Characterization of Gold Nanoparticle Antimicrobials

By

Kelsey Higgins

B.S Texas Tech University 2012

A thesis submitted to the Faculty of the Graduate School of the University of Colorado in partial fulfillment of the requirement for the degree of

Master of Science

Department of Chemistry and Biochemistry

2014
This thesis entitled:
Characterization of Gold Nanoparticle Antimicrobials
Written by Kelsey Higgins
Has been approved for the Department of Chemistry and Biochemistry

__________________________________________
Dr. Gordana Dukovic

__________________________________________
Dr. Daniel Feldheim

__________________________________________
Dr. Wei Zhang

Date ________________________________

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.
Nanoparticles have been gaining recognition for their potential in drug delivery; however, a new niche is growing. Nanoparticles are no longer simply carrying a drug, but rather being designed as the therapeutic itself. The Feldheim group has designed gold nanoparticles functionalized with an array of organothiol ligands varying in intermolecular forces, hydrophilicity and functional groups. Optimization of this library of drug nanoparticles produced drug nanoparticles with a bacteriostatic effect against bacteria, such as E. Coli and Klebsiella. There are still many pieces of the puzzle to address. This thesis presents research conducted to 1) Explore which parameter during the synthesis process effects potency of the nanoparticle 2) Develop a new characterization method for determining the composition of the self-assembled monolayer.
CONTENTS

CHAPTER

I. INTRODUCTION: GOLD BACTERIOSTATIC NANOPARTICLES

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Background</td>
<td>1</td>
</tr>
<tr>
<td>Thiol Modified Gold Nanoparticles</td>
<td>2</td>
</tr>
<tr>
<td>Nanoscale Structure Activity Relationships</td>
<td>3</td>
</tr>
<tr>
<td>Strategy</td>
<td>3</td>
</tr>
<tr>
<td>Materials Characterization</td>
<td>4</td>
</tr>
<tr>
<td>HPLC Method Development</td>
<td>6</td>
</tr>
<tr>
<td>Applications</td>
<td>7</td>
</tr>
<tr>
<td>Summary</td>
<td>7</td>
</tr>
<tr>
<td>Thesis Organization</td>
<td>7</td>
</tr>
<tr>
<td>References and Notes</td>
<td>8</td>
</tr>
</tbody>
</table>

II. THE OPTIMAL FEED RATIO

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>9</td>
</tr>
<tr>
<td>Hypothesis</td>
<td>9</td>
</tr>
<tr>
<td>Strategy</td>
<td>10</td>
</tr>
<tr>
<td>Method</td>
<td>10</td>
</tr>
<tr>
<td>Effects of Total Amount of Ligand during the Exchange</td>
<td>13</td>
</tr>
<tr>
<td>Optimal Feed Ratio</td>
<td>17</td>
</tr>
<tr>
<td>Applications</td>
<td>18</td>
</tr>
<tr>
<td>Summary</td>
<td>18</td>
</tr>
</tbody>
</table>
### TABLES

#### Tables

1. **Minimum Inhibition Concentration**  
   Page 18

2. **Ratio of Cysteamine to SMPS**  
   Page 29
FIGURES

Figures

1. Thiols of Interest ..........................................................53
2. Staple Motif ..........................................................53
3. TEM of Gold Nanoparticles .............................................53
4. Method Diagram HPLC ..................................................53
5. TEM of Gold Nanoparticles .............................................53
6. TEM of Thiol Exchanged Nanoparticle Batch T1 .......................53
7. Average Diameter ..........................................................53
8. Distribution of Size ..........................................................53
9. Distribution of Size ..........................................................53
10. Optimal Feed Ratio ..........................................................53
11. Method Diagram HPLC ..................................................53
12. Cysteamine Bonded to Dye HPLC ......................................53
13. SMPS Bonded to Dye HPLC ..............................................53
14. Calibration Curve of Cysteamine ........................................53
15. Calibration Curve of SMPS ....................................................5
Chapter 1

Introduction: Gold Bacteriostatic Nanoparticles

1.1 Background

An estimated 11,285 fatalities occur each year due to Methicillin-resistant Staphylococcus aureus (MRSA). This fact is an example of the outcomes of bacteria developing resistance to known therapeutics, an issue that challenges humanity. Bacteria are constantly evolving systems and mechanisms to combat the very therapeutics that allow us to live without the fear of lethal infections.

There are various ways nanoparticles are currently being utilized, including as cancer therapeutics and as drug carriers. Recent research suggests that nanoparticles, particles in the 1-100 nanometer range, could be the next class of antimicrobials. Nanoparticles have a cornucopia of interesting characteristics which contribute to the possibilities of creating a new class of antimicrobials. Research conducted by Stellacci found that gold nanoparticles could travel into the cell, either through perhaps through the cell membrane or maybe through efflux pump channels, though since they are a much different size than traditional molecule drugs they may not be pumped back out of the cell through efflux pumps. This could contribute to the lack of resistance apparent for E. Coli defending against the bacteriostatic nanoparticles. Another intriguing aspect to gold nanoparticles is that they can mimic the mechanism of antibodies. Similarly how antibodies have unique fingerprints that bind to targets, gold nanoparticles can be decorated with various ligands to produce binding sites for targets. The combinations possible to create of decorated gold nanoparticles are exponential even starting with a fairly small library. With these properties in mind, the Feldheim lab has conducted research to create this new class of antimicrobials. The nanoparticles developed are no longer simply being employed as drug carriers, they are now being designed as the antimicrobial agent.

In 2007, mono-disperse gold nanoparticles were synthesized with a self assembled monolayer of para-mercaptopbenozic acid and a crystal structure determined. In the Feldheim lab and other labs para-mercaptopbenzoic acid stabilized gold nanoparticles were further developed to produce gold nanoparticles with organo-thiol ligand monolayers that contain other ligands of interest. An original library of ligands was selected, containing twelve unique thiols which vary
in functional group, charge, hydrophilicity and size. Using this library and a single pot exchange mechanism hundreds of combinations were produced. Some of these combinations produce a bacteriostatic effect when incubated with various gram negative bacteria. The nanoparticles further discussed in this thesis are products of this original research and are synthesized with a combination of cysteamine and 3-mercaptop-1-propanesulfonate on their surface.

Figure 1.1
a. Cysteamine
b. Sodium 3-mercaptop-1-propanesulfonate (SMPS)

The research conducted for this thesis is a pilot investigation of nanoscale structure activity relationships (NSAR). NSAR studies are vital in creating potent bacteriostatic gold nanoparticle and furthering nanoparticles into marketable products. The research conducted for this thesis is inspired by of some of the questions brought to light by the discovery of these novel bacteriostatic gold nanoparticles. This thesis also explores new HPLC methods for characterization of these novel materials.

1.2 Thiol Modified Gold Nanoparticles

Figure 1.2 “Staple motif” where cyan atoms are sulfur and gold atoms are yellow.
The gold to sulfur bond provides the backbone for the chemistry behind the exchange reactions and gold nanoparticle synthesis. The nature of the exact interaction between gold and sulfur has been debated. Current research unveils that there is a keen “staple” motif between the gold and sulfur atoms.\textsuperscript{7} This motif consists of a single sulfur atom coordinating with two gold atoms.\textsuperscript{7} There is also evidence that the presence of gold will cleave disulfide bonds and support formation of a self-assembled monolayer on the surface of the gold.\textsuperscript{8} This unique bond is the grounding for self-assembled thiol monolayer gold nanoparticles. For this thesis, gold nanoparticles were synthesized in the presence of a sole ligand, para-mercaptobenzoic acid. These were labeled as pmba nanoparticles. To form the gold bacteriostatic nanoparticles, these pmba nanoparticles undergo a ligand exchange mechanism step to swap out some of the pmba monolayer with other ligands of interest.

### 1.3 Nanoscale Structure Activity Relationships

One of the most intriguing aspects of these gold bacteriostatic nanoparticles is the delicate nature of the feed ratio. The feed ratio is defined as the amount of moles of the ligand of interest compared to the amount of gold nanoparticles in solution during the exchange reaction. Bresee and colleagues discovered by changing the feed ratio one could produce nanoparticles that inhibited the growth of bacteria, while if the ratio was changed the product nanoparticles did not produce a bacteriostatic effect.\textsuperscript{6} These intriguing results lead to the formulation of a couple of questions to focus on: is there a range of ratios that produce nanoparticles that have minimum inhibition concentrations of varying potency; does size of the nanoparticles effect minimum inhibition concentrations; and does composition of the organo-thiol self assembled monolayer affect minimum inhibition concentrations? These questions were explored through NSAR. The feed ratio was chosen as the variable to change as it was predicted that changing the feed ratio could change both size of the final nanoparticles product as well as change the composition of the monolayer. By choosing to manipulate only one variable, the feed ratio, the results provided informative and conclusions could be drawn based upon information gathered. These results will be discussed in the following chapters.

### 1.4 Strategy
Three separate test groupings of nanoparticles were synthesized. The first grouping was designed with nine unique feed ratios that were predicted to change the average size of the nanoparticle. These nine different nanoparticles batches were labeled T2-T10, and there was a control nanoparticle labeled T1. T1 was used as a control for all three groups of nanoparticles. The control parameters were selected from previous experimental data from the Feldheim group as being the most potent, proven through minimum inhibition concentration assays. T2-T10 were developed varying on the total amount of ligand in solution during the exchange process, while the amount of the ligands with respect to each other were held constant. This batch was tested for minimum inhibition concentration and average size using transmission electron microscopy.

The second grouping of nanoparticles was labeled LS1-LS7. The parameter that was changed in these test nanoparticles was the feed ratio ratios, or the amount of the ligand cysteamine compared to the amount of SMPS in the exchange solution. The minimum inhibition concentration was determined for LS1-LS7. Also a HPLC method was developed to attempt to determine the composition of the self-assembled monolayer.

The results from the first grouping of nanoparticles led to a third set being synthesized, assigned the names SS1-SS6. This third set was designed to further explore the optimal ratio of producing a bacteriostatic effect at low concentrations. Therefore for this third set, only the minimum inhibition concentration was determined.

1.5 Materials Characterization
Figure 1.3 TEM image of gold nanoparticles with an organo-thiol monolayer

Nanoscale material has always proved more challenging to characterize than macro scale materials. The gold nanoparticle preparation selected for the experiments discussed in this thesis yields nanoparticles that have a gold core of one hundred and forty four atoms, are about 2-3 nanometers in diameter and are stabilized by approximately eight-seven thiol ligands. The methods used to characterize the nanoparticles include UV-Visible spectroscopy (UV-VIS), transmission electron microscopy (TEM) and high performance liquid chromatography (HPLC). UV-VIS allows quantification of the nanoparticles, by incorporating Beer-Lambert’s law. Beer-Lambert’s law is an equation that expresses the fact that absorbance is linear with concentration and with the path length and molar absorptivity as known values the concentration can be deduced from UV-VIS data. Size and morphology can be determined by the use of transmission electron microscopy, which collects images that can be analyzed to determine an average particle diameter. In addition, the composition of the self-assembled ligand monolayer can be analyzed with a new high performance liquid chromatography method.
1.6 HPLC Method Development

![Figure 1.4 Method for determining composition of organo-thiol monolayer](image)

High performance liquid chromatography (HPLC) was utilized to determine the composition of the thiol monolayer. This was a result of some troubleshooting with nuclear magnetic resonance (NMR)\(^\text{10}\), X-ray crystallography\(^\text{7}\) and infrared spectroscopy\(^\text{6}\) (IR). Current IR methods do not produce quantitative data.\(^\text{6}\) This proves an issue for determining a ratio of the ligand of interest percentage of the monolayer. NMR proves challenging for various reasons; solid state NMR of whole nanoparticles provides very little signal and takes large amounts of resources and time,\(^\text{10}\) also NMR of etched nanoparticles has issues with disulfides and signal overlap. X-ray crystallography has been useful in determining the surface structures of gold nanoparticles, but can take years to achieve one result.\(^\text{7}\) The motivation for development of this new method is to achieve both qualitative and quantitative data through the use of HPLC. By first removing the ligands of interest off the gold nanoparticles and then reacting the ligands with a thiol reactive
fluorescent maleimide to achieve a signal from the photodiode detector. This HPLC method was developed to attempt to quantity the exact number of ligands per nanoparticle, in hopes to produce a standardized method that is precise, affordable and speedy.

1.7 Applications

There is a growing niche for nanoparticles; the research topics nanoparticles cover include energy materials, catalysts and therapeutics. Nanoparticles could grow to provide novel applications in many sectors of daily life. Developing a new method of characterization for the self-assembled monolayer will prove key in further nanoscale structure activity relationships and characterization of these complex materials. By researching nanoscale structure activity relationships the gold nanoparticle can be fine tuned to produce an active, potent therapeutic that is a new class of its own. The need for a new class of therapeutic is upon us. As there has not been a new class of therapeutic developed in decades. Current therapeutics on the market are beginning to face complications as bacteria develop resistance. Without the development of a new antimicrobial, there could be difficulties combating the evolution of bacteria.

Endless possibilities exist as nanoparticles are currently being researched and designed to achieve a cornucopia of properties. But to understand these properties, there needs to be a quicker, effective and reproducible method to determining the structure of the self-assembled monolayer of the nanoparticles.

1.8 Summary

As the therapeutics currently on the marketplace become less effective against bacteria, it is vital to be continuing research to design new or improved antimicrobials. This thesis presents further studies in smart design of nanoparticle antimicrobials to hopefully develop an entire new class of antimicrobials where the nanoparticle is in itself a drug. Also this research discusses a novel method for determining the composition of the organo-thiol monolayer that surrounds the gold core of the nanoparticle.

1.9 Thesis Organization

This thesis is separated into three chapters. The first chapter provides insight, background information and an overview. The second chapter discusses the data which suggests that there is
an optimal feed ratio for minimum inhibition concentration. This chapter also explores the effect of average size of the nanoparticle on minimum inhibition concentrations. Finally, the third chapter delves into the HPLC method designed to explore the self-assembled monolayer’s ligand composition.

1.10 References and Notes

Chapter 2

The Optimal Feed Ratio for Inhibiting *E. Coli* Growth

2.1 Introduction

Under the premise that gold nanoparticles with different compositions of self-assembled monolayers could behave in a similar manor to antibodies, Breese developed a library of twelve ligands to use in an associative exchange mechanism to create gold nanoparticles with unique self-assembled monolayers. One hundred and twenty different combinations of decorated gold nanoparticles were synthesized by Breese. Using the library of twelve ligands in combinations of pairs and triples during the exchange process Breese slowly began to discover that certain combinations of ligands produced gold nanoparticles with a bacteriostatic effect against *E. Coli*. This work set up the base for the work conducted for this thesis.

Once it was established that certain combinations of ligands during the exchange process could produce bacteriostatic gold nanoparticles the next step was to determine how changing parameters of the exchange process could affect the efficacy of the gold nanoparticles. A parameter of the exchange process that was accurately and precisely controlled was the feed ratio, the amounts of one ligand to another ligand. One can control both the total amount of ligand in solution during the exchange process as well as the ratio of one ligand to another ligand. It was also important to explore what properties of the nanoparticle related to their activity; was it size, composition of monolayer or polydispersity? These properties could be explored through changing the feed ratio. As it was hypothesized that changing the total amount of ligand in solution could alter the size of the final product nanoparticle as well as the polydispersity. The composition of the monolayer was hypothesized to be under the influence of the feed ratio as well. It follows logic that the ligand in higher concentration during the exchange process would be more abundantly found on the gold nanoparticle product.

2.2 Hypothesis

This chapter follows the following hypothesis; there is an optimal feed ratio, the amount and ratio of ligands in solution during the exchange process, to produce the most effective
nanoparticles. Finding this optimal feed ratio is key for developing methods for fine-tuning the potency of gold bacteriostatic nanoparticles. Focusing on this premise experiments were designed to investigate how varying the feed ratio effects the minimum inhibition concentration. An optimal feed ratio was discovered in comparison to various feed ratios, validating the hypothesis within the selected range of feed ratios. Another parameter predicted to affect the minimum inhibition concentration was size; the final average size of the bacteriostatic gold nanoparticles was found to be dependent on the total amount of ligands in solution during the exchange process. This parameter was varied to explore the effects of size on minimum inhibition concentration, proving the hypothesis to be null within a certain range of size.

2.3 Strategy

Nine batches of experimental nanoparticles were synthesized where the variability came in the amount of total ligands in solution. A prediction was made that changing this total amount of ligand in solution could change the size of the nanoparticle and perhaps change the minimum inhibition concentration needed to produce bacteriostatic effects against E. Coli. This prediction was made on the hypothesis that the total amount of ligand in solution could possibly affect the average size of the nanoparticles by inducing etching or Oswald’s ripening. The ratio of cysteamine to sodium 3-mercapto-1-propanesulfonate (SMPS) was held in a narrow range to limit the amount of variability of the batches. Each batch was tested for a minimum inhibition concentration to find if average size or distribution of size affected the MIC. Fourteen additional batches were synthesized in which the feed ratio ratio was changed, defined as the ratio of cysteamine to MPS in solution during the exchange mechanism. These fourteen batches were tested for their minimum inhibition concentration to try to achieve an optimal feed ratio. A control gold bacteriostatic nanoparticle was synthesized with a set of parameters matching the most potent gold bacteriostatic nanoparticle found by Bresee.¹

2.4 Method

_Synthesis of base gold nanoparticles_

For these experiments base gold nanoparticles were utilized that were synthesized with a para-mercaptobenzoic acid monolayer. For this synthesis 11.1 mM HAuCl₄ from SigmaAldrich, 37.8 mM p-mercaptobenzoic acid (p-MBA) from TCI-America, and 178 mM NaOH in 55.6%
methanol was combined and stirred for eighteen hours. This solution was divided into three separate flasks and diluted to a final concentration of Au\(^{3+}\) of .48 mM by the addition of 260mL methanol and 740 mL H\(_2\)O. A reduction was performed on the Au\(^{3+}\) upon the addition of 10mL of .25 M NaBH\(_4\) with the reagent being purchased from SigmaAlrich. In conclusion for this step of synthesis, the final methanol concentration was brought to 24.8% with the addition of 100mL of H\(_2\)O. This reduction occurred over a twenty four hour time point, with constant stirring. To precipitate and collect the base nanoparticle product, 68 mmoles of NaCl and .15L of methanol was added to each of the flasks. The resulting solutions were then transferred to centrifuge tubes and underwent centrifugation at 3200xG for five minutes. The tubes were decanted and the nanoparticle precipitant was left to dry for twenty four hours. After air drying the precipitant was reconstituted in H\(_2\)O. The product was washed in a .22 micron filter and concentration was quantified by UV-Vis. For the UV-Vis method, the wavelength recorded was the absorption at 510 nm and the molar absorptivity value used was 409,440 M\(^{-1}\) cm\(^{-1}\). This information in combination with Beer-Lambert’s law allows for calculation of the final concentration of the nanoparticles. An important note for this procedure; the source of the reagents is vital to producing nanoparticles that produce bacteriostatic effects. These pmba nanoparticles were then tested for activity against \(E.\ Coli\) and found to not produce any bacteriostatic effect at any concentration tested (1x,8x,10x,100x). Also using TEM the average diameter of the pmba nanoparticles was discovered to establish a control for experiments T2-T10.
Figure 2.1 This figure displays a TEM image of pmba nanoparticles.

Synthesis of Thiol Modified Gold Nanoparticles T1-T10

One pot place exchange reactions were conducted with the addition of varying amount of total thiol in centrifuge tubes. For T2-T10 a 1.36:1 or 1:1 ratio of cysteamine to MPS was selected though the total amount of ligand in solution was varied. The control nanoparticle batch was labeled T1 and was chosen based upon the work of Bresee. The control nanoparticle has a feed ratio of 45:33 and a ratio of cysteamine to MPS of 1.36:1. For nanoparticle batches TS1-TS8 and SS1-SS6 the ratio of cysteamine to MPS was varied. The thiols were added to .0296 micromoles pmba gold nanoparticles in 4 mL of sodium phosphate buffer at the pH of 9.5. Reactions were then shaken on a plate shaker in the 19 °C fridge for 24 hours. To harvest the exchange product 40 mmoles of NaCl and 15 mL of methanol is added to each exchange reaction. Each reaction was then centrifuged at 3200 xG for 30 minutes. The product was reconstituted with H2O and washed once more with additional NaCl and methanol to remove any free thiol. The nanoparticle exchanges were dried overnight at room temperature and then brought up in H2O. To remove any excess salt the exchanges were washed with H2O and a 10k MWCO filter. The final product concentration was determined using the same UV-Vis method as for pmba nanoparticles. Each of these batches of nanoparticles was investigated for average diameter and distribution of size through the use of TEM (Philips CM12, 100 kV). In addition minimum inhibition concentrations (MIC) were determined through incubating cultures of E.coli with varying concentrations (1 micromolar to 100 micromolar) of nanoparticle batches T1-T10. The MIC assays were prepared in the following steps: preparation of stock solution of E. Coli, preparation of a antibiotic stock solution, inoculation, incubation, plating dilutions of the incubated stock solution, and counting the colonies.
2.5 Effects of the total amount of ligand in solution during the exchange

Upon changing the total amount of ligand in solution during the exchange a phenomenon occurred where the batches of nanoparticles varied in average size and distribution of size. This is displayed below in figure 2.3.
Figure 2.3

This graph displays the average diameter of nanoparticle of batches T1-T10 and pmra.

The average diameter of the nanoparticle batches ranged from 1.577nm to 3.098nm. Through testing the MIC it was found that the nanoparticles in the size range from 1.577nm to 3.098nm did not affect the MIC to a very large degree. The MIC did not even increase by a factor of ten. Indicating that as long as the average diameter stays below the value of ~3 nanometers the mode of action is not irrupted. It should be noted that nanoparticles that were insoluble in H2O were not tested for MIC due to their insolubility in the necessary solvent. The following table displays the MIC values of batch T1-T10.
<table>
<thead>
<tr>
<th>TEST</th>
<th>Feed Ratio (cysteamine: MPS)</th>
<th>MIC $^{\log99.9}$</th>
<th>Feed Ratios Ratio (cysteamine: MPS)</th>
<th>Total multiple amount of thiols in solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>T2</td>
<td>33:33</td>
<td>2</td>
<td>1:1</td>
<td>66</td>
</tr>
<tr>
<td>T3</td>
<td>45:45</td>
<td>2</td>
<td>1:1</td>
<td>90</td>
</tr>
<tr>
<td>T4</td>
<td>33:45</td>
<td>2</td>
<td>1:1.36</td>
<td>78</td>
</tr>
<tr>
<td>T5</td>
<td>22.95:16.95</td>
<td>2</td>
<td>1.36:1</td>
<td>39.9</td>
</tr>
<tr>
<td>T6</td>
<td>91.8:67.8</td>
<td>Insoluble</td>
<td>1.36:1</td>
<td>159.6</td>
</tr>
<tr>
<td>T7</td>
<td>15.3:11.27</td>
<td>5</td>
<td>1.36:1</td>
<td>26.57</td>
</tr>
<tr>
<td>T8</td>
<td>11.48:8.45</td>
<td>Insoluble</td>
<td>1.36:1</td>
<td>19.93</td>
</tr>
<tr>
<td>T9</td>
<td>41.31:30.42</td>
<td>5</td>
<td>1.36:1</td>
<td>71.73</td>
</tr>
<tr>
<td>T10</td>
<td>37.18:41.31</td>
<td>5</td>
<td>1:1.1</td>
<td>78.5</td>
</tr>
<tr>
<td>Control (T1)</td>
<td>45:33</td>
<td>1</td>
<td>1.36:1</td>
<td>78</td>
</tr>
</tbody>
</table>

**Table 2.1**

A table displaying the ratios of cysteamine to MPS, total amount of ligand in solution during the exchange process and MIC for T1-T10. The MIC is the minimum inhibition concentration, the concentration in which the gold nanoparticle prevents log 99.9 growth of the *E.Coli*. The total amount of ligand in solution during the exchange process is defined as the amount of MPS in...
addition to the amount of cysteamine in multiples compared to the original concentration of gold nanoparticles.

Figure 2.4
This figure details the distribution of size of T1-T10 and pmba gold nanoparticles.
Figure 2.5
This figure displays the distribution of size for a sampling of a hundred T6 and pmba nanoparticles.

Based upon figure 2.5 and 2.6, polydispersity is apparent in all batches of test nanoparticles while pmba only nanoparticles have a much narrower size range. The polydispersity of the bacteriostatic nanoparticles may contribute to a mode of action.

2.6 The Optimal Feed Ratio

To discover an optimal feed ratio the ratio of cysteamine to MPS was altered. After the first batch of data was recorded from the first set of parameters another six batches of nanoparticles were synthesized with a narrower range of ratio of cysteamine to MPS. The graph below shows the percent efficacy found for select batches of nanoparticles, where 100% efficacy is defined as a MIC of 1 micro molar. Certain nanoparticle batches were not included in the graph due to having 0% efficacy. Nanoparticles that are not displayed in the graph had the following feed ratios of cysteamine to SMPS; 3:1, 4:1, .33:1 .25:1.
Figure 2.6

A graph that displays the relationship of the feed ratio of cysteamine to MPS in relationship to efficacy against *E. Coli*.

2.7 Applications

Antimicrobials can be purposed in various ways, such as for therapeutics and being utilized in coatings. Antimicrobial coatings are used in many sectors of industry, such as in medical implants\(^2\), surgical equipment\(^3\), and surfaces\(^4\). Gold bacteriostatic nanoparticles could be classified as a new class of antimicrobials and could be used for any of the applications traditional antimicrobials are currently utilized for. This could be a breakthrough in the field of therapeutics as the nanoparticles have been shown by previous research conducted by Breese that details the lack of ability for bacteria to develop resistance to gold nanoparticle therapeutics.\(^1\) Establishing that there is indeed an optimal ratio of cysteamine to SMPS for creating the most potent gold bacteriostatic nanoparticle is a step towards bringing these materials to the consumer market.

2.8 Summary

Through the analysis of results the optimal ratio of cysteamine to SMPS was found to be 1.36:1. This produces batches of nanoparticles with an MIC of 1 micro molar. It was also
discovered that the altering the feed ratio to even 2:1 cysteamine to SMPS produces nanoparticles with little efficacy. Therefore the following hypothesis was formed; the feed ratio of cysteamine to SMPS was producing nanoparticles with different surface coverage. This hypothesis originated from the research conducted in this chapter that provides data to support the conclusion that changing the total amount of ligand in solution changes size but does not change the MIC with much degree as long as the feed ratio of cysteamine to SMPS is held constant. While it was found the MIC is dependent on the feed ratio of cysteamine to SMPS. This hypothesis will be explored in chapter three.

2.9 References and Notes


Chapter 3

Self-assembled Monolayer Composition

3.1 Introduction

An intriguing question arises from working with these gold bacteriostatic nanoparticles; what is the composition of the self-assembled monolayer? If these gold bacteriostatic nanoparticles do indeed have a similar mode of action to antibodies then one could make the connection that the surface patterning of the gold nanoparticles must be important. While there are experiments that attempt to explore the actual patterning of the surface, seeing if the ligands form islands or even stripes, the first question to work on is determining whether the feed ratio effects how much of each ligand attaches to the gold nanoparticle.\(^1\) There are a few qualitative methods for determining what ligands are attached to the nanoparticles, but each of these methods has its draw-backs. For example IR has been used to determine if certain ligands were present on the nanoparticles, but the signal produced can be ambiguous and weak, as well as it is possible for the signals to overlap. NMR has also been used to characterize gold nanoparticles; this process is extremely time intensive and the signal to noise ratio is poor. MALDI has also been employed, but only works in certain cases, such as quantization of proteins on gold nanoparticles.\(^2\) Therefore it was key to develop a new method for characterizing the self-assembled monolayer that forms around the gold nanoparticles. High performance liquid chromatography was selected as the platform to develop the new method on based upon its reproducibility, selectivity, and sensitivity.

3.2 Strategy

The strategy for characterizing the self-assembled monolayer is displayed in figure 3.1. It was deemed necessary to “etch” the gold nanoparticles, as whole gold nanoparticles would not be able to bind to a dye molecule nor was it theorized that whole gold nanoparticles would run at different retention times in a HPLC method even if their self-assembled monolayer was different. Many different techniques were attempted to etch away the gold core and leave the thiols that had once created the self-assembled monolayers in solution. Cyanine and Iodine were two catalysts added to solution to attempt to etch. Both resulting solutions were examined with NMR
and found to decompose the ligands, producing a mess of signals and by-products. These results pushed the strategy in another direction. It was hypothesized that it could be possible to push the reaction of the formation of self-assembled monolayer gold nanoparticles backwards by adding in a large excess of a thiol. This produced a large amount of by-products, predicted to be polymers of gold and thiol. This was not viable for the method. Therefore the next attempt proceeded with a small excess of the original thiol that made up the base nanoparticles, pmba. With 100x pmba in solution it was hypothesized that another exchange reaction would occur and the ligands of interest would exchange with the pmba. The extent to which the ligands of interest would exchange with the pmba is unknown and a factor contributing to error. After this etching process the next step was to add TCEP to break apart disulfides that had formed during the etching process. Next the solution was run through a cellulose filter to rid the solution of the gold nanoparticles. Then the solution was prepped with buffer, NaCl and EDTA before addition of the malamide dye. The malamide dye was then added in a 10x concentration to the original concentration of nanoparticles. This solution was then analyzed by HPLC under specific conditions and compared to standards to determine the concentration of thiols in solution.

**Figure 3.1** The strategy for determining the composition of the self-assembled monolayer using HPLC.
3.3 HPLC Method

A new HPLC method was designed for analysis of thiols. This method was employed in the overall strategy for determining the composition of the self-assembled monolayer of thiols. In the beginning stages of design for this method the thiols of interest, cysteamine and SMPS were run under various isocratic HPLC solvent systems with water and acetonitrile to attempt to identify if any signal could be obtained. Various concentrations were used to insure the compounds would be within detection limits. Both cysteamine and SMPS did not produce signal at the wavelengths 254, 280 or 495. Because of this it was necessary to attach a dye to the thiols. Fluorescein-5-maleimide was selected as the dye molecule; maleimides are the standard in reagents for thiol-selective modification. In this reaction, a thioether is produced as the thiol is added across the double bond contained in the maleimide. Fluorescein-5-maleimide is a selective dye, though it can produce hydrolysis byproducts at pH greater than 8. Fluorescein-5-maleimide emits a signal at 495. The first step in creating the method is accurate and precise identification of the peaks. In this process multiple gradients and isocratic solvent systems were tested; the one chosen was a gradient solvent system that uses water and acetonitrile with a 0.1% TFA additive to protonate the molecule to prevent damage to the HPLC column. The column chosen was an Agilent eclipse plus C18, 3.5 micrometer packing, 4.6 x 100 mm dimensions. Under these conditions, 5 microliters of the sample was injected and observed under the wavelength 495. The following figures show the peak from cysteamine attached to the dye and SMPS attached to the dye. SMPS bonded to the dye eludes at 5.4 minutes under the chosen gradient. Cysteamine bonded to the dye molecule elutes at 5.9 minutes.
Figure 3.2
The chromatogram of a solution of cysteamine bonded to the dye molecule.

Figure 3.3
The chromatogram of SMPS bonded to the dye molecule.
Once the solvent conditions were set and the peak retention times known, standards were run in seven different concentrations to create calibration curves for each of the thiols of interest. The calibration curves are attached in the following figures.

Figure 3.4
The calibration curve for Cysteamine bonded to the dye molecule.
3.4 Experimental

Once a HPLC method had been established samples with unknown concentrations of thiols etched from gold nanoparticles could be examined. The first step was to “etch” the gold nanoparticles. For this step 100 times the molar concentration of nanoparticles of 0.1M pMBA was added to the gold nanoparticles suspended in H$_2$O in a conical. The solution was placed in a 19°C shaker for 24 hours. After 24 hours the solution was removed from the shaker and the solution was adjusted to 0.05M TCEP, which was allowed to react for 4 hours. This solution was then run through a 0.22 micro amicron filter to removed the solid nanoparticles from solution. After filtration the following were added to 250mL of solution, 50 milli molar EDTA, 5 milli molar NaCl and sodium phosphate buffer. The solution was then adjusted to a pH of 7-7.5. After being adjusted to the correct pH the solution was placed in the 19°C shaker for 24 hours. This
results in a solution of dye molecules bonded to the thiols that have been etched from the gold nanoparticle self-assembled monolayer. Five microliters of the resulting solution is run through the HPLC at a gradient with a reverse-phase system with the detector set up at 495nm. The gradient begins at 20% ACN 80% H2O, both solvents containing a 0.1% TFA additive throughout the method, then shifts to 22% ACN 78% H2O over 5 minutes, and finally rests at 25% ACN 75% H2O after 6 minutes. The resulting chromatographs are then analyzed for peak area of the known peaks for the thiols bonded to the dye molecule.

3.5 Data

<table>
<thead>
<tr>
<th>Feed Ratio: Amount of cysteamine: Amount of SMPS</th>
<th>Concentration of Cysteamine on N.P (molar)</th>
<th>Concentration of SMPS on N.P (molar)</th>
<th>Ratio of Cysteamine: SMPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:10</td>
<td>.00018 ± .00001</td>
<td>.00065 ± .00001</td>
<td>1 : 3.7</td>
</tr>
<tr>
<td>10:20</td>
<td>.00021 ± .00001</td>
<td>.00075 ± .00001</td>
<td>1 : 3.6</td>
</tr>
<tr>
<td>10:30</td>
<td>.00014 ± .00001</td>
<td>.00066 ± .00001</td>
<td>1 : 4.8</td>
</tr>
<tr>
<td>10:40</td>
<td>.000070 ± .00001</td>
<td>.00054 ± .00001</td>
<td>1 : 7.7</td>
</tr>
</tbody>
</table>

**Table 3.1**

Data acquired from the HPLC and etching method of gold bacteriostatic nanoparticles.

Figure 3.6 details the data acquired from the HPLC and etching method of gold bacteriostatic nanoparticles. These batches of nanoparticles varied in the feed ratio of cysteamine to SMPS.

3.6 Explanation of Results
The trend revealed from the HPLC areas of the peaks of interest provides evidence that SMPS binds preferentially to gold nanoparticle and that by increasing the ratio of a thiol in solution the resulting nanoparticle has a larger amount of that thiol in comparison a nanoparticle exchanged with a smaller ratio of that thiol in solution. This could be due to differences in sterics, binding affinity or size. Also the data supports the inference that SMPS binds preferentially even when there is a large excess of another ligand, (4:1).

3.7 Applications

This method could be developed into a quantitative method to determine the composition of the thiol self-assembled monolayer on any capped nanoparticle. This could be a novel, sensitive way to characterize self assembled monolayers, replacing such costly or only qualitative methods such as IR or NMR. The applications of capped nanoparticles extend across many fields; therefore the impact of a new method of characterization is broad. In the Feldheim lab alone there are gold nanoparticles with a variety of ligands that could now be characterized in the future. These gold nanoparticles are bacteriostatic against bacteria such as *E. Coli* and *Klebsiella*. There are also nanoparticles in the Feldheim lab that have been synthesized with various drug molecules of interest, to target infections such as HIV. With this research there is a promising development towards a method.

3.8 Summary

This chapter explores a new method for determining the composition of the self-assembled monolayer that forms around bacteriostatic gold nanoparticles. This method could be utilized in all the fields that currently investigate nanoparticles as a characterization technique. It could be possible to characterize any gold nanoparticle that has a self-assembled monolayer with the methods detailed in this chapter. This is a new, exciting, timely, cost-effective way of learning more about the characteristics of the self-assembled monolayer. Through better understanding of the self-assembled monolayer there is hope of breaking the secret of the mode of action for the Feldheim lab gold bacteriostatic nanoparticles and creating a new class of antimicrobials.

3.9 References and Notes


BIBLIOGRAPHY


