficial for molecular compounds, but they are limited with nanoparticles for two reasons: (i) the polydispersity and heterogeneity of nanoparticles and (ii) excess ligands, surfactants, residual reactants, and side products of colloids.¹ Therefore, simple questions such as “What protects the nanoparticle surface?”, “How are the surface stabilizers bonded to the inorganic core?”, and “What determines the stability of nanoparticles?” remain unknown. Understanding the makeup of the surface layer is crucial for the many applications of nanotechnology. Second, whereas there is a deeper understanding for larger plasmonic nanoparticles, ultrasmall nanoparticles’ (<3nm in diameter) structure and properties remain poorly understood. At one time, it was thought that the relationship between sensitivity and size of the nanoparticle would not yield different results. However, when exactly precise nanoparticles were synthesized and studied, it was demonstrated that metallic nanoparticles’, of less than three nanometers, behaviors were affected. Conventional gold and silver nanoparticles exhibit surface plasmon resonances which account for their absorption, scattering, and surface-enhanced Raman scattering properties.¹ In metallic nanoparticles, the surface plasmon resonance peak is broad, but the electron dynamics in ultrasmall nanoparticles is unknown. It is known that the surface plasmon resonance properties of gold nanoparticles are size dependent.¹ When metal nanoparticles enter the ultrasmall size region (<3nm), the strong quantum size effects are encountered thus making the ultrasmall nanoparticles sensitive to

particle size. Even a single atom difference adds to this sensitivity. The evolution of structure and properties from discrete atoms to the metallic state is still a mystery. Third, the mechanisms for controlling the shape of nanoparticle synthesis still remain unclear at the atomic level. In order to address these unknown areas, atomically precise nanoparticles are of importance as well as determining the total structure—core and surface—of the nanoparticles. Once the structure of the nanoparticle of different sizes and shapes are determined, it will be possible to understand and learn how ligands protect the core, the material properties—electronic, optical, and catalytic—at the atomic level, and further address the size dependence and shape control.¹

Later, ultras-small nanoparticles less than 2 nm in diameter were discovered to be molecule like in their properties; their electronic band structure becomes discontinuous and breaks into discrete energy levels similar to a molecule. Because of this discovery, these ultras-small nanoparticles, referred to as nanoclusters, are known as the bridge between molecules and nanoparticles. Nanoparticles are the bridge between nanoclusters and bulk metals. These relationships are displayed in Figure 1.

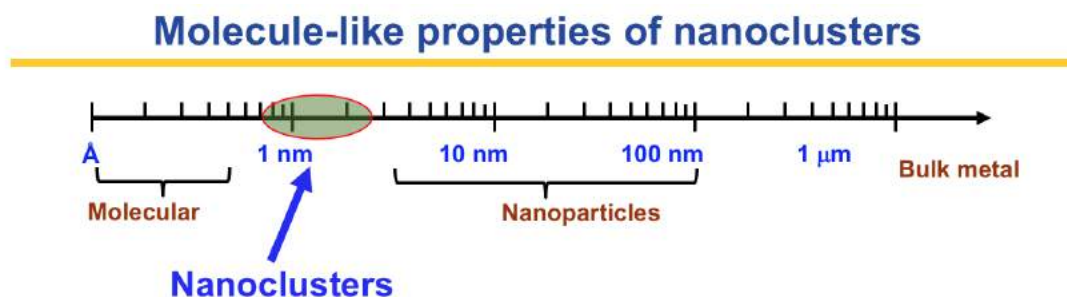


Figure 1. Size range of molecules to clusters to particles to bulk. *The figure above shows the size differentiation between molecules, clusters and particles. It is included to further clarify the above introduction so that the ranges given above can be put into context.*

1.2 Protein Templated Silver Nanoclusters

Nanoclusters are a group of nanoparticles made of a specific number of atoms or molecules that can be calculated. Although most research has been conducted with gold, silver nanoparticles are another option, and in recent years, research on them has advanced. Even though silver and gold are located in the same group of the periodic table, silver nanoclusters exhibit more complex surface structures.¹ Also, silver nanoclusters are more robust when compared to their gold nanoparticle analogues.² Since there are limited reports on silver, the research is trivial. However, there are several reports regarding the synthesis of silver nanoclusters producing clusters that are highly luminescent in the solution and solid states of matter.² Non-luminescent silver clusters have been observed in frozen solutions and zeolite conditions as well. Since silver clusters usually aggregate and grow into larger clusters/nanoparticles, their surface energy must be reduced.² In order to synthesize stable entities, it is necessary to have templates that are preformed or capping agents. Molecular events that occur inside these silver cluster cells can be seen, measured, and tracked using fluorescence microscopy since they have luminescent properties.² In one study that was published in the *Journal of Materials Chemistry*, the research lab noticed how the amount of reducing agent played a pivotal role in the formation of clusters. Through the controlled addition of reducing agent, the solutions showed different colors from blue to red under UV light allowing the luminescence of the silver clusters to be observed in the visible spectrum. It was also found that the stability of the silver clusters was dependent on a specific temperature, time, and pH.² Due to its exciting photophysical

properties, silver nanoclusters are interesting material for study for various new applications.

1.3 Potential Applications

At the present, much research is designed towards the applications of nanoparticles. Current areas of interest include catalysis, chemical sensing, optical imaging, biological labeling and biomedicine, and light-emitting devices. Nanoclusters would make a unique class of catalysts because of their distinct properties such as high specific surface area, a high fraction of low-coordinated atoms, quantum size effects, tunable compositions, and unique surface structures.¹ Moreover, photoluminescent nanoclusters have been used in chemical sensing. Atomically precise nanoclusters have provided advantages and opportunities when compared to conventional nanoparticle-based sensors because the polydisperse nanoparticles in the conventional sensors do not give a clear picture of the sensing mechanisms. Because of the broad range of applications involving nanoparticles, this field is diverse and important to study.

Chapter 2: Protein Induction and Purification for Wildtype CSP1

The protein that was used is known as wildtype Csp1. Csp1 is a copper storage protein. It is a tetramer of four-helix bundles with each monomer binding up to 13 Cu(I) ions through mainly the cysteine residues that make the core of the bundle. Csp1 is the first example of a protein that contains a metal within an established protein-folding motif.³

The sequence below is the gene sequence for WTCSP1.

```
TGGAGCCACCCGCAGTTCGAGAAGGGTGGCAGCGGTGAAGACCCGCACGCGGGCCACAAAAT
GAGCCACGGTGC GAAGTACAAAGCGCTGCTGGACAGCAGCAGCCACTGCGTGGCGGTTGGTG
AAGATTGCCTGCGTCACTGCTTTGAAATGCTGGCGATGAACGACGCGAGCATGGGTGCGTGCA
CCAAGGCGACCTATGATCTGGTGGCGGCGTGC GGTGCGCTGGCGAAACTGGCGGGTACCAAC
AGCGCGTTCACCCCGGCGTTTGC GAAGGTGGTTGCGGACGTTTGC GCGGCGTGCAAGAAAGA
GTGCGATAAGTCCCGAGCATCGCGGAGTGCAAAGCGTGCGGTGAAGCGTGCCAAGCGTGCG
CGGAGGAATGCCACAAAGTGGCGGCGTAA
```

Below, indicates the amino acid sequence.

```
WSHPQFEKGGSGEDPHAGHKMSHGAKYKALLDSSSHCVAVGEDCLRHC FEMLAMNDASMGAC
TKATYDLVAACGALAKLAGTNSAFTPAFAKVVADVCAACKKECDKFPSIAECKACGEACQACAE
ECHKVAA
```

The sequence highlighted in yellow refers to Strep Tag. This sequence codes for a synthetic peptide; it allows the protein to be purified and detected by affinity chromatography. The sequence highlighted in blue refers to the spacer. The sequence highlighted in green refers to the actual protein amino acid sequence. In the protein sequence, the cysteine (C) residues are highlighted in red. These 13 cysteine residues are potential sites for Ag coordination because sulfur has a high binding affinity.

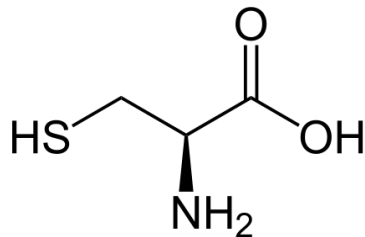


Figure 2. Structure of Cysteine: *This figure shows the chemical structure of the amino acid, Cysteine.*

2.1 Transformation Protocol

The wildtype Csp1 protein genes were transformed in BL-21 DL3 competent cells. In order to achieve this, the BL-21 competent cells were removed for -80°C and were kept on ice for 10 minutes to allow thawing. The gene was then diluted in 40µL of distilled water. One microliter of the distilled gene was taken in a fresh 10mL culture tube. Then, the cells and gene were incubated on ice for 30 minutes. The cells then underwent a heat shock at 42°C for 45 seconds. They were then kept on ice for 10 minutes. For the culture tube, one milliliter of LB media was added and incubated on a shaker for one hour at 37°C. This allows the cells to grow. 50µL of the cells were plated on an LB-Agar plate which has 50 mg/mL of kanamycin. This plate is kept overnight in the incubator at 37°C. The following day, the colonies are observed (see Figure 3), and the plate is stored at 4°C.

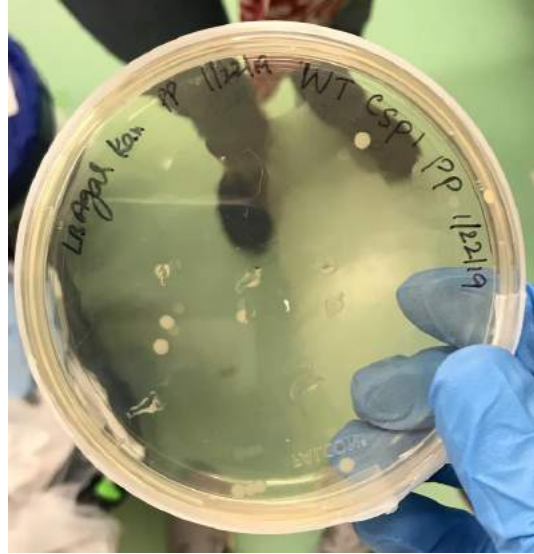


Figure 3. Bacteria Colonies on Agar Plate: *This figure shows the bacterial colonies that were observed on the LB Agar plate. These colonies contain the gene of interest. One of these colonies will be picked and used to further the protein purification process.*

2.2 Protein Induction

The colonies that were produced in the transformation are used to prepare a primary culture and to further the induction of the protein. The induction procedure began by combining 10mL of LB media and 10 μ L of 50 mg/mL Kanamycin in a 10mL culture tube. Then, a single fully grown colony on the culture plate was picked and added to the 10mL LB media. The primary culture was then grown overnight at 37°C and 180 rpm and is shown in Figure 4.



Figure 4. Primary Culture: *This figure shows the primary culture after being grown overnight at 37°C and 180 rpm.*

The next day, the secondary culture was prepared in one liter of LB media with one milliliter of Kanamycin. This secondary culture is then inoculated with 10mL of the primary culture. It is then grown for three to four hours until the OD600 reading is between 0.4 and 0.6. Once the reading is between the allotted parameters, the cells are used to make a glycerol stock. 5mL of these un-induced cells are kept in a 10mL culture tube and are used as a control. The one-liter culture is then induced with 1mL of 1M IPTG; the overall final concentration is 1mM. This culture is then grown for another four to six hours at 30°C. After this, the induced cells are harvested at 4°C, 5K for 10 minutes. The cells are then stored at -20°C.

2.3 Protein Purification

After induction is complete, the cells are then sonicated with buffer. The buffer is specific to the type of protein, wildtype or the redesigned protein. The sonication buffer

created contained 50mL of 20mM Tris at pH 8.5, 0.5mg of DNase at 10 μ g/mL, 1mM PMSF, 1mM EDTA, and 7.7 mg of 1mM DTT. The 1mM PMSF was created by dissolving 100mM (17.4mg) PMSF in 1mL of IPA; 500 μ L was put in the sonication buffer. The 1mM EDTA was created from the 100mM stock in the fridge and 500 μ L was added to the buffer. The induced cells as well as the un-induced cells were sonicated in this buffer using the following sonication cycle: 3 minutes' total with pulsing on for 10 seconds and pulsing off for 20 seconds. The sonicated cells were centrifuged at 14K for 45 minutes in a SS-34 rotor in 4°C. Figure 5 shows the pellet that is created after sonication and centrifugation in the SS-34 rotor are completed.

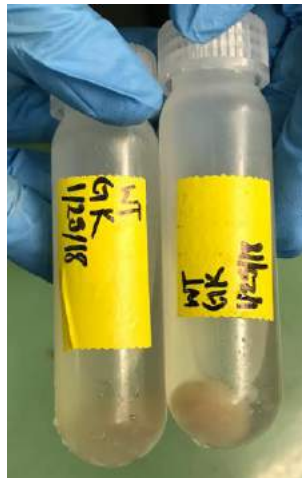


Figure 5. Protein and Pellet: *This image is of the protein and pellet collected after sonication and centrifugation in the SS-34 rotor for 45 minutes at 4°C and 14K. The supernatant is collected from these tubes and will be loaded into the Strep-Trap column.*

A Strep-Trap column is then conducted (see Figure 6). A 5mL column which is stored in the fridge at 4°C with a flow rate of 4 mL/min is used. The column is stored in Buffer W which consists of 100mM Tris-HCl, 150mM NaCl, and 1mM EDTA at pH 8. The protein is then loaded into the column at a flow rate of 4 mL/min. The timer is set according to the volume of the supernatant that is collected (shown in Figure 7). The flow through is collected. The column is then washed again with Buffer W for five to six

minutes. The flow through is collected. While this flow through is collected, 2.5mM of des-thiobiotin is created by combining 30mL of elution buffer and dissolving 16mg of des-thiobiotin. The protein is then eluted with Buffer W with 2.5mM of des-thiobiotin for seven minutes. After this, the column is washed with water for five minutes. It is then washed with 500mM NaOH, which is 600mg in 300mL of water, for six minutes. After these six minutes, the column is immediately washed with water for another five to six minutes. During this washing step, the pH of the column is checked until the column has reached a neutral pH level. After reaching a neutral pH reading, the column is again washed with Buffer W for five to seven minutes and stored at 4°C. The machine's system is then purged with water, 70% EtOH, and then water once the procedure is completed.



Figure 6. Strep-Trap Column Setup: *This figure shows the Strep-Trap column setup and the Econo Gradient Pump machine which carries the protein to the column.*



Figure 7. Wildtype CSP1 Supernatant: *This figure is an image of the Supernatant that was collected after the rest of the protein was loaded into the Strep-Trap column. This leftover supernatant will be used as one of the samples loaded into the gel.*

The purified protein is then subjected to dialysis in 50 mM Tris, 150 mM NaCl, 2.5 grams of Chelex at pH 7.8, which are the components of the dialysis buffer. The membrane chosen to hold the purified protein has a 6000-8000 MCOW (see Figure 8). The buffer used contains 1 mM DTT for the first two exchanges and left overnight stirring around 50 rpm. The next morning, the buffer is changed but no DTT is added. After two to three hours, the protein is filtered with a 0.22 μ M filter. The UV is checked specifically at the absorbance value of 280nm. The protein is then stored at -80°C after aliquoting in small eppies and flash freezing in liquid Nitrogen.

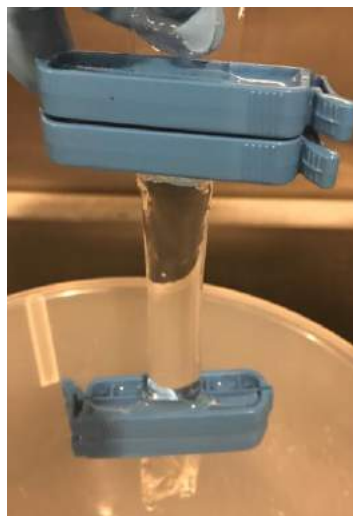


Figure 8. Dialysis: *This is the eluted protein from the Strep-Trap column. It is held in the dialysis membrane of a 6000-8000 MCOW.*

In order to check and see if the protein was achieved, a sample was run on a gel. After centrifugation, the supernatant was collected to check for induction using a 12% Tris-Tricine SDS-Page gel with a high molecular weight marker. The uninduced protein was kept earlier in the procedure to be used as a control for the gel. The gel involves two components: the resolving gel and the stacking gel. The protocol recipe for the resolving gel includes 3.3mL of 3M Tris-HCl-SDS, 4mL of 30% Bis-Acrylamide, 1mL of deionized water, 1.7mL of glycerol, 40 μ L of APS, and 8 μ L of TEMED. The recipe for the stacking gel includes 1.85mL of deionized water, 400 μ L of 30% Bis-Acrylamide, 750 μ L of 3M Tris-HCl-SDS, 20 μ L of APS, and 3 μ L of TEMED. Once the resolving and stacking gels solidified, the gel could be loaded with samples—the high molecular weight ladder, the proteins before and after purification, and flow through 1 and 2 collected during purification. The samples were prepared by taking 20 μ L of the sample, 5 μ L of the loading dye and heating them at 90°C for 10 minutes. After heating, the samples were loaded on the gel. Approximately 12 μ L were loaded in each well. The running buffer used was 1X of the Tris-Tricine SDS buffer. The gel was run for about 2 hours until the samples

completed their run down to the bottom of the gel. An image showing the gel and samples that were run is seen in Figure 9. In this SDS gel, there the protein of interest is located in lane 4. The single band that appeared in this lane corresponded to WT CSP1 protein at 13,500 Da.

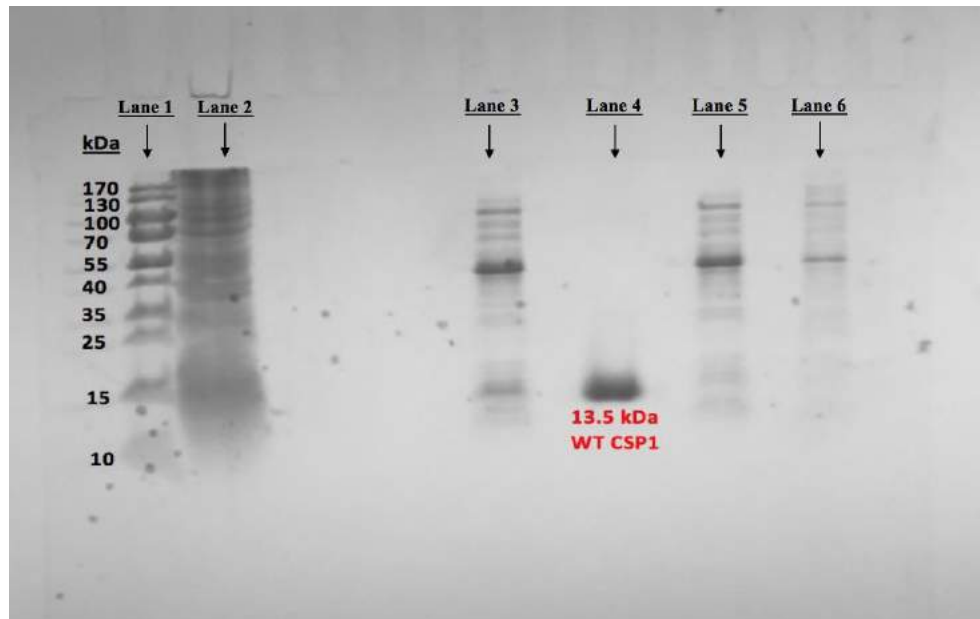


Figure 9. Gel After Protein Purification: *This is an image of the gel for the wildtype CSP1 protein. Lane 1 is the high molecular weight ladder, lane 2 is the pellet, lane 3 is before purification, lane 4 is after purification, lane 5 is flow through 1, and lane 6 is flow through 2.*

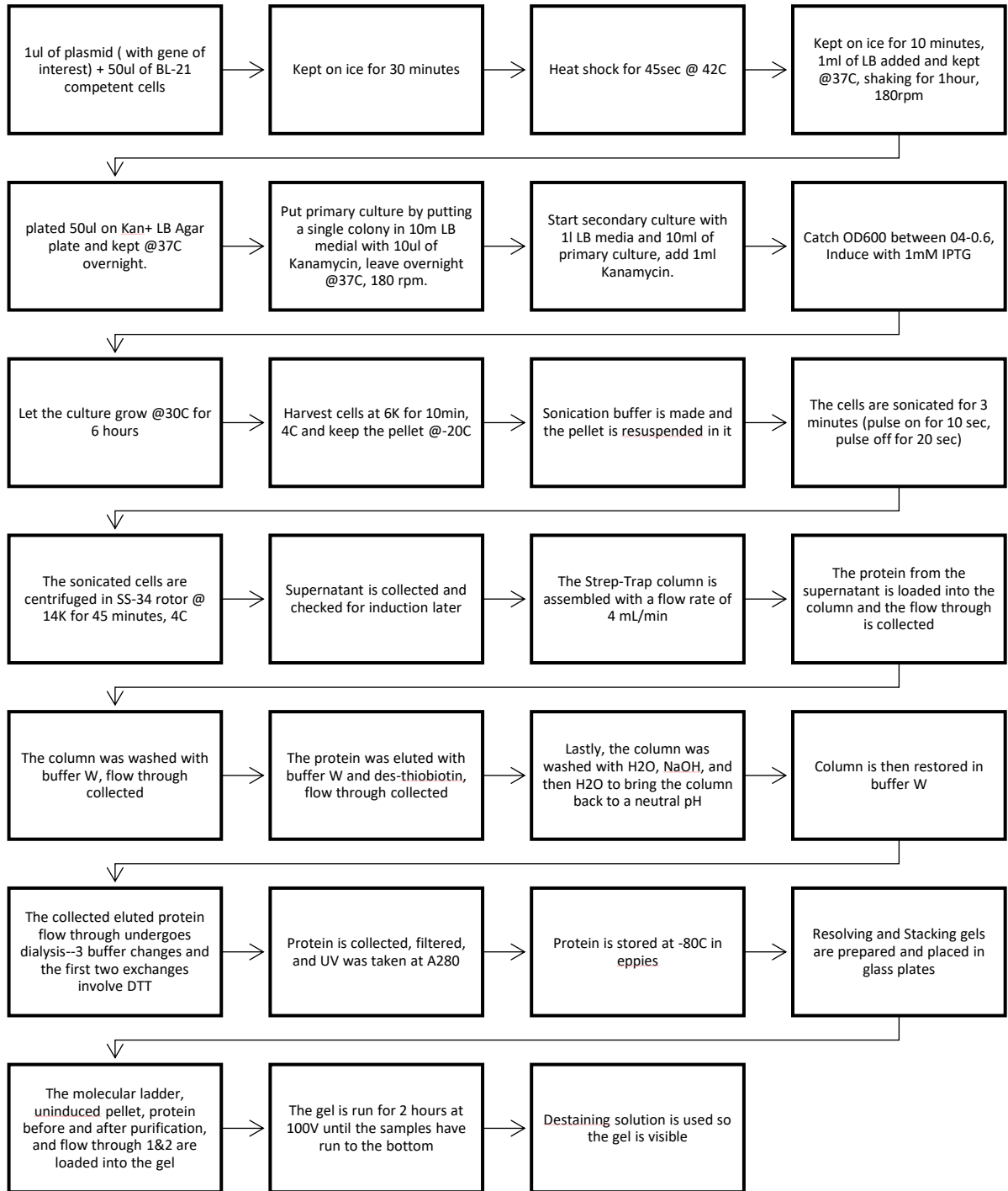


Figure 10. Flow Chart of Entire Process: *This is a flow chart depicting all the steps for the protein purification process: transformation, induction, and purification. These steps were previously stated, but for a visual and easier representation for one to follow, a flow chart was constructed.*

Chapter 3: Synthesis of Silver Nanoclusters

While it has been stated that silver clusters are more reactive compared to their gold analogues, there are limited reports that exist about this because they are difficult to make compared to the gold nanoclusters. Because not much is known about silver nanoclusters, different conditions were tried in order to create them.

3.1 Synthesis of Silver Nanoclusters

For these experiments, the method created in the Chakraborty lab for gold nanoparticle synthesis was used and modified for silver. First, the wildtype CSP1 protein that had been purified was concentrated to 50 μM . A 10K filter was used and the protein was centrifuged at 12K and 4°C for 5 minutes. After, the concentration was checked by taking the UV and dividing by 8480, the molar extinction coefficient. In order to get the protein to 50 μM , dialysis buffer—50mM Tris, 150mM NaCl at pH 7.8—was added. Once the protein concentration was obtained, 100 μL of the protein is used for each experiment.

Protein Concentration (μM)	Protein Used (μL)	Ag Concentration	Equivalents of Ag	Amount of Ag (μL)	H ₂ O Added (μL)	5% 1M NaOH (μL)
50	100	10 M	10	5	95	10
50	100	10 M	20	10	90	10
50	100	10 M	30	15	85	10
50	100	10 M	40	20	80	10
50	100	10 M	50	25	75	10
50	100	10 M	60	30	70	10
50	100	10 M	70	35	65	10

Table 1. 10-70 equivalents of Ag Samples: *The table above explains the contents and amounts in each vial. The equivalents of silver were increased by increments of 10 to see a cluster formation.*

The table above details the experiments conducted at 0 and 50°C. 10 equivalents and 40 equivalents of silver were done at 50°C, and 10-70 equivalents were done at 0°C. In each vial, the amount of water and silver were mixed and stirred together for a couple of minutes. Next, 100 μL of protein was added to each vial and stirred for a couple of minutes and then reduced with 5% 1M NaOH.

After the addition of protein to each vial, a white precipitate formed. The addition of NaOH caused the formation of a dark grey color. Due to the formation of a precipitate when the experiment was conducted at 50°C in both the 10 and 40 equivalents of silver, the temperature was lowered to 0°C (as seen in Figure 12). The 10-70 equivalents of silver were held at 0°C and stirred overnight and is shown in Figure 11.

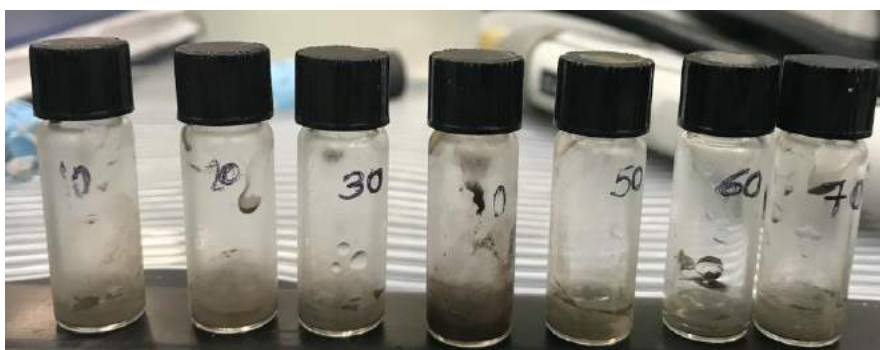


Figure 11. 10-70 equivalents of 10M Ag: *This image shows the 10-70 equivalents of silver after stirring overnight at 0°C.*

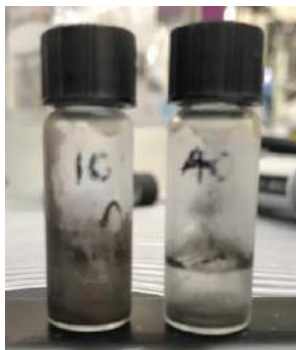


Figure 12. 10 and 40 equivalents of 10M Ag: *This image is of 10 and 40 equivalents of silver at the end of the experiment at 50°C.*

Since precipitation occurred in the previous experiments, two separate vials, containing 10 equivalents of 10M Ag and 95 μ L of either water or dialysis buffer (50mM Tris, 150mM NaCL at pH 7.8), were prepared. In the vial containing dialysis buffer, the silver precipitated before the addition of protein. The vial containing water and silver did not precipitate. This is represented in Figure 13.



Figure 13. Buffer Versus Water: *The vial on the left contains 10 equivalents of 10M silver and 95 μ L of dialysis buffer that precipitated before protein was added to the mixture. The vial on the right contains 10 equivalents of silver and 95 μ L of water.*

Because the vial containing dialysis buffer and 10 equivalents of silver precipitated before protein was added, another vial was prepared containing 100 μ L of protein and 95 μ L of dialysis buffer. This solution was heated at 37°C for 30 minutes before the 10

equivalents of silver were added. By adding the protein and the buffer together first instead of metal and buffer together, it was to see if maybe the different combination would prevent precipitation from occurring. But, precipitation still occurred and the solution became a milky, grey color and is shown in Figure 14.

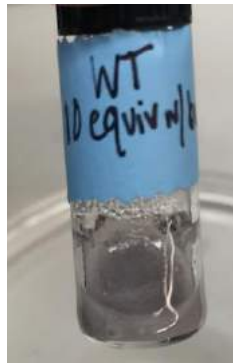


Figure 14. Dialysis Buffer Precipitation: *Image of the vial containing 100 μ L of protein, 95 μ L of dialysis buffer, and then 10 equivalents of silver. This combination still produced a precipitation like the previous reactions.*

Since the dialysis buffer and 10 equivalents of silver vial created a precipitation before protein was added and the vial containing water and 10 equivalents of silver did not precipitate, HEPES buffer was used in the next reaction instead of dialysis buffer. The decision to change the buffer was done to see if this would avoid precipitation. So, 40 μ L of concentrated wildtype protein and 160 μ L of HEPES buffer were combined and mixed at 37°C. This solution was heated for 10 minutes on an oil bath and then 5 μ L of 10M Ag was added; a white precipitate formed and is shown in Figure 15.



Figure 15. HEPES Buffer Precipitation: Image displaying the white precipitate that formed after 10 equivalents (5 μ L) of 10M AgNO₃ was added to the solution with HEPES buffer and protein.

The precipitation of silver is undesirable. Due to numerous precipitations occurring, it was decided to lower the silver concentration from 10M to 0.1M AgNO₃ solution. The 10M concentration of silver was allowing for the formation of larger sized nanoparticles that were not soluble in the solution.

Protein Concentration (μ M)	Protein Used (μ L)	Ag Concentration	Equivalents of Ag	Amount of Ag (μ L)	H ₂ O Added (μ L)	5% 1M NaOH (μ L)
51	100	0.1M	10	0.51	99.49	10
51	100	0.1M	20	1.02	98.98	10

Table 2. Lowered AgNO₃ Concentration: This table explains the contents contained in the two vials with a lowered silver concentration to 0.1M instead of 10M.

The table above explains the ingredients that were in each of the vials stirred at 37°C. In each vial, water and silver were mixed for a couple of minutes in an oil bath at 37°C. Then, the protein was added and stirred for 5 minutes, and then reduced by 5% 1M NaOH. By lowering the concentration to 0.1M AgNO₃, no precipitate formed in these reactions. While there are no visible color changes or fluorescence in the two vials (see Figure 16), there was a spectral difference observed. In the energy plot (Figure 17), the peak of the protein was viewed at 280nm (4.43eV). Even though there was a spectral

difference observed in the UV graph, a MALDI was not performed, so it is unknown if a silver nanocluster was formed.

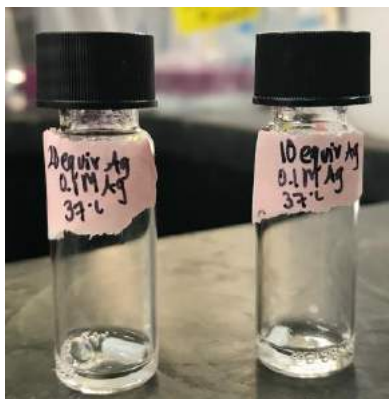


Figure 16. 10 and 20 Equivalents of 0.1M Ag: This is the image of the vials containing 10 or 20 equivalents of silver, 100 μ L protein, H₂O, and 5% 1M NaOH. No precipitate formed in these reactions.

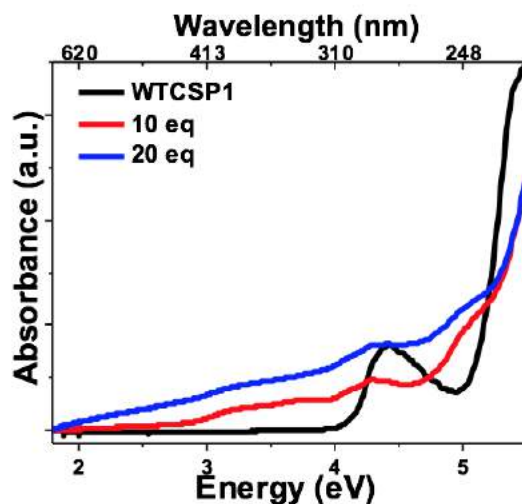


Figure 17. Energy Plot of 10 and 20 Equivalents 0.1M Ag: Energy plot comparing the wildtype protein to when it has 10 equivalents of silver or 20 equivalents of silver. The peak of the protein is seen at 280nm which correlates to an energy of 4.43eV. A MALDI was not performed for this experiment, so it is unknown for what the silver formed.

3.2 Changing pH

Unlike the previous experiments that used dialysis buffer, 5mM Tris buffer was used to concentrate the proteins down. The dialysis buffer that the protein is concentrated

in has a pH of 7.8, but the pH was altered in this experiment to see if this change would have effects on nanocluster formation.

Protein Conc. (μM)	Protein Used (μL)	Ag Conc.	Equivalents of Ag	Amount of Ag (μL)	H ₂ O Added (μL)	pH	Temp ($^{\circ}\text{C}$)
52	100	0.1M	10	0.52	99.5		50
52	100	0.1M	10	0.52	99.5	1	50
52	100	0.1M	10	0.52	99.5	13	50
52	100	0.1M	10	0.52	99.5		37
52	100	0.1M	10	0.52	99.5	1	37
52	100	0.1M	10	0.52	99.5	13	37

Table 3. Silver Samples with Different pH Values: *The vials made in this experiment were at pH 1, 7.8, which is the pH of dialysis buffer, or 13. The experiment was conducted at temperatures of 37°C and 50°C.*

For the vials that were at pH 1 or 13, the protein was concentrated in those different pH values. The protein was concentrated by taking 200 μL of protein with 200 μL of the specific pH and placed in a 10K filter. The protein was then centrifuged for 5 minutes. After the first round of centrifuging, an additional 200 μL of pH 1 or 13 was added and centrifuged for another 5 minutes, then this process was repeated again and centrifuged for a final 10 minutes. Depending on the final amount of protein that was obtained, pH was added to the protein to maintain a 200 μL volume. Because we were changing the pH in some of the vials, those vials did not get reduced with NaOH, but the two vials that were not held at pH 1 or 13 did get reduced with 5% 1M NaOH. Even though the pH was changed, there was no spectral or fluorescence change observed (see Figure 18).



Figure 18. Ag Samples at Varying pH: Left image: 10 equivalents of 0.1M AgNO_3 at 50°C with respect to pH 1, 7.8, and 13. Right image: 10 equivalents of 0.1M AgNO_3 at 37°C with respect to pH 1, 7.8, and 13.

3.3 Mixing Gold with Silver

Since no fluorescence was appearing with silver alone, it was decided to mix gold and silver together and observe the effects. It is thought that a potential reason for no fluorescence being observed with silver alone is due to the energy difference gap between the highest occupied molecular orbital (HOMO) and lowest occupied molecular orbital (LUMO) is not large enough for excitation to be visible. When combining gold and silver together, fluorescence was able to be seen.

Protein Conc. (μM)	Protein (μL)	Ag Conc.	Equiv. of Ag	Ag (μL)	Au Conc.	Equiv. of Au	Au (μL)	H_2O Added (μL)	5% 1M NaOH (μL)
52	100	10mM	1	0.52	10mM	8	4.16	95.32	10
50	100	10mM	2	1	10mM	7	3.5	95.5	10
50	100	10mM	3	1.5	10mM	6	3	95.5	10

Table 4. Combining Gold and Silver to Observe Fluorescence: The table above explains the contents in the experiment of 3 different vials. Each vial contains different equivalents of silver and gold. These metals were mixed in order to observe changes in fluorescence due to the different equivalents of silver in each vial.

The table above represents the contents of the three vials at 37°C. The protein was concentrated in dialysis buffer (50mM Tris, 150mM NaCl at pH 7.8). When mixing metals, the total amount of gold equivalents was added to the solution. Then, the silver equivalents were added to the solution one equivalent at a time with 15-minute stirring increments in between each silver equivalent addition. Similar to the previous experiments, the metals and water were stirred first and then the protein was added and then reduced 5-10 minutes later. In the vial containing 8 equivalents of gold and 1 equivalent of silver, the fluorescence was of a hotter pink color compared to the other two vials that had a brighter orange hue. Since the other two vials have an orange fluorescence and have more equivalents of silver, the silver component may be playing a greater role when compared to the 8 equivalents of gold and 1 equivalent of silver and causing the fluorescent color changes to be observed. The fluorescence is shown in Figure 19.

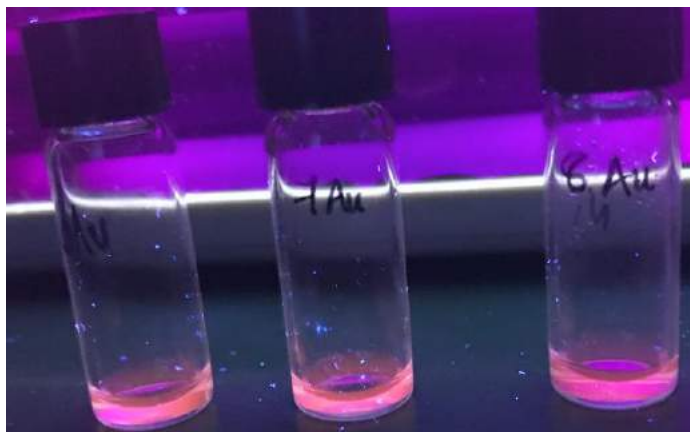


Figure 19. Fluorescence of Silver with Gold: *The image above shows the fluorescence of the silver and gold vials. From left to right, the vials are 6 Au equivalents with 3 Ag equivalents, 7 Au equivalents with 2 Ag equivalents, and 8 Au equivalents with 1 Ag equivalent.*

Because changes in fluorescent occurred in different gold and silver combinations, it was decided to perform two experiments. Both experiments involved concentrating the protein to 50µM. 200uL of protein was used, so the water amount that was added this time

was 200 μ L. The UV was taken after the addition of water to check the concentration, which should be around 25 μ M. For the first experiment, 9 equivalents of 10mM Au stock solution, which is 9 μ L, were added to the vial containing water and protein; the solution was stirred for 10 minutes, and then, the UV was taken. 5% 1M NaOH was added to the water, protein, and gold solution and stirred for 30 minutes. The UV was recorded after stirring was complete. Then, 1 equivalent of 10mM AgNO₃ was added in 15 minute increments and the UV was checked after each addition. Silver addition was added until 9 equivalents was reached.

The other experiment that was conducted was a silver-gold ratio. After the protein and water were mixed, the UV was taken to measure the concentration, which was 26 μ M. Then, 1 equivalent of 10mM gold stock solution was added and stirred for 10 minutes. The UV was checked, and then 1 equivalent of silver from 10mM AgNO₃ was added, was stirred for 10 minutes, and the UV was taken. This process was repeated until 5 equivalents of both gold and silver were added to the solution. This solution was then reduced with 5% 1M NaOH and stirred overnight.

Both experiments—the 9 equivalents of gold and 9 equivalents of silver, and the silver-gold ratio—were performed two different ways to see if either synthesis worked for creating a silver nanocluster. In the 9 equivalents gold and 9 equivalents of silver, after all of the equivalents of gold were added to the protein and water mixture and stirred, the solution was reduced with 5% 1M NaOH. It was reduced before adding the silver equivalents because a gold nanocluster was formed, and the reduction would allow silver to bind easier. In the silver-gold ratio, the solution was reduced after all the silver and gold

equivalents were added to the solution. Even though these experiments were performed in different ways, there was no formation of a silver nanocluster.

The absorbance was measured throughout both experiments to observe spectral changes (see Figure 20 and Figure 21). In the 9 equivalents gold and 9 equivalents silver experiment, each addition of silver followed the similar trend in absorbance. In the silver-gold ratio experiment, each addition of gold and silver followed the similar trend in absorbance. When the UV was taken the day after of the overnight silver-gold ratio sample, there was a spectral change that appeared and the peak is observed at 250nm. Both experiments—the 9 equivalents of gold and 9 equivalents of silver, and the silver-gold ratio—were concentrated in a 10K tube to get rid of any impurities that may have formed overnight. After the impurities were removed, the same pattern observed for the absorbance was restored. The absorbance for these experiments is shown in Figure 18 for the 9 equivalents gold and 9 equivalents silver experiment and in Figure 19 for the silver-gold ratio experiment.

The day after the experiments were performed, the fluorescence of both experiments was observed. The 9 equivalents of gold with 9 equivalents of silver had an orange hue. The silver-gold ratio vial had an orange/brown appearance.

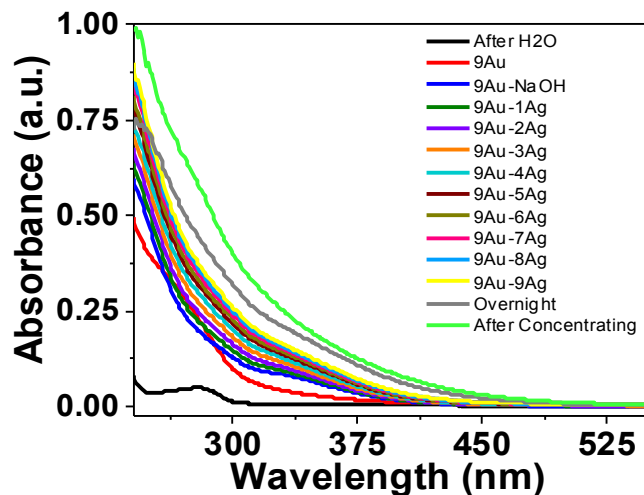


Figure 20. Absorbance of 9 Equivalents Au with 9 Equivalents Ag: *This figure shows the UV for the 9equivalents gold with 9 equivalents of silver experiment. The Overnight line refers to the solution stirring overnight and the UV was taken the next day after the experiment was conducted. The After Concentrating line refers to when the solution was centrifuged to remove further impurities that could have been created overnight.*

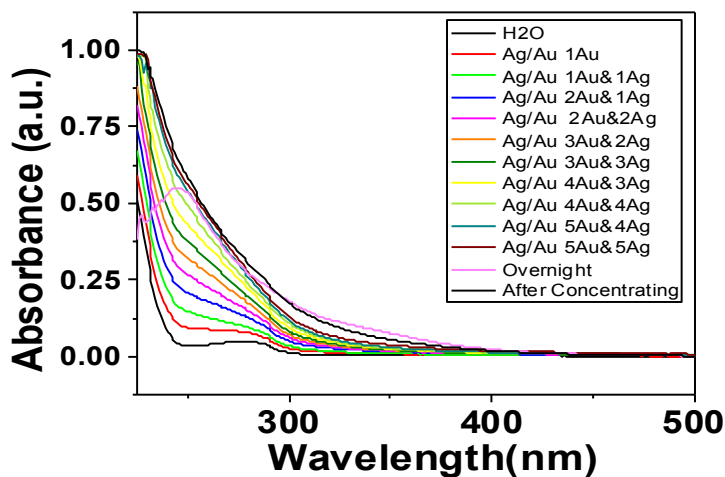


Figure 21. Absorbance of Ag/Au Ratio: *This is a graph showing all the UVs taken during the silver-gold ratio experiment. The Overnight line refers to the solution stirring overnight and the UV was taken the next day after the experiment was conducted. The After Concentrating line refers to when the solution was centrifuged to remove further impurities that could have been created during the overnight stirring.*

3.4 Changing Reducing Agents

While the two previous experiments were two different ways of attempting to form the nanocluster, there was not a great difference spectrally between the two experiments. It was then decided to experiment with a different reducing agents. As an endogenous reducing agent, NaOH changes the pH of the amino acids in the wildtype protein internally. Compared to NaOH, NaBH₄ is a faster reducing agent and is an exogenous reducing agent since it donates hydrogens (H⁺) or electrons (e⁻) externally. NaBH₄ was decided as another reducing agent option because when creating gold nanoclusters, it was a successful reducing agent.

Protein Conc. (μM)	Protein (μL)	Ag Conc.	Equiv. of Ag	Ag (μL)	Au Conc.	Equiv. of Au	Au (μL)	H ₂ O (μL)	Reducing Agent
47	100	0.1M	10	0.47	0	0	0	99.53	NaOH
47	100	0.1M	10	0.47	0	0	0	99.53	NaBH ₄
47	100	10mM	2	0.94	10mM	7	3.29	95.77	NaOH
47	100	10mM	2	0.94	10mM	7	3.29	95.77	NaBH ₄
47	100	10mM	3	1.41	10mM	6	2.82	95.77	NaOH
47	100	10mM	3	1.41	10mM	6	2.82	95.77	NaBH ₄

Table 5. Samples with Different Reducing Agents: *The table above explains the contents contained in the 6 different vials. The vials were done with either NaOH or NaBH₄ as the reducing agent.*

The table above explains the different experiments that were conducted at 37°C. Within the three different experiments, two vials were created containing the same ingredients with either NaOH or NaBH₄ as the reducing agent. Similar to the previous experiments, 5% 1M NaOH was added. The method in which NaBH₄ was added was 10X the amount of the silver metal, and the concentration of NaBH₄ created was 50mM. In the 10 equivalents silver experiment, 9.4μL of NaBH₄ were added; in the 7 equivalents gold and 2 equivalents silver experiment, 1.88μL of NaBH₄ were added; in the 6 equivalents gold and 3 equivalents silver experiment, 2.82μL of NaBH₄ were added. It is important to

note that the amount of NaBH_4 that should have been added to each of the vials with NaBH_4 as the reducing agent should be 10X the total amount of metal, not 10X the amount of silver.

The absorbance was taken for each experiment to allow comparisons to be spectrally visible. In the 10 equivalent 100mM Ag sample, there was an extra peak observed with NaOH as the reducing agent. The extra peak was formed at 289nm and indicated that something had formed within the sample and is shown in Figure 22 and Figure 23. For the 7 equivalents gold with 2 equivalents silver sample and the 6 equivalents gold with 3 equivalents silver sample, there were no spectral changes observed in the UV graphs (see Figure 24 and 25). But for these two experiments, the amount of NaBH_4 that was added to each vial was 10X in respect to silver alone. If it was added in 10X the amount of total metal (both gold and silver), the results may be different.

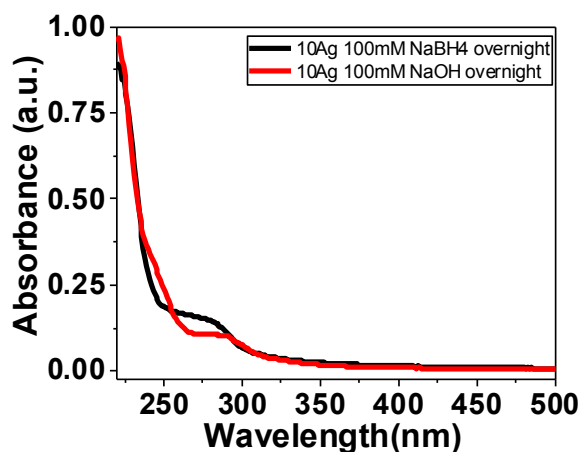


Figure 22. Reducing Agent Comparison with 10 equivalents Ag: *This is a UV graph comparing the 10 equivalents of silver from 100mM (0.1M) silver solution with NaBH_4 and NaOH after stirring overnight.*

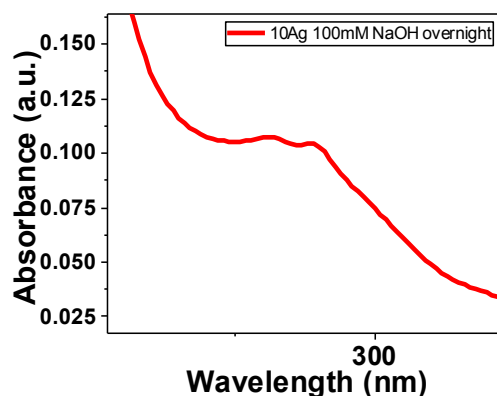


Figure 23. Spectral Change Observed in 10 equivalent Ag: *This is a zoomed in graph from the previous graph of the 10 equivalents of silver from 100mM (0.1M) solution containing NaOH. An extra peak is observed at 289nm in the sample.*

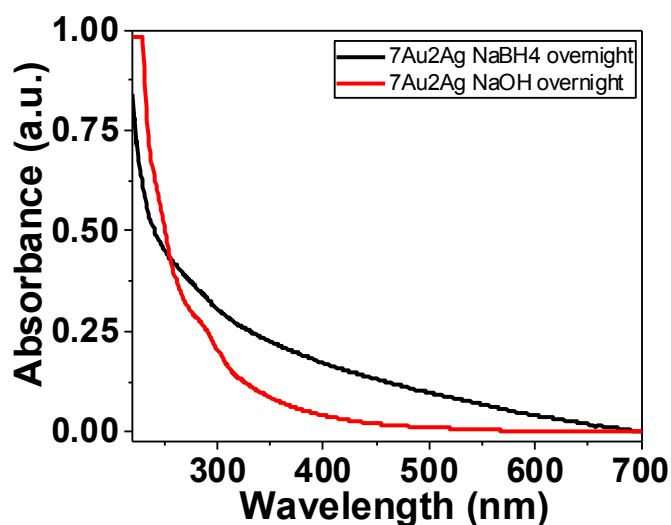


Figure 24. Reducing Agent Comparison with 7 Equivalents Au and 2 Equivalents Ag: *This is a graph comparing the UV of 7 equivalents gold and 2 equivalents silver with NaBH_4 vial to the 7 equivalents gold and 2 equivalents silver with NaOH.*

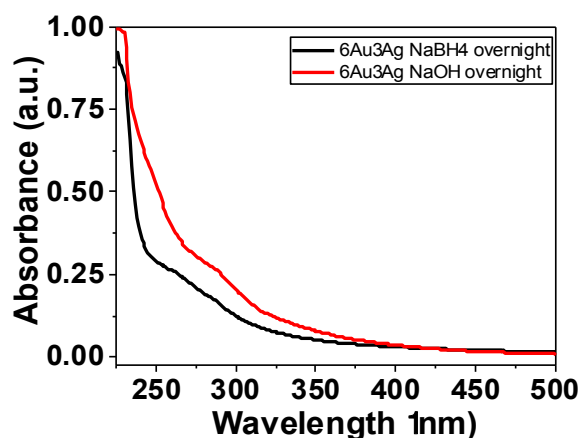


Figure 25. Reducing Agent Comparison with 6 Equivalents Au and 3 Equivalents Ag: This is a graph comparing the UV of 6 equivalents gold and 3 equivalents silver with NaBH_4 vial to the 6 equivalents gold and 3 equivalents silver with NaOH .

3.5 Silver Nanocluster Formation

Until now, there was no observable color change with respect to the synthesis conditions used. From the paper published in the *Journal of Materials Chemistry*, the protocol that they used for silver nanocluster formation was a rapid, room temperature synthesis. We modified their protocol to tailor to our protein and materials. The experiment began by concentrating the wildtype protein to around 50uM in dialysis buffer. Then, 100μL of water and 100μL of 10mM AgNO_3 were mixed and stirred at 400rpm for 10 minutes at 37 °C. 100μL of 50μM wildtype protein was added and stirred for 10 minutes. After this, 6% of 1M NaOH was added and then stirred for another 10 minutes. The final step was drop wise addition of 10mM NaBH_4 until a noticeable color change to a brown solution was created, and then the solution stirred for 30 minutes. The brown color change indicates that the silver nanocluster was formed.

During this experiment, the change in color was observed. When water and silver were stirred, the solution was clear. After the addition of protein and stirring, the solution

turned a milky, opaque color and remained this color through the addition of NaOH. It only took one drop of NaBH₄ to solution to change to a brown color and is shown in Figure 26. This drop was measured to be 4μL of 10mM NaBH₄.

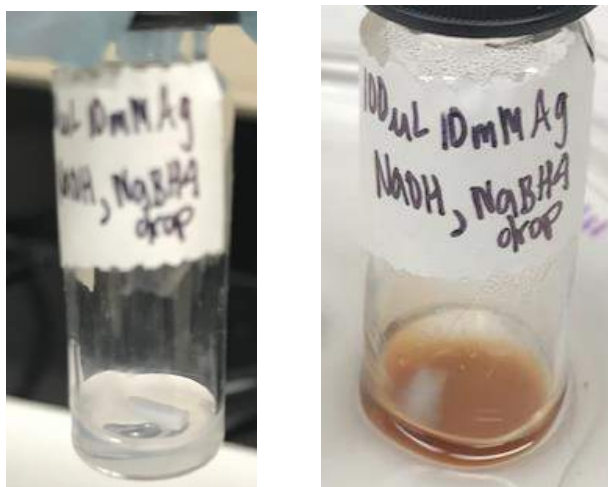


Figure 26. Color Changes Observed: *Left: This photo shows the milky color change after the addition of protein to vial containing 100μL of water and 100μL of silver. Right: This photo was after the addition of one drop of NaBH₄ to the vial. The brown color indicates the formation of a silver nanocluster.*

Since the dropwise addition of NaBH₄ proved to be successful in creating a silver nanocluster, the same protocol was used. As previously stated, it only took one drop of NaBH₄ to cause the brown color change, and the drop was measured to be 4μL. The next experiment used the same protocol, but different amounts of 10 mM NaBH₄—0.5μL, 1μL, 1.5μL, and 2μL. Throughout the experiment, the color changes were observed. When water and silver were stirred, the solution was clear. After the addition of protein and stirring, the solution turned a milky, opaque color and remained this color when NaOH was added. When NaBH₄ was added in different amounts, the degree of brown was different, ranging from lighter to darker brown as the amount of NaBH₄ increases; this is shown in Figure 27. The sample containing 2μL of NaBH₄ was the darkest brown in color.



Figure 27. Increasing Amount of NaBH₄: This image shows the different hues of brown created when different amounts of NaBH₄ was added to the solutions. From left the right, the amount of NaBH₄ increases in 0.5 μL increments.

Due to the formation of silver clusters, a MALDI and native gel were created from the 10mM Ag samples. Since 10mM is a larger concentration, there were three different levels of the concentration created—low, medium, and high—for the MALDI. These were made by centrifuging and diluting the silver sample with dialysis buffer. Samples were made in either 0.5 μL or 1 μL amounts and mixed with a matrix. The matrixes used were alpha-cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid (SA). Unfortunately, the mass of the clusters was not able to be determined from this MALDI because the molecular weights were too high.

Resolving Gel	Stacking Gel
5.89mL of 0.375M Tris HCl pH 8.8	4.275mL of 0.375M Tris HCl pH 8.8
4mL of 30% Bis-Acrylamide	670 μL of 30% Bis-Acrylamide
100 μL of 10% Ammonium Persulfate	50 μL of 10% Ammonium Persulfate
10 μL of TEMED	5 μL of TEMED

Table 6. Native Gel: The table provides the ingredients and amounts used in the resolving and stacking gels of the native gel.

In the native gel, the samples containing different amounts of NaBH₄—0.5 μL, 1 μL, 1.5 μL, and 2 μL—as well as the first sample containing one drop of NabH₄, 4 μL, were run. The samples were prepared by adding 2 μL of loading dye to the samples in their

natural state or by adding 3 μ L of 5% glycerol to the samples that were concentrated by centrifuging for 10 minutes. The samples were run against a low and high molecular weight ladder at 200 volts for 30 minutes on ice and is shown in Figure 28. After the samples ran, it was observed that not all of the samples came out of the wells; this is seen at the top of the gel where the wells are shaded from samples still being there. There were some visible bands above the 130kDa band on the high molecular weight ladder indicating the samples were of a higher molecular weight. Since a native gel was used instead of an SDS gel, the samples were not denatured; the wildtype protein was still intact as a tetramer and is visible in lane 2 of the gel.

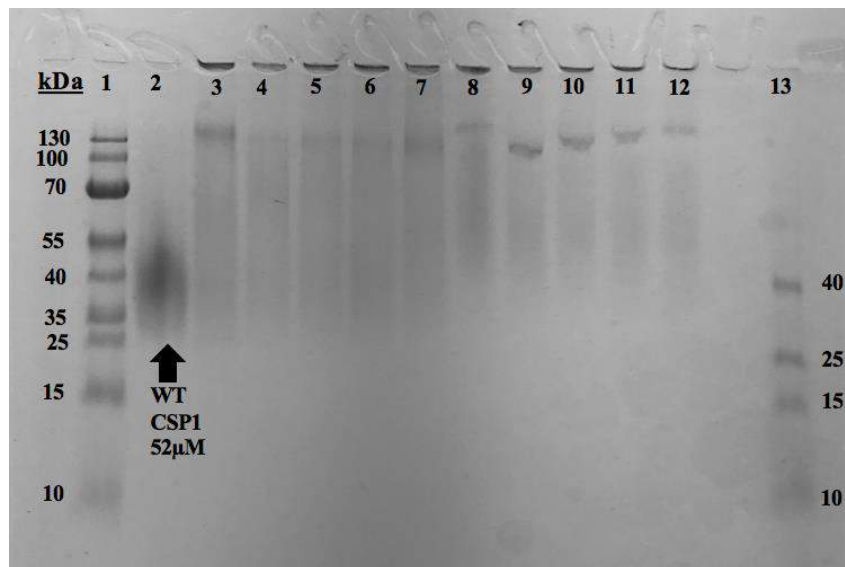


Figure 28. Native Gel of 10mM Ag Cluster: Lane 1 contains the high molecular weight ladder, lane 2 contains Wildtype CSP1 52 μ M protein, lane 3 contains 10mM Ag with 0.5 μ L NaBH₄, lane 4 contains 10mM Ag with 1 μ L NaBH₄, lane 5 contains 10mM Ag with 1.5 μ L NaBH₄, lane 6 contains 10mM Ag with 2 μ L NaBH₄, lane 7 contains 10mM Ag with 4 μ L (one drop) NaBH₄, lane 8 contains concentrated 10mM Ag with 0.5 μ L NaBH₄, lane 9 contains concentrated 10mM Ag with 1 μ L NaBH₄, lane 10 contains concentrated 10mM Ag with 1.5 μ L NaBH₄, lane 11 contains concentrated 10mM Ag with 2 μ L NaBH₄, lane 12 contains concentrated 10mM Ag with 4 μ L (one drop) NaBH₄, and lane 13 contains the low molecular weight ladder.

Even though the change in brown color formation occurred, both the MALDI and native gel results indicated the current concentration of 10mM Ag was too high. It was then

decided to lower the concentration to 5mM Ag and observe those effects for cluster formation. A cluster was formed, and a UV was taken. A graph was created to compare the absorbance of the 5mM Ag cluster with the 10mM Ag cluster and is shown in Figure 29. From the graph, the 10mM Ag cluster followed a pattern similar to nanoparticles not nanoclusters; whereas, the 5mM Ag cluster had a broader peak. The 10mM Ag cluster followed a surface plasmon resonance trend, and this trend confirms the presence of silver nanoparticles. Surface plasmon resonance results from the strong interaction of the silver nanoparticle with light. This occurred because the conduction electrons on the metal surface, which is from silver, underwent a collective oscillation when excited by light at specific wavelengths.⁴ According to a paper published by Dr. Oldenberg, they observed a surface plasmon resonance peak for silver nanoparticles around 425nm. This peak is similar to wavelength location of the 10mM Ag peak which is at 420nm, indicating that the 10mM concentration is assignable to plasmonic Ag particles. Since the 5mM Ag had a broader peak after lowering the concentration, the absorbance graph shows that the nanocluster trend is followed.

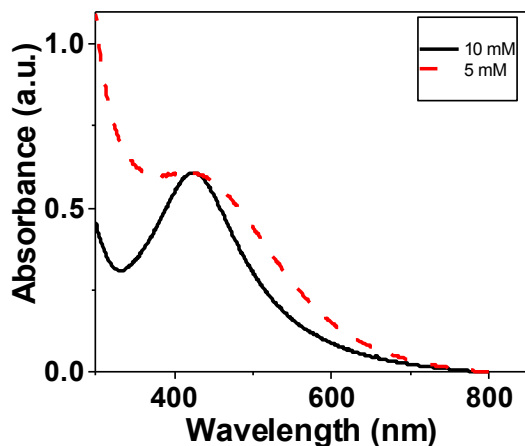


Figure 29. Absorption Comparison between 5mM and 10mM Ag Cluster: *From the absorption graph, the 10mM Ag cluster has a sharp absorption pattern due to surface plasmon resonance. The 5mM Ag cluster has a broader peak.*

Because the concentration of the silver solution was too high, the 10mM AgNO₃ concentration was reduced to 0.5mM, 1mM, 2mM, and 5mM. 100μL of water and 100μL of AgNO₃ at the different concentrations were combined in vials and stirred for 10 minutes at 400rpm and either at 37°C or room temperature. Then, 100μL of wildtype protein 52μM was added and stirred for 10 minutes. 6% 1M NaOH was then added and stirred for 10 minutes. Finally, 0.5μL of 10mM NaBH₄ was added to each vial and stirred for another 10 minutes. The color changes were observed after each addition and are shown in figures 30 and 31. In the vials containing 0.5mM Ag, there was no color change observed throughout the entire experiment. A potential explanation for this is due to such a low concentration of silver, there might not be enough silver to allow a cluster to form.

Vial	After H ₂ O & AgNO ₃	After Protein Addition	After NaOH Addition	After NaBH ₄ Addition
0.5mM Ag RT	Clear	Clear	Clear	Clear
1mM Ag RT	Clear	Clear	Clear	Light Brown
2mM Ag RT	Clear	Clear	Clear	Light Brown
5mM Ag RT	Clear	Opaque	Opaque	Light Brown
0.5mM Ag 37°C	Clear	Clear	Clear	Clear
1mM Ag 37°C	Clear	Clear	Clear	Light Brown
2mM Ag 37°C	Clear	Clear	Clear	Light Brown
5mM Ag 37°C	Clear	Opaque	Opaque	Light Brown

Table 7 Color Changes Observed in Formation of Silver Clusters: *The table explains the observations in color after each addition in the experiment when forming silver clusters. The vials were done at two different temperatures—37°C or room temperature—and at different silver concentrations.*



Figure 30. Ag Clusters at Room Temperature: *The photo shows the color changes of the silver clusters made at room temperature. From left to right, the brown color increases in hue due to a higher silver concentration.*



Figure 31. Ag Clusters at 37°C: *The photo shows the color changes of the silver clusters made at 37°C. From left to right, the brown color increases in hue due to a higher silver concentration.*

The absorbance was taken for all of the samples and is shown in figure 32 and 33. Figure 32 shows the absorbance values for the samples at room temperature and Figure 33 shows the absorbance values for the samples at 37°C. Regardless of the temperature being at room temperature or 37°C, the absorbance increased as the concentration of silver increased. At room temperature, the 2mM sample had a peak at 430nm (2.88eV), the 5mM sample had a peak at 445nm (2.78eV). The 1mM concentration's broad peak at 450nm (2.75eV) was more active and visible at 37°C when compared to the room temperature

sample. At 37°C, the 2mM sample showed a peak at 460nm (2.69eV), and the 5mM sample showed a peak at 445nm (2.78eV). The absorbance for the 0.5mM silver concentration was not spectrally active in either temperature conditions. An energy plot was created to help further explain the temperature comparison of the silver nanoclusters and is shown in Figure 34. Even though the experiments were performed at two different temperatures, the clusters follow the same trend line. When heat is provided, the 1mM, 2mM, and 5mM silver concentrations vials at 37°C had a higher absorbance than the room temperature vials did.

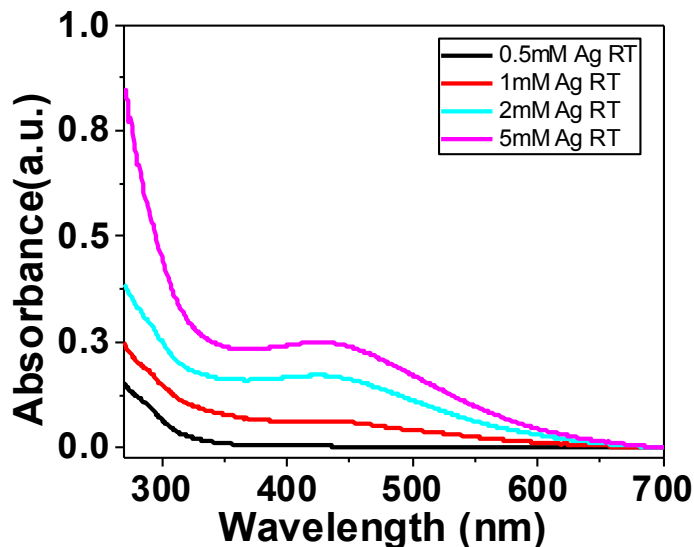


Figure 32. Absorbance of Clusters at Room Temperature: *This graph shows the UV Absorbance of the silver nanocluster samples at room temperature.*

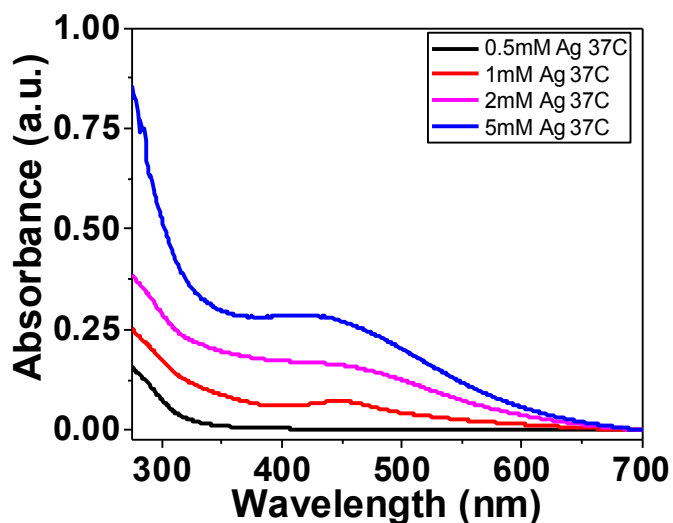


Figure 33. Absorbance of Clusters at 37°C: *This graph shows the UV Absorbance of the silver nanocluster samples at 37°C.*

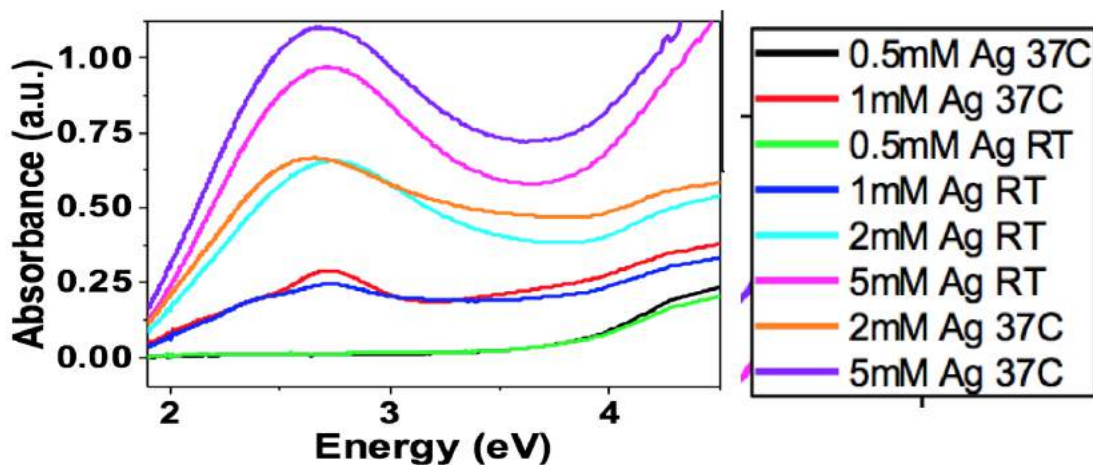


Figure 34. Energy Plot of Silver Clusters: *This energy graph compares the silver cluster vials experimented at room temperature and 37°C.*

From all the different concentrations of silver that were made and used for creating clusters, MALDI was taken again. The concentration that worked was 2mM Ag at 37°C and is shown in Figure 36. The absorbance of the 2mM Ag sample with at peak at 450nm is shown in Figure 35. This mass ionization technique allowed the mass/charge of the wildtype CSP1 protein alone to be found as well as the mass/charge of the silver cluster to

be discovered. When the charge is +1, the protein peak and mass is 13902. When the charge is +2, the protein peak moves to the peak on the far left following the blue line, and the mass is 6951. The silver nanocluster mass ionization peak when the charge is +1 is 16330. When the charge is +2, the silver nanocluster peak moves to the peak on the far left following the red line, and the mass is 8165. The number of silver atoms in our protein is 25.28 atoms.

$$\frac{16330 - 13902}{107.87 \text{ grams}} = 25.28 \text{ atoms of Ag}$$

Equation 1: Atoms of Silver

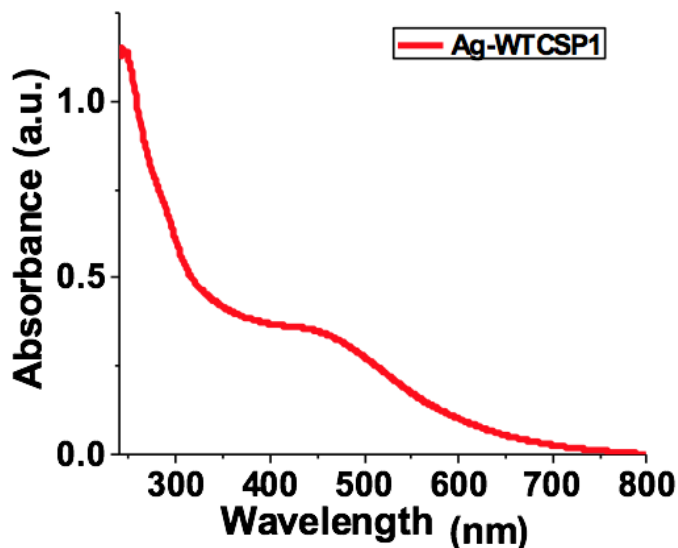


Figure 35. Absorbance of 2mM Ag Cluster: *This figure is from the UV of the 2mM Ag cluster that showed results when the MALDI was taken. The peak at 450nm indicates the formation of the cluster.*

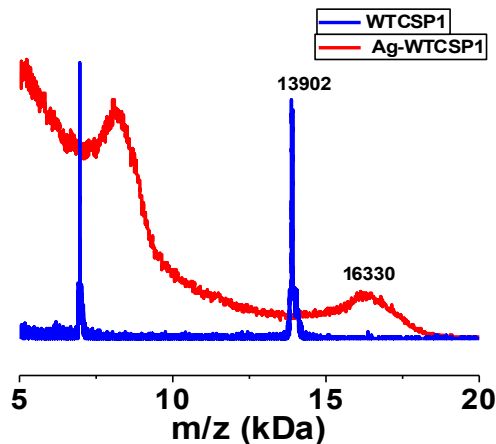


Figure 36. MALDI of 2mM cluster: *The graph above came from the MALDI findings in the 2mM Ag cluster.*

While results from the MALDI were received for the 2mM Ag cluster, a native gel was made. All of the different silver concentration samples—0.5mM, 1mM, 2mM, and 5mM— at room temperature and 37°C were run. From the native gel, there were three single bands corresponding to CSP1-AgNCs at 70,000 Da. These three samples were from 0.5mM Ag cluster at room temperature, 1mM Ag cluster at room temperature, and 1mM Ag cluster at 37°C. There was no clear band from the 2mM Ag cluster. This native gel, represented in Figure 37, was taken the day after the experiments were completed, so this could be a potential reason for why the 2mM Ag cluster did not appear but had positive results in the MALDI. The 5mM clusters that were made did not have a visible band. At the top of lane 2 and 6, it is visible that not all of the sample travelled through the gel, so the 5mM cluster concentration could be too high for results to be seen.

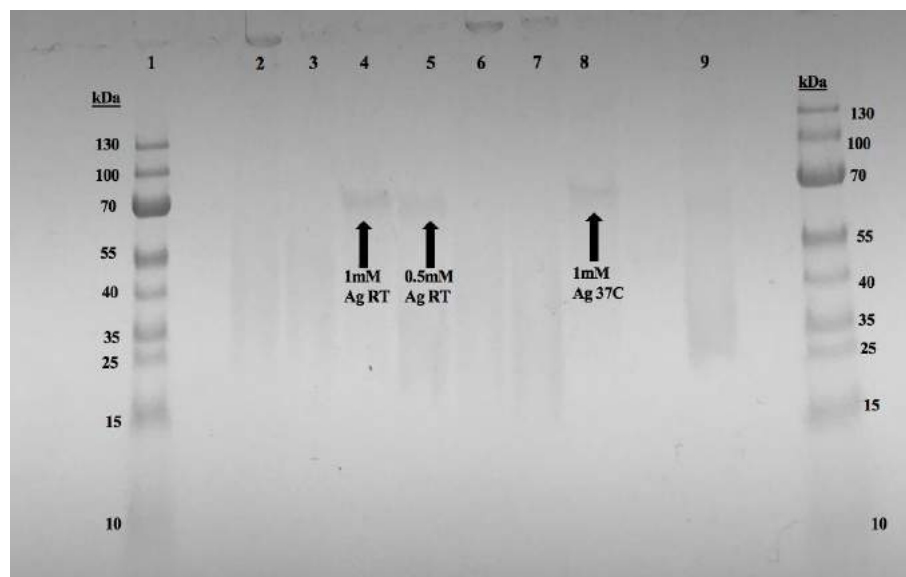


Figure 37. Native Gel of Different Ag Cluster Concentrations: *In the native gel, the samples that were run from right to left were lane 1 is the high molecular weight ladder, lane 2 is 5mM Ag at room temperature, lane 3 is 2mM Ag at room temperature, lane 4 is 1mM Ag at room temperature, lane 5 is 0.5mM Ag at room temperature, lane 6 is 5mM Ag at 37°C, lane 7 is 2mM Ag at 37°C, lane 8 is 1mM Ag at 37°C, and lane 9 is 0.5mM Ag at 37°C. The bands that were seen were from the 0.5mM Ag sample at room temperature, and the 1mM Ag sample at 37°C and room temperature.*

3.6 Conclusion

The purpose of this thesis was to find a protocol for synthesizing silver nanoclusters. In the beginning, the protocols used mimicked the protocol that was used for synthesizing gold nanoclusters. Since silver and gold are found in the same column of the periodic table, both elements share similar properties in the way that they react. But, silver is easily degradable and oxidizes faster than gold, so it doesn't work using the same nanocluster protocol as gold does.

After following the experiment that was published in the *Journal of Materials Chemistry*, the formation of silver nanoclusters was achieved. The original 10mM Ag concentration was of a higher concentration which was concluded from the results of both

the MALDI and native gel. When the 10mM Ag was lowered to 5mM Ag, the absorbance was compared between the two samples. This furthered the conclusion that the 10mM Ag samples were too large. The absorbance pattern from the 10mM Ag sample followed the same trends seen when creating nanoparticles because the peak was from surface plasmon resonance. Unlike the 10mM Ag sample, the 5mM Ag absorbance peak was broader resembling nanocluster formation. Once formation of nanoclusters was observed, various lower concentrations of silver were created. Another MALDI and native gel were done. From the MALDI, the 2mM Ag nanocluster at 37°C had positive results. From the native gel, bands were visible for the 0.5mM Ag nanocluster at room temperature and for the 1mM Ag nanoclusters at room temperature and 37°C. This showed that it is possible to make silver nanoclusters of different sizes and conditions.

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