Composite Gold-Silver Alloy Nanoparticles for GFP Interactions

Daniel Ling
East Greenwich High School
300 Avenger Drive
East Greenwich, RI 02818
Abstract: Gold nanoparticles (AuNPs) are widely used for their optical sensitivity and convenience. The unique optical properties of AuNPs arise from their surface plasmon resonance (SPR) when interacting with light of various wavelengths in the visible range. We are developing a novel gold and silver composite nanoparticle (AuAg-NP) with tunable optical properties, synthesized by reduction of AgNO$_3$ and HAuCl$_4$ with oleylamine and octadecene as reducing agents. With precise synthesis and modification, we are able to fine-tune the SPR peak of the AuAg-NPs through adjustments in the Au/Ag composition, size, and surface conjugations. By controlling the characteristics of the AuAg-NPs, we study the energy transfer between the AuAg-NPs and other optically active molecules, specifically the green fluorescent protein (GFP). The GFP, known for its distinctive green fluorescence, has extensive use in cellular and molecular imaging. Both the AuAg-NPs and the GFP have similar light-activated properties in the same optical range, allowing for resonant energy transfer. We are investigating ligand addition and silica coating on the AuAg-NPs and studying their effects on the NP-GFP coupling. We have observed a wide range of interactions, including quenching of the GFP fluorescence.
ACKNOWLEDGEMENTS

First I want to thank the Department of Chemistry at Brown University for allowing me to conduct my research with access to their facilities. In addition, I would like to acknowledge the assistance of Mr. Don Ho and Professor Shouheng Sun of the Department of Chemistry at Brown University. Finally, I would like to express my gratitude to my family for their love and support during the intensive process of scientific research.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>1</td>
</tr>
<tr>
<td>List of Figures</td>
<td>1</td>
</tr>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>4</td>
</tr>
<tr>
<td>Results</td>
<td>5</td>
</tr>
<tr>
<td>Discussion and Conclusions</td>
<td>10</td>
</tr>
<tr>
<td>References</td>
<td>13</td>
</tr>
</tbody>
</table>
LIST OF TABLES

TABLE 1  “DNH2015” Surface Plasmon Resonance Peaks
TABLE 2  “DNH2011-4” Surface Plasmon Resonance Peaks Over Time
TABLE 3  “DNH2011-4” Surface Plasmon Resonance Peaks with Silica Shell

LIST OF FIGURES

FIGURE 1  Nanoparticle conjugated with block polymers
FIGURE 2  Structure of PEG-diamine
FIGURE 3  Structure of Lipoic Acid-PEG carboxylic acid
FIGURE 4  UV-Vis spectra of “DNH2015” at various stages of solubilization and purification
FIGURE 5  “DNH2011-4” characterized for stability after synthesis
FIGURE 6  “DNH2011-4” characterized for silica shell comparison
FIGURE 7  Fluorescence of nanoparticle solutions from 500 nm to 700 nm
FIGURE 8  Fluorescence of PBS and nanoparticle solutions from 500 nm to 700 nm
INTRODUCTION

Nanoparticle research has garnered interest in the applied sciences for the past few decades. In particular, metal nanoparticles are attractive due to their unique biomedical, catalytic, and optical properties. There exist numerous methods of preparation of metal nanoparticles. However, since geometry greatly affects the optical properties of the dispersion, certain ligand addition reactions and surface modifications are necessary for desired functionality. One potential application of these metal nanoparticles is the resonant transfer of energy through surface plasmon resonance to other optically active molecules. An optically active molecule of interest is the green fluorescent protein (GFP). The GFP molecule has extensive use in biological imaging for its distinctive green fluorescence, acting as optical sensors for understanding various cellular activities. However, the optical properties of AuAg alloy nanoparticles and GFP have never been combined for greater fluorescence capabilities and advanced cellular visualization. Although metal nanoparticles have been applied to biological applications such as calorimetric detection of DNA and proteins, fluorescence enhancement has not been studied extensively.

Here we propose to investigate the conjugation of AuAg nanoparticles and GFP (NP-GFP) in addition to NP-GFP interactions. We report a one-pot synthesis by reducing salts, HAuCl₄ and AgNO₃. Varied precursor amount and reaction time can control the Au/Ag ratio and nanoparticle size. These atomic systems of such small dimension can be finely tuned in size and shape to exhibit desirable properties. AuAg-NPs have interesting optical responses due to the mobile conduction electrons that are present throughout the metal. These conduction electrons can oscillate and give rise to surface plasmon resonance (SPR). Through ultraviolet-visible spectroscopy (UV-Vis), the intrinsic frequency at which the delocalized electrons in a nanoparticle resonate, giving rise to its SPR peak, can be ascertained by testing various frequencies of light. This property of resonant energy absorption and emission is exhibited not only by AuAg alloy nanoparticles, but also biological molecules. A biological molecule, GFP, has an intrinsic excitation peak similar to that of AuAg nanoparticles. For AuAg nanoparticles, it is possible to tune the frequency of the SPR peak exactly to the frequency of GFP fluorescence. In an earlier study by Kostov et al., it was found that GFP resonance can excite an SPR mode in a 50 nm silver film when a 3 nm GFP monolayer is placed on it, separated by a 5 nm SiO₂ layer. Here we study the feasibility of the reverse process, a transfer of energy from AuAg-NPs to GFP, by coupling the SPR of the AuAg-NPs with the intrinsic GFP fluorescence peak. Even a small enhancement of the GFP fluorescence signal by the AuAg-NPs would be of great interest to the biomedical field.

In order to determine an effective method of enhancing GFP fluorescence using nanoparticle SPR, an optimum orientation and geometry of the nanoparticle conjugated with GFP must be resolved. We will vary the gold and silver percent composition, nanoparticle size, and surface modifications to assess the efficiency of various syntheses and eventually determine the most effective composite for GFP enhancement through fluorescence spectroscopy. One surface modification is the addition of a silicon oxide coating during synthesis of the nanoparticle. It should be noted that SiO₂ was also used by Kostov et al. for their studies. This SiO2 layer can be used to fine-tune the distance between the nanoparticle and GFP. Moreover, ligand addition necessary for water
solubilization, such as oleylamine-polyethylene glycol (OAm-PEG) and lipoic acid-polyethylene glycol (LPA-PEG), will contribute to the geometry of the structure. The desired structure of our system is shown in Figure 1.

![Fig. 1 Nanoparticle conjugated block polymers](image)

Finally, the particles will be targeted with anti-GFP antibodies (Ab), for specific and selective recognition of GFP in solution. We will carry out fluorescence measurements of the AuAg-NPs with antibody conjugation and GFP attachment and the results will be compared with those on free GFP in phosphate buffered saline (PBS).

Metal nanoparticles have been studied in recent years for their optical sensitivity and efficacy. Many means of synthesis of metal nanoparticles have been developed in recent decades such as laser ablation\(^1\), phase transfer\(^2\), and the co-reduction of AuCl\(_4\) and AgNO\(_3\) with other organic compounds\(^3\). These metal nanoparticles have vast applications in analytical chemistry and bio-imaging. In particular, gold and silver nanoparticles are ideal tools for biological applications because of their optical efficiency and unique properties: they are nontoxic to cells and their interactions with biological molecules can be easily controlled. The optical sensitivity of the AuAg alloy nanoparticles has been demonstrated in the detection of attached biomolecules and therapeutic applications\(^4\,5\).

Recent pioneering studies by Kostov et al.\(^6\) have shown fluorescence enhancement. A monomolecular layer of GFP has been shown to exhibit 12-fold enhancement of its fluorescence based on surface plasmon-coupled emission (SPCE)\(^6\). If these findings are accurate, the hypothesized fluorescence enhancement through NP-GFP
coupling, which employs the surface plasmon resonance properties of AuAg-NPs, should be feasible.

An enhancement of GFP fluorescence through AuAg nanoparticle coupling will be useful for scientists in various biological disciplines\cite{15-19}. The GFP molecule has particular value in the field of biochemistry due to its low aggregation, high quantum yield, and high sensitivity. The GFP fluorophore absorbs energy of incoming light to emit green fluorescent light. Due to its fluorescence, GFP is considered the “microscope” of the 21st century and is one of the most widely used tools in molecular and cellular biology after it was discovered and developed by Nobel laureates Osamu Shimomura, Martin Chalfie, and Roger Tsien\cite{15, 20}. The advantage of GFP over other fluorescent molecules is its accurate protein labeling, non-toxicity to living cells, and cell tagging. When the GFP gene is expressed, a protein attached with GFP can be easily identified and tracked using the GFP’s fluorescence, making the invisible dynamics of a cell visible\cite{19}. GFP can be used for analyzing gene expression, tracing intercellular dynamics, and measuring protein-protein interactions. Therefore, the development of GFP in the past decade has provided another dimension to cellular visualization. If the NP-GFP coupling proves viable, the enhancement of the GFP fluorescence will enable researchers to observe more complex biological structures, and even examine individual molecules, adding to the ongoing revolution in cell biology that the discovery of GFP has initiated.

**MATERIALS AND METHODS**

*Nanoparticle Synthesis*

All chemicals used in this study were purchased from Sigma-Aldrich Chemical Company. All reactions were carried out in ventilated fumehoods. A solution of AgNO\textsubscript{3} was formed by dissolving 2 mmol of AgNO\textsubscript{3} in 20 mL of oleylamine\cite{21}. The solution was slowly heated to 100 °C under inert nitrogen flow. A HAuCl\textsubscript{4} solution (0.2 mmol HAuCl\textsubscript{4} in 5 mL of octadecene with 1 mL of oleylamine) was injected into the hot solution. The solution temperature was kept at 120 °C for 0.5 hours before cooling to room temperature. Using energy-dispersive spectroscopy (EDX), the Au/Ag ratio was measured to be 0.82/0.18. By altering precursor amount and reaction time, the Au/Ag ratio can be varied. A solution of 2 mmol of AgNO\textsubscript{3}, 0.2 mmol of HAuCl\textsubscript{4}, and a growth time of 1 hour (at 120 °C), yielded Au\textsubscript{0.60}Ag\textsubscript{0.40} NPs. 4 mmol of AgNO\textsubscript{3} reacted with 0.2 mmol of HAuCl\textsubscript{4} for 1 hour to yield Au\textsubscript{0.52}Ag\textsubscript{0.48} NPs, while a 2 hour reaction time produced Au\textsubscript{0.39}Ag\textsubscript{0.61} NPs.

*Nanoparticle Functionalization*

Block polymer synthesis consisted of various ligands. PEG-diamine, shown in Figure 2, was dissolved (2 equivalents) in 20 mL of dichloromethane (DCM) at 4 °C.

![Fig. 2 Structure of PEG-diamine](image-url)
Lipoic acid-N-Hydroxysuccinimide (LPA-NHS) was dissolved (1 equivalent) in 5 mL of DCM over 1 hour. The solution was warmed to room temperature, stirred overnight, and the solvent was evaporated, yielding the resultant ligand shown in Figure 3.

Fig. 3 Structure of Lipoic Acid-PEG carboxylic acid

AuAg NPs in hexane were dried and re-suspended in 5 mL of chloroform. Ligand was dissolved in 5 mL of chloroform and combined with solution of NPs. The resulting solution was placed in shaker for 24 hours. In addition, a silica coating was added to provide rigid protection and vary separation between the fluorophore and the particle. To ensure accurate characterization, the AuAg-NP dispersions were purified through centrifugation and dialysis. Centrifugation was utilized at 8500 revolutions per minute (RPM) for 8 minutes to precipitate nanoparticles and yield a supernatant solution. Some accumulated nanoparticles were filtered out. The particles were allowed to dry and were resuspended in 10 mL of deionized water. The nanoparticles were dialyzed using a semipermeable membrane. The membranes were then placed in water and stirred gently overnight.

Nanoparticle Characterization

First the nanoparticles were characterized using dynamic light scattering (DLS). The laser was allowed to stabilize for 30 minutes after being turned on. A quartz cuvette was filled with the nanoparticle solution and analyzed using the DLS machine.

The intrinsic SPR peak of the nanoparticles was ascertained through Ultraviolet-visible (UV-Vis) spectroscopy. The UV-Vis lightbulb was allowed to warm for 15 minutes after turning on the PerkinElmer Lambda 35 Spectrophotometer. A glass cuvette was filled with the nanoparticle solution and analyzed in the 200 nm to 900 nm wavelength range. The absorption level was set at 1.0 arbitrary units (A.U.) for controlled comparison of SPR peaks. Using UV-Vis spectroscopy, the nanoparticles were analyzed for absorption at various stages of purification, stability over a one-month period, and comparison with a silica coating. All absorption measurements were graphed and normalized using Origin 8.

The fluorescence peaks of the GFP-AuAg-LPA-PEG-Ab solution, GFP-AuAg-LPA-PEG-Ab with excess GFP solution, free GFP solution, and PBS solution were determined with a PerkinElmer Fluorescence Spectrometer. A quartz cuvette was filled with the solutions and was analyzed in the 500 nm to 700 nm wavelength range. The fluorescence was measured in intensity counts (kHz).

RESULTS
Characterization through UV-Vis spectroscopy consisted of various examinations. First, one solution of nanoparticles of a 60 to 40 Au/Ag ratio, labeled DNH 2015, was analyzed through spectroscopy as shown in Figure 4.

The nanoparticle composition of DNH2015 was first analyzed in hexane, labeled “DNH2014 Hexane” and denoted as the black curve as shown in Figure 4. The same composition of nanoparticles was solubilized in water with the block polymer OAm-PEG, labeled “DNH2015 OAm-PEG_Pre-Dialysis” and denoted by the red curve. After centrifugation and dialysis, the supernatant solution and pellet solution were both characterized by the UV-Vis spectrometer, labeled “DNH2015 Supernatant Sample” (blue curve) and “DNH2015 Aggregate Sample” (green curve) respectively. The SPR peaks of the four samples are shown in Table 1.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Wavelength of SPR Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNH2014 Hexane</td>
<td>436.17 nm</td>
</tr>
<tr>
<td>DNH2015 OAm-PEG_Pre-Dialysis</td>
<td>446.15 nm</td>
</tr>
<tr>
<td>DNH2015 Supernatant</td>
<td>445.91 nm</td>
</tr>
<tr>
<td>DNH2015 Aggregate</td>
<td>529.79 nm</td>
</tr>
</tbody>
</table>

Table 1
“DNH2015” Surface Plasmon Resonance Peaks

A set of nanoparticles of another composition, labeled “DNH2011-4” was analyzed for stability over time using UV-Vis spectroscopy, as represented by Figure 5.
The sample, “DNH2011-4”, is a supernatant solution in water and was analyzed one week following synthesis, denoted by the black curve, and one month following synthesis, denoted by the red curve. Table 2 describes the SPR peaks of the two measurements.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Wavelength of SPR Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNH2011-4 (1 week)</td>
<td>493.12 nm</td>
</tr>
<tr>
<td>DNH2011-4 (1 month)</td>
<td>498.23 nm</td>
</tr>
</tbody>
</table>

Table 2
“DNH2011-4” Surface Plasmon Resonance Peaks Over Time

The sample “DNH2011-4” was also analyzed through UV-Vis spectroscopy for comparison of SPR peaks without a silica coating and with a silica coating, illustrated by Figure 6.
Fig. 6 “DNH2011-4” characterized for silica shell comparison

“DNH2011-4 Supernatant Sample” in water one week after synthesis and purification is denoted by the black curve in Figure 6. The same composition of nanoparticles was coated with silica and solubilized in ethanol. The absorption measurements of “DNH2011-4” with the silica shell is denoted by the red curve. The corresponding SPR peaks are shown in Table 3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Wavelength of SPR Peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNH2011-4 Supernatant</td>
<td>493.12 nm</td>
</tr>
<tr>
<td>DNH2011-4 Si in Ethanol</td>
<td>503.08 nm</td>
</tr>
</tbody>
</table>

Table 3
“DNH2011-4” Surface Plasmon Resonance Peaks With Silica Shell

After characterization through UV-Vis spectroscopy, solutions were prepared for fluorescence spectroscopy. A solution of GFP dispersed in PBS, a NP-GFP solution, and a NP-GFP solution with excess GFP were all analyzed by the fluorescence spectrometer.
As illustrated by Figure 7, the fluorescence of GFP decreases when conjugated with the AuAg-NPs in comparison to free GFP in solution, shown by the red curve and black curve respectively. When an equal molar amount of GFP is added to the NP-GFP solution as shown by the blue curve, the resulting solution restores its fluorescence intensity.

For further comparison, a solution of PBS was analyzed by the fluorescence spectrometer and graphed along with the solution of GFP and a solution of NP-GFP with a longer ligand on the same pair of axes. However, note the difference in the scale of intensity.
Fig. 8 Fluorescence of PBS and nanoparticle solutions from 500 nm to 700 nm

The AuAg-NPs were modified with a longer ligand of 20 kiloDaltons (kD). The fluorescence of the GFP is unmistakable, as seen in the red curve, when compared to the PBS solution alone, shown by the black curve. But once again, the intensity of the fluorescence of the GFP decreases considerably when attached to the AuAg alloy nanoparticle, as seen in the blue curve.

DISCUSSION AND CONCLUSIONS

In this study, our AuAg-NPs were synthesized by means of simultaneous reduction of gold and silver salts with organic solvents. The nanoparticles were characterized through UV-Vis spectroscopy, and GFP enhancement was tested through fluorescence spectroscopy. As evidenced in Figure 7 and Figure 8 in the Results section, the NP-GFP solution exhibited decreased fluorescence in comparison to GFP alone in PBS solution. Therefore, the optical properties of the AuAg-NPs apparently quenched the fluorescence of the GFP molecule, contrary to the desired outcome.

The UV-Vis spectra measurements that were performed reveal features of the synthesized nanoparticles in this study. As seen on Figure 4, the SPR peaks of the same sample in various stages of purification and solvation were 436.17 nm in hexane, 446.15 nm solubilized in water before dialysis, 445.91 nm as a supernatant solution, and 529.79 nm as a precipitate solution. Disregarding the precipitate, the SPR peaks of sample “DNH2015” did not shift a considerable amount on the 200 nm to 900 nm optical range. The aggregate sample appears to have measured a significant shift in the SPR peak due to
variable accumulations and irregular sizes of nanoparticles, which may have caused distorted absorption.

Figure 5 displays absorption at different times after synthesis of the AuAg-NPs. The wavelength of the SPR peak of “DNH2011-4” after one week following synthesis is 493.12 nm. The SPR peak of “DNH2011-4” after one month following synthesis is 498.23 nm. Evidently, the sample “DNH2011-4” exhibited minimal shift in its SPR peak over time when considering the extensive range at which it was characterized. These measurements demonstrate a relative stability of the synthesized AuAg-NPs.

The SPR peak of “DNH2011-4 Supernatant Sample” was compared to that of the same composition of nanoparticle coated in silica and solubilized in ethanol. The wavelength of the SPR peak of the supernatant is 493.12 nm and the SPR peak of the silica-coated sample occurs at 503.08 nm. The silica coating had a reasonable effect on the absorption of sample “DNH2011-4”. The shift in SPR, however, can also be attributed to the different solvents used.

Excess GFP was added to the solution of NP-GFP and the fluorescence restored its intensity to match that of the original solution of GFP in PBS, confirming the quenching result. As illustrated in Figure 7, the NP-GFP in solution with excess GFP nearly equaled the solution of GFP in fluorescence intensity. Since excess GFP was added in an equal amount to that of GFP alone in PBS, it can be interpreted that the NP-GFP composite did not contribute to the fluorescence. However, since the NP-GFP solution measured a reasonable intensity of fluorescence to begin with and the excess GFP was added in an equal molar amount, one may question why the fluorescence of the NP-GFP with excess GFP did not exceed that of the GFP alone. One possible answer is that increased light scattering with the addition of excess GFP may decrease the fluorescence efficiency of the system. Another possible explanation is a distorted geometry of the composite given an increased amount of GFP in solution and increased possibility for unintentional GFP attachment. The SPR processes involving electron oscillations require proper geometry for the resonance to occur; therefore, an irregular orientation of the nanoparticle and GFP complex due to excess GFP could also cause quenching.

Effective fluorescence enhancement requires a specific spatial orientation of the AuAg-NP and GFP\textsuperscript{22}. Therefore, the proximity of the GFP molecule on the AuAg-NP must be considered. A close proximity between the two molecules may have caused the decrease of GFP signal compared to that of isolated GFP. This conjecture is called into question when comparing data sets of Figures 7 and 8. The difference between the experimentation illustrated in Figure 7 and Figure 8, as noted in the Results section, is the length of the ligands on the nanoparticles. In Figure 8, the LPA-PEG ligand utilized was 20 kD in comparison to the 2 kD of the ligands in Figure 7. The fluorescence peaks of the data sets of NP-GFP with LPA-PEG of 2 kD on Figure 7 and NP-GFP with LPA-PEG of 20 kD on Figure 8 are similar in comparison. Therefore, it appears that the molecular weight and length of ligands do not have a substantial effect on fluorescence.

Our observation of quenching of GFP fluorescence seems to contradict with previous findings of fluorescence enhancement\textsuperscript{9, 22}. We note that in the study of Kostov et al.\textsuperscript{9}, the GFP proteins form a monolayer\textsuperscript{9} on top of the silver firm. In our system, the GFP molecules are coupled to AuAg-NPs individually. Instead of acting as antennas for the GFP molecules, the AuAg-NPs apparently act as absorbers, drawing energy out of the
GFP. Whether or not the enhancement effect observed earlier is a collective phenomenon of many GFP molecules will require further systematic studies.

Although a decrease in fluorescence was observed, the study indicates a strong interaction between the GFP and the AuAg-NP. This apparent interaction may aid microscopists that rely on specificity with imaging. With future developments in GFP fluorescence, scientists may achieve advanced control in bioimaging by combining the optical properties of gold and silver nanoparticles with the fluorescence capabilities of other optically active molecules such as GFP.

Future studies will be directed toward developing and modifying AuAg-NP synthesis and functionalization. Ongoing efforts will focus on understanding fluorescence interactions to a greater degree. Further investigation of GFP interactions using AuAg nanoparticles can conceivably extend to applications involving other biological molecules that have similar optical properties.
REFERENCES


