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Analysis of Gold Nanoparticles and Their Use with Laser Desorption/Ionization Mass Spectrometry

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ANALYSIS OF GOLD NANOPARTICLES AND THEIR USE WITH LASER DESORPTION/IONIZATION MASS SPECTROMETRY

A Dissertation Presented

by

ALYSSA LINDA MARIE MARSICO

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

February 2017

Chemistry
ANALYSIS OF GOLD NANOPARTICLES AND THEIR USE WITH LASER DESORPTION/IONIZATION MASS SPECTROMETRY

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ALYSSA LINDA MARIE MARSICO

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DEDICATION

This work is dedicated to my beloved mother, father and brother.
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Most importantly, I would like to express my sincerest gratitude to my research advisor, Professor Richard Vachet. Words cannot express how thankful I am for the opportunity to study and learn under his constant guidance and support. He has been an amazing role model, not only as an exceptional scientist but also as a person. He was always there to give me both research and life advice. My graduate career has been filled with plenty of high and low moments, but Richard has always been there for me as a patient and understanding mentor. I have had the chance to grow during my time under his guidance and I would not be who I am today if it was not for Richard. I plan to pass along his ambition for teaching and passion for science into my future career, and I will constantly strive to be like him.

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ABSTRACT

ANALYSIS OF GOLD NANOPARTICLES AND THEIR USE WITH LASER DESORPTION/IONIZATION MASS SPECTROMETRY

FEBRUARY 2017

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Directed by: Professor Richard W. Vachet

Gold nanoparticles (AuNPs) have many unique properties that make them attractive for use in various biological applications. Laser desorption/ionization mass spectrometry (LDI-MS) has been used to monitor AuNPs in complex biological samples but there are still many ways in which AuNPs can be used with MS. In this dissertation, the use of AuNPs to assist in analyte ionization has been investigated. Their ability to enhance signal from biomolecules based on their surface chemistry, size, aggregation and method of deposition has been studied. The first use of an inkjet printer to create surfaces from which analytes can be sampled is discussed and revealed that the aggregation of the NPs is crucial to analyte enhancement. In addition, the investigation of the use of both AuNPs and a small amount of matrix that is typically used in matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) in order to detect previously difficult to ionize ligands in biological samples is reported and revealed the existence of a synergistic relationship between the two components. The method by which AuNPs dry and form a coffee ring is also taken advantage of to easily locate and enhance signal from larger biomolecules due to the concentration of the analyte, AuNP and matrix to the formed ring. Finally, desorption efficiencies and energies required for these ionization processes are all investigated and compared in order to better understand the mechanism of ionization.
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CHAPTER 1

INTRODUCTION

1.1 Gold Nanoparticles in History

Gold is globally known for its unique bright yellow color and is commonly recognized as a symbol of beauty, wealth and power. It is a precious metal that is rare but found in nature in its native solid form. Since its discovery thousands of years ago, people have been fascinated by the beauty of gold. Its malleable properties allow it to be easily fabricated into currency, jewelry and various forms of art. One of the first known uses of gold as a form of currency was back in 600 BC when Croesus instructed gold coins with a lion on them to be produced in Lydia, or current day Turkey. Since then, many civilizations have used gold not only as currency but have also incorporated it into their attire to demonstrate their wealth and power. Due to this, goldsmiths knew how to process solid gold, not only shaping it into coins but also hammering it into very thin leaves. Little did they know, this processing would have a crucial impact on the advancement of modern science.

It is not surprising that people began experimenting with gold as their fascination with its beauty grew. Further advancements resulted in the ability to process gold into nanometer sized structures that had different properties than bulk gold. These structures no longer displayed the signature bright yellow color that had come to define gold, but instead exhibited a range of colors from red to blue. These new discoveries opened even more possibilities for gold to be used in art, more specifically in stained glass. One of the first discovered gold stained pieces of glass was located in St. Paul’s Monastery in Jarrow, England, which was founded in 686 AD (Figure 1.1). Due to its wide range of possible colors, artists continued to use gold to create beautiful pieces of art.
for centuries.\textsuperscript{4,6} Unbeknownst to them, they had produced some of the first examples of nanomaterials.

\textbf{Figure 1.1:} Glass window stained using gold in St. Paul’s Monastery in Jarrow, England.\textsuperscript{6}

Due to the lack of analytical techniques, the newly discovered nanometer-sized gold structures went uncharacterized for years. No one really knew exactly what was causing these color changes until gold nanoparticles were first reported by Michael Faraday in 1857. He reacted phosphorous dissolved in CS\textsubscript{2} with aqueous HAuCl\textsubscript{4} to reduce the gold salt and reported observing “fine particles”, or what he ended up calling “activated gold”. The suspension of these particles demonstrated a ruby red color, quite unique compared to the bright yellow color commonly observed from bulk gold (Figure 1.2). He even recognized that the observed red color was due to the miniature size of the particles that were formed and how they scattered light.\textsuperscript{4,7}
Since Faraday first discovered gold nanoparticles (AuNPs), their study and uses have increased exponentially. Today, manufactured nanoparticles (not solely gold) are used in over 1,800 consumer products ranging from health and fitness products to automotive technology. Gold nanoparticles (AuNPs) in particular are extremely attractive due to some of their unique properties, including that they are biologically inert and extremely stable. They can also be functionalized with a wide variety of thiol containing self-assembled monolayers (SAMs) through a strong bond between the gold and sulfur (Figure 1.3). Further research into these SAMs, or ligands, have given rise to monolayers that are soluble in water, biocompatible and have different surface functionalities. They have also been increasingly investigated in the research community, resulting in applications in areas such as drug delivery, imaging, sensors and therapeutics.
1.2 Gold Nanoparticle Characterization

The increase in uses of nanomaterials has established a need for better analytical tools to characterize their physical properties, like their size, shape and surface chemistry. Several techniques have been used to characterize the physical properties of AuNPs, but some of these methods have limitations. Transmission electron microscopy (TEM)\textsuperscript{24,25}, atomic force microscopy (AFM)\textsuperscript{26,27} and scanning tunneling microscopy (STM)\textsuperscript{28} have been used to reveal information regarding the NPs core, including size and shape. However, they do not provide any insight into the ligands that are attached to the surface of the NPs. Their limited field of view also does not give a complete representation of a larger sample. In addition to these techniques, both X-ray diffraction (XRD)\textsuperscript{29} and small angle X-ray scattering (SAXS)\textsuperscript{30} have been used to characterize NPs but these methods still provide a challenge when trying to characterize the ligands on the NPs. The characterization of the attached ligands is crucial to understand the interaction of NPs with biological compounds because the ligands control the surface charge and many properties of the
NPs. Therefore, different methods to characterize both the core and the ligands of NPs are essential.

Some analytical techniques do exist to provide some information regarding the ligands, including thermogravimetric analysis (TGA) and nuclear magnetic resonance (NMR). The ligand-to-core mass ratio can be obtained using TGA but it does not provide any information regarding the structure of the ligands. To obtain structural information on the ligands, NMR can be used but the peak broadening that occurs when this analysis is done and the large sample size required are limitations when using this technique. To combat these drawbacks, UV-Vis and Fourier transform infrared spectroscopy (FT-IR) can be used to identify the ligands, but broad peaks make it difficult to identify atoms that are close to the NP core.

1.3 Mass Spectrometric Characterization of Gold Nanoparticles

Mass spectrometry (MS) is often viewed as a universal analytical tool to characterize various molecules, making it a promising candidate to analyze and characterize NPs. Techniques like laser desorption/ionization (LDI), matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI) and ion mobility (IM) MS have been previously used to characterize NPs.

When LDI-MS was first used to analyze AuNPs, the intact gold clusters were detected while little to no information was gathered about the attached ligands. In an attempt to obtain ligand information, both LDI-MS and MALDI-MS were used to analyze AuNPs in both positive and negative ion modes. In these experiments, gold clusters, intact ligands and ligand fragments were all detected. At first, these studies were limited to specific types of nanoparticles with a certain number of gold atoms and ligands. Recently, however, MALDI-MS has been used to analyze a wide range of nanoparticles with different core sizes and surface chemistries.
Other MS techniques like ESI and IM have also been used to analyze and characterize intact NPs. When ESI was used to analyze NPs, the intact gold core with the ligands attached were analyzed and detected. This allowed information regarding the NP composition to be obtained.42,43 However, ESI MS analysis of NPs is limited to a small set of NPs with certain core metal atoms and specific types of attached ligands. IM-MS can be used to provide insight into ligand segregation and can be useful in quantifying the surface components of NPs. However, using IM-MS to detect and characterize NPs in complex mixtures becomes challenging due to the presence of many other compounds that can also be ionized.44,45

1.4 Detection of Gold Nanoparticles

Not only is the characterization of NPs important, but it is also crucial to be able to detect them sensitively and selectively to understand their biodistribution and fate in the environment.46-49 Nanomaterials have been monitored in systems like bacteria50, plants51, cells52,53 and certain animals54 to investigate their fate. Reliable, sensitive and selective analytical methods must be used in order to obtain useful information regarding the biodistribution and environmental fate of NPs. Not only must these methods be able to detect the NPs but they also need to have a high tolerance for biomolecules and not allow these compounds to interfere with the analysis of the NPs. The techniques described above are all useful when analyzing pure NP samples but some of them may not be feasible for the analysis of NPs in complex biological samples.

Several optical methods, like confocal microscopy55, can be employed to characterize NPs in biological systems. However, this technique only works for NPs with unique optical properties and even with them, it cannot be used for accurate quantification. To aid in the detection of these NPs, some labelling methods can be used but these also have some drawbacks.56,57 The labels are
typically attached via some reaction, subsequently altering the ligands attached to the NPs. Considering these ligands control the surface functionality, the added labels may change the behavior of the NPs. Also, several different labels would need to be designed in order to react and attach to NPs with varying surface chemistries, which is not only time consuming but challenging.

Certain elemental analysis methods, like inductively coupled plasma mass spectrometry (ICP-MS)\textsuperscript{58,59} and atomic absorption spectroscopy (AAS)\textsuperscript{60}, are currently used to quantify NPs in complex biological systems. ICP-MS has been used to investigate the biodistribution of NPs in cells\textsuperscript{61,62}, plants\textsuperscript{63} and fish\textsuperscript{64}. For example, Zhu et al. investigated the effect that different AuNPs in water have on Japanese medaka fish.\textsuperscript{64} Various NPs with different ligands attached were added to the water in which the fish swam (Figure 1.4a). Once exposed, the NPs were taken up by the fish and distributed amongst several organs depending on their surface chemistry. After a given time, the fish were sacrificed and certain organs were analyzed for gold using ICP-MS (Figure 1.4b). The results indicated that positively charged NPs were taken up more readily than neutral and negatively charged NPs. Also, that there was an increase in uptake for NPs with hydrophilic NPs but then a decrease, leading to the conclusion that they were finally excreted. On the other hand, hydrophobic NPs entered the circulatory system and eventually led to fish mortality (Figure 1.4c). Although ICP-MS is a useful technique to investigate the biodistribution of NPs in biological samples, it monitors the metal only and destroys the ligands. Therefore, if the ligands were modified by the biological sample, ICP-MS would not be able to provide this information.
Figure 1.4: AuNP uptake by Japanese medaka fish, a) Structures of AuNPs the fish were exposed to; b) Analysis process of fish organs; c) Gold uptake by fish after several exposure time points.

As described earlier, mass spectrometry has shown extreme promise in analyzing pure NP samples, and it has previously been used for multiplexed analyses of various analytes, making it a good candidate for detection in biological systems. While MS techniques like IM have not yet been used to detect and characterize the ligands attached to the NPs, both MALDI and ESI have been. However, the conditions used in MALDI and ESI may also ionize biomolecules that are present in the sample, subsequently introducing a significant amount of interference in the mass spectra. These interferences may make it difficult to detect small amounts of NPs as the high concentration of competing biomolecules may suppress the signal from the ligands. LDI, however, has shown great promise in detecting and characterizing NPs in complex biological samples without the ionization of other compounds in the sample.
1.5 Nanoparticles and Laser Desorption/Ionization Mass Spectrometry

A laser is used to irradiate the sample in LDI-MS, typically with a wavelength of either 337 nm (N\textsubscript{2} laser) or 355 nm (Nd:YAG laser). When LDI-MS is used to analyze NPs, the core of the NPs absorbs this laser energy and transfers it to the attached surface ligands, which are subsequently desorbed and ionized\textsuperscript{72,73}. Using LDI-MS to analyze NPs allows for the characterization of a wide range of NPs with various core materials because most of these materials absorb the laser energy efficiently. For example, when LDI-MS is used with AuNPs, the gold core absorbs the laser energy, which is readily transferred to cleave the Au-S bond that attaches the surface bound alkanethiol compounds, allowing them to be desorbed and ionized\textsuperscript{74}.

The ionization process involved when using LDI-MS to analyze NPs allows this technique to be used to detect NPs in complex biological systems. The localized energy transfer that occurs from the core of the NPs to the attached ligands provides a highly selective ionization method that minimizes interferences from other compounds present in the sample. Our group has previously used this method to detect AuNPs in biological samples like cells\textsuperscript{38} and tissues\textsuperscript{68}. For example, Yan et al. demonstrated the multiplexed detection of AuNPs with different ligands in tissues using LDI-MS in an imaging format. This method enabled the selective ionization and detection of AuNPs with different ligands in a mouse tissue with minimal biomolecule interferences, which provided information regarding the biodistribution of these different NPs\textsuperscript{68}.

Due to the fact that these NPs can be functionalized with a certain desired ligand which can be selectively ionized by LDI-MS, they have been attractive to researchers to use for various applications other than to detect NPs in biological samples\textsuperscript{75,76}. For example, Nagahori et al. used AuNPs tagged with carbohydrate-functionalized alkanethiol monolayers to monitor enzymatic glycosylation reactions. Stepwise sugar elongation reactions resulted in mass changes in the ligands attached to the AuNPs, which could then be detected using LDI-MS\textsuperscript{77}. Alternatively, Lee et
al. used AuNPs to detect and investigate antigen-antibody binding. A small molecule was used as a reporter of the target protein in this study. The target protein was captured specifically by the ligands, or antibodies, attached to the AuNPs. These NPs also carry the small reporter molecules, which are analyzed by LDI-MS after the capture of the target protein. This method allows for the detection of antigen-antibody binding at an attomolar level. These applications demonstrate how LDI-MS can be used to characterize and detect NPs with high selectivity, a broad range of applications and high tolerance to interfering biomolecules.

The fact that NPs efficiently transfer the laser energy used to ionize analytes in LDI-MS is comparable to the function of a matrix in MALDI-MS, which is a small organic molecule that absorbs the laser energy and transfers it to the analyte. This similarity led to the investigation into the replacement of NPs as the organic matrix in a technique that is known as surface-assisted laser desorption/ionization mass spectrometry (SALDI-MS). Various types of nano-structured surfaces and nanoparticles have been investigated as alternate matrices for use in SALDI-based analyses. Gold nanoparticles particularly have many attractive attributes for use with LDI-MS, including the ease with which they can be functionalized. Several studies have been done using AuNPs to assist in the ionization of various types of compounds. For example, Russell et al. explored the effect the size of the gold core and the NP surface chemistry had on the ionization of peptides and proteins. Alternatively, Cheng et al. synthesized AuNPs with α-cyanohydroxycinnamic acid (CHCA) ligand, which is a common matrix used in MALDI-MS. They found that this ligand improved peptide ionization efficiencies compared to other ligands. While AuNPs have been used to assist in the ionization of various compounds and both their size and surface chemistry have been investigated, the method by which they are deposited and subsequently dried has been minimally studied. Part of the research described in this dissertation
will demonstrate the first use of inkjet printed AuNP surfaces to enhance the LDI-MS detection of low molecular weight analytes.

The cross between SALDI and MALDI that Cheng et al. investigated opens new possibilities of using both nanomaterials and matrices in LDI-MS. Liu et al. used quantum dots and CHCA to enhance peptide detection in MALDI. However, the combination of NPs and matrices has not yet been investigated. Much of the work described here involves using both AuNPs and added matrix to not only enhance biomolecule detection, but also to assist in the ionization of ligands attached to the AuNPs that were previously difficult to detect by LDI-MS. Finally, thermometer ions and their fragmentation patterns are used to investigate the energy involved in these various AuNP assisted LDI-MS ionization processes in an attempt to provide some insight into the energy transfer process that occurs.

1.6 Dissertation Overview

LDI-MS has been shown to detect and monitor AuNPs in complex biological samples like cells and tissues by ionizing and detecting the ligands attached to the gold core. This is possible due to the ability of the gold core of the NPs to absorb the laser energy and efficiently transfer it to the attached ligands, allowing them to subsequently be desorbed and ionized. This method allows for the highly selective, sensitive and simultaneous detection of different AuNPs in complex samples. The concept of using the efficient laser absorbance ability of the gold core of AuNPs to ionize the ligands attached to the AuNPs to track them in biological systems instead of an additional label is extremely attractive. It is also attractive as a way to enhance the detection of various biomolecules in place of a traditional organic matrix used in MALDI-MS. In this dissertation, new applications of LDI-MS for the detection and characterization of AuNPs with
difficult to ionize ligands, and for use with AuNPs to assist in ionization of biomolecules will be described.

The first use of inkjet printed AuNP surfaces to enhance the detection of low molecular weight biomolecules will be described in Chapter 2. Gold nanoparticles with different surface chemistries were investigated for their use in enhancing amino acid signals with LDI-MS. The findings show that both the surface charge of the AuNPs and the method in which they were deposited affect the enhancement seen with amino acids. In addition, it was determined that the amount of AuNPs deposited using an inkjet printer affect the enhancement due to the aggregation of the AuNPs.

In Chapter 3, a method to use LDI-MS to ionize and detect AuNPs with ligands that were previously difficult to ionize by LDI-MS will be described. It was determined that adding a small amount of matrix to the AuNP samples allowed for the ionization of the ligands attached to the AuNPs. Further investigation allowed for the discovery that this ionization arises from a synergy that exists between the gold core of the AuNP and the small amount of added matrix. These two components both allow for the transfer of energy to the attached ligands, further allowing their desorption and ionization. This new method not only allows for the detection of AuNPs that were previously difficult to ionize, but it does so without ionizing biomolecules also present in the sample. This maintains the selective ionization that is crucial for the monitoring of AuNPs in biological samples using LDI-MS.

Chapter 4 introduces the utilization of the manner in which NPs dry when deposited to enhance signal observed for biomolecules. It was determined that when a mixture of AuNPs, biomolecules and a small amount of matrix was deposited and allowed to dry, a visible ring was formed. This ring allowed for the concentration of the three parts of the solution to an area that
could easily be located. The combination of this concentration effect and the ease in which the analyte could be found and sampled resulted in a signal enhancement for biomolecules.

Finally, to better understand the process by which AuNPs absorb and transfer the laser energy in LDI-MS, thermometer ions were used to investigate the different processes described in the previous chapters. Both a benzylpyridium salt and a peptide, leucine encephalin, were used to provide insight into the energy of each of these processes. The ratio of the signal from the intact protonated ion to the signal from the fragments of each of these thermometer compounds was calculated to investigate the energy involved in various processes that were studied throughout this dissertation.

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CHAPTER 2

INKJET PRINTED GOLD NANOPARTICLE SURFACES FOR THE DETECTION OF LOW MOLECULAR WEIGHT BIOMOLECULES BY LASER DESORPTION/IONIZATION MASS SPECTROMETRY


2.1 Introduction

Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) is a powerful analytical tool that has been used to analyze many different biomolecules.\(^\text{1-5}\) Using MALDI to ionize small molecules for mass spectrometric detection is beneficial because of the technique’s inherent sensitivity and minimal sample consumption; however, analyzing small molecules by MALDI can be challenging due to matrix ion interferences in the low \(m/z\) region of the mass spectrum. Thus, finding alternate matrices that can facilitate ionization of low molecular weight compounds without producing interfering ions is of continued interest.\(^\text{2}\) Nanomaterials have been investigated as alternatives to traditional organic matrices because they often have the proper optical properties to absorb UV laser light and facilitate ionization, while at the same time producing fewer interfering ions. Various types of nano-structured surfaces, including silicon and platinum,\(^\text{6-14}\) and different nanoparticles (NPs), including carbon-, silicon-, iron oxide-, titanium dioxide-, zinc oxide-based and noble metal NPs (gold, silver and platinum) have been studied as alternate matrices.\(^\text{6, 15-25}\)
An attractive attribute of gold NPs (AuNPs), in particular, is the ease with which they can be functionalized using self-assembled monolayers. Such monolayer-protected NPs have tremendous potential as matrices because of the multidimensional control over their chemical and physical properties, while maintaining some of the inherent attributes that make them promising alternate matrices. AuNPs can be synthesized to have various monolayer ligands that offer different surface chemistries and variable core sizes. Russell and co-workers, for example, explored these attributes of AuNPs and found that surface chemistry and size have notable influences on ionization efficiencies of proteins and peptides.26,27 Cheng and co-workers synthesized AuNPs with monolayers of \( \alpha \)-cyanohydroxycinnamic acid (CHCA), a common matrix used in MALDI, and found that this monolayer coating improved peptide ionization efficiencies as compared to AuNPs with other monolayer coatings.28 The CHCA monolayer, however, still gave rise to ion interferences in the low \( m/z \) region of the mass spectrum, mitigating to some extent the value of these particular NPs for the analyses of low molecular weight compounds.

Further studies of monolayer chemistry and size will allow for some additional level of control over the arrangement of these NPs on surfaces, which is important for optimizing how the laser energy is transferred to enable analyte ionization.29,30 Depending on the core size, monolayer ligand length, and monolayer chemistry, NPs can be assembled on surfaces to facilitate analyte ionization. Moreover, monolayer ligand chemistry could further be used to control which analytes interact with NP matrices, enabling selective ionization. In the work presented in this chapter, we describe the use of monolayer-protected AuNPs with varying surface chemistries as matrices for low molecular weight compounds. These AuNPs are deposited on surfaces to which analytes are then added, and thus this represents another example of surface assisted laser desorption/ionization (SALDI). We investigate how NP deposition method and NP monolayer chemistry influence the ionization efficiency of amino acids, which have been chosen as test
analytes because they are biologically relevant small molecules. We find that inkjet-printing\textsuperscript{25, 31-35} is a convenient and reproducible means of producing NP-coated surfaces that enable the very efficient ionization of these molecules.

![Figure 2.1: AuNPs with the indicated monolayer are used for these experiments.](image)

### 2.2 Results and Discussion

#### 2.2.1 Nanoparticle Deposition

Two different methods of depositing the AuNPs on surfaces were investigated. First, pipette spotting of the AuNPs with subsequent analyte addition on top was explored. This deposition method is simple and facilitates ionization of added amino acids. As an example, when 1 µL of a 100 nM solution of 2 nm AuNPs coated with TEGOH (see Figure 2.1) are spotted onto a target surface and 100 fmol of arginine is then added, peaks corresponding to protonated arginine ($m/z$ 175) and sodiated arginine ($m/z$ 197) are observed during SALDI-MS analysis (Figure 2.2). Analytes ions that could be isobaric with Au\textsuperscript{+}, such as sodiated arginine can still be resolved from this interference, as organic molecules often have positive mass defects while Au has a negative mass defect. Comparable experiments in which no AuNPs are added result in no arginine-related ions during LDI-MS analysis, indicating how the functionalized 2 nm AuNPs enhance the ionization of this amino acid. Unfortunately, simple spotting of these AuNPs with a pipette does not give reproducible ion signal (Figure 2.3). The irreproducibility is attributed to “hot spots” that are generated as a result of the “coffee-ring effect” that occurs when the AuNPs dry after deposition.
on the surface, leaving a surface of AuNPs that is not homogeneous. These hot spots enhance ion signals in some areas but not others, depending on where the AuNPs deposit after drying (Figure 2.4a).

**Figure 2.2:** Detection of 100 fmol of arginine with the assistance of 2 nm TEGOH AuNPs; The asterisks (*) indicate peaks from an unknown contaminant seen when using the pipette spotting method. Inset: resolved Au\(^+\) and (Arg+Na\(^+\)) peaks.
Figure 2.3: Comparison of different AuNPs used to detect both arginine and histidine. These experiments were done using the pipette spotting method and the error bars shown are due to the inconsistency that arises from the “hot spots” that are formed when spotting the AuNP solution on the surface.

As an alternative to pipette spotting, we investigated inkjet printing as a means of depositing a more homogeneous layer of AuNPs. Inkjet printing has been used by our group and others to deposit NPs on a surface.\textsuperscript{25, 31-35} Upon printing 2 nm TEGOH AuNPs we find that the AuNPs are more homogenously distributed than pipette spotting (Figure 2.4), and as a result analyte ions can be detected by SALDI-MS anywhere on the printed surface. When printing AuNPs as a matrix, a wide range of amino acids can be successfully detected, including arginine, histidine, cysteine, glycine, glutamic acid, leucine, methionine, phenylalanine and serine (Figure 2.5). This method of AuNP deposition also lets us readily test how various NP surface densities influence analyte ion signal (see below).

Figure 2.4: a) Gold ion signal (m/z 197) obtained by LDI-MS when using pipette spotted AuNPs. b) Gold ion signal obtained by LDI-MS on a surface inkjet printed with AuNPs.
Figure 2.5: Detection of a) 1 pmol glutamic acid, b) 250 fmol cysteine, c) 250 fmol phenylalanine and d) 250 fmol methionine using a printed surface of RGB 160-170 2 nm TEGOH AuNPs. Asterisks mark peaks that arise from the stainless steel surface.

2.2.2 AuNP Density and Physical Characteristics

Having established inkjet printing as a better way to deposit AuNPs for SALDI-MS, we next considered how AuNP monolayer chemistry and AuNP core size influenced analyte ionization efficiency. To do this, we synthesized and printed NPs with positively charged (TTMA), negatively charged (TEGCOOH) and neutral (TEGOH) monolayer functional groups (Figure 2.1). For all the AuNPs studied, ion signals for the amino acids could be detected at much lower concentrations than are possible without the AuNPs (i.e. LDI-MS), but the NP monolayer identity noticeably influenced the result. AuNPs with the neutral TEGOH monolayer were found to provide the best signal. As an example, TEGOH could detect down to 50 fmol of arginine while TEGCOOH AuNPs could barely detect 250 fmol of arginine (Figure 2.6). The AuNPs with the TTMA monolayer performed better than the AuNPs with TEGCOOH, allowing amino acids to be detected as low as
50 fmol, but there were more interfering ions present from the positively-charged TTMA monolayer than the TEGOH monolayer because of the ease with which the TTMA monolayer is ionized relative to the TEGOH monolayer.\textsuperscript{36,37}

![Figure 2.6: A comparison of SALDI mass spectra acquired using (a) TEGOH AuNPs to detect 50 fmol of arginine and (b) TEGCOOH AuNPs to detect 250 fmol of arginine.]

The effect of AuNP core size on ionization enhancement was also considered. Core sizes of 2 nm, 4 nm, 6 nm and 8 nm with TEGOH monolayers were investigated. We also studied 13 nm AuNPs that were stabilized with citrate; it is difficult to synthesize and stabilize 13 nm AuNPs with the same monolayers shown in Figure 2.1. Analyte detection efficiency was explored for the different sized AuNPs by inkjet-printing different NP concentrations to find optimum NP densities on the printed surfaces. This was accomplished in a more high-throughput manner by printing AuNP gradients that went from high density to low density using different RGB values from the printer software, as described in the experimental section. After printing the NP gradients, rows of amino acid solutions at different concentrations were spotted onto the resulting surface and analyzed using MS imaging and ImageJ (Figures 2.7 and 2.8). With this approach, the optimal AuNP densities could be readily determined. As an example, the data in Figure 2.7 demonstrate that for 2 nm TEGOH AuNPs there is an optimum NP density corresponding to RGB values around 160-170 that enhances the analyte ion signals to the greatest extent. This RGB range corresponds to a
density of NPs of around 2-5 ng Au/cm² (Table 2.1), which was determined using ICP-MS. Each of the different sized NPs was printed in this same way. The optimal RGB values for the other NP sizes were 225-235 for the 4 nm AuNPs, 115-125 for the 6 nm AuNPs, 85-95 for the 8 nm AuNPs, and 230-240 for the 13 nm AuNPs. Each of these different core sizes enhanced the ion signal for amino acids when compared to LDI-MS without AuNPs, but the 2 nm core materials routinely gave better signal, enabling 25 fmol of analyte to be detected in many cases.

**Figure 2.7:** Intensity of signal from 250 pmol of spotted arginine and histidine across a RGB gradient of printed 2 nm TEGOH. a) Signal from the m/z 197 peak, which arises from both the Au⁺ ion and the sodiated arginine ion. b) Ion signal from protonated arginine. c) Ion signal from protonated histidine. d) Ion signal from sodiated histidine.
Figure 2.8: Signal intensities obtained from 100 pmol (a), (b) and (c) and 50 pmol (d), (e) and (f) of both arginine and histidine that were spotted on a printed gradient of 2 nm TEGOH AuNPs. The signal from the m/z 197 peak is shown in both (a) and (d), the signal from the sodiated arginine peak can be seen in both (b) and (e) and the signal from the sodiated histidine peak is shown in both (c) and (f).

2.2.3 Surface Characterization

Because the 2 nm TEGOH AuNPs reproducibly gave the best spectra and sensitivity, we further characterized the surfaces that were created upon inkjet-printing these AuNPs to understand how the NPs assembled on these surfaces. ICP-MS and TEM were used to determine how many AuNPs were present at specific RGB values and how the NPs were arranged on the surface. Three representative regions of the printed NPs were chosen for characterization: (i) the high end of the gradient, where the gold signal is the highest and some analyte signal is observed; (ii) the optimal region where analyte signal is most enhanced; and (iii) the low end of the gradient where some analyte signal is seen but no gold signal is observed. The results for these three regions are summarized in Table 2.1 and Figure 2.9. The ICP-MS results reveal that the gold surface densities are very different in each region, with the optimal region showing relatively low AuNP densities (i.e. 2-5 ng/cm²). Interestingly, the TEM images show that the AuNPs are clustered
together to a greater extent in the optimal region (Figure 2.9b), indicating that AuNP clusters are essential for efficient ionization. The high end of the AuNP gradient shows a homogenous distribution of individual and clustered AuNPs, but the average cluster size and cluster density is less than in the optimal region, which may explain why ionization is slightly less efficient in this higher concentration region. Linear arrangements, or “strings”, of AuNPs are also present in a greater number in the high end of the gradient, but these were not classified as clusters of AuNPs. Not surprisingly, the mass spectra from the high end of the gradient give higher Au\(^+\) signals, and suppression from these higher Au\(^+\) signals may explain the relatively lower analyte ion signals. Overall, the inkjet-printed gradient and the corresponding ICP-MS and TEM images demonstrate that the nature of the NP distributions on the surface influences ionization efficiency. It is likely that the larger NP clusters more effectively distribute the laser energy in a way that maximizes analyte desorption/ionization without extensive heating that might cause analyte degradation. The larger clusters may also spread out the energy such that fewer Au\(^+\) ions are produced, thereby decreasing the abundance of this interference.

**Figure 2.9:** TEM images of (a) the high end of the inkjet-printed 2 nm TEGOH AuNP gradient, (b) the optimal region, (c) and the low end of the gradient where arrows point out AuNPs. The larger splotches in the low-end image are due to residual printing solution.
Table 2.1: Summary of the characterization results from surfaces inkjet-printed with 2 nm TEGOH AuNPs.

<table>
<thead>
<tr>
<th>Area of Slide</th>
<th>ICP Results (ng Au/cm²)ᵃ</th>
<th>Average Number of clusters per μm²ᵇ</th>
<th>Average NPs/clusterᶜ</th>
<th>Average Diameter of clusters (nm)ᵈ</th>
</tr>
</thead>
<tbody>
<tr>
<td>High End of Gradient</td>
<td>400 – 500</td>
<td>20 ± 10</td>
<td>4.4 ± 0.8</td>
<td>20 ± 3</td>
</tr>
<tr>
<td>(RGB = 0 – 20)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Optimal Region of Gradient</td>
<td>2 – 5</td>
<td>38 ± 9</td>
<td>7 ± 4</td>
<td>30 ± 10</td>
</tr>
<tr>
<td>(RGB = 160-170)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Low End of Gradient</td>
<td>0 – 1</td>
<td>0</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>(RGB = 245 – 255)</td>
<td></td>
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</tr>
</tbody>
</table>

ᵃ Results were obtained by printing specific RGB values on glass slides and determining the printed gold by ICP-MS as described in the experimental section. The resulting gold amounts were then converted to ng Au/cm² using the area assayed by ICP-MS.

ᵇ These values were obtained by counting the number of clusters of AuNPs in a TEM sample area of approximately 0.25 μm².

c These values were calculated by counting the number of AuNPs in each cluster.

ᵈ These values were obtained from the diameter of each NP cluster.

2.2.4 Comparison of SALDI to LDI and MALDI

When 2 nm TEGOH AuNPs are inkjet-printed at the optimal densities, amino acids such as arginine and histidine can be detected at levels as low as 25 fmol. This limit of detection is 2-3
orders of magnitude lower than previous approaches that have used NP-based matrices to detect amino acids.\textsuperscript{14,38} LDI-MS cannot detect amino acids anywhere near this level. MALDI-MS can detect amino acids at similar concentrations, but the spectra are more congested in the lower \(m/z\) region (Figure 2.10). This greater number of low \(m/z\) interferences in MALDI-MS is due to the presence of the organic matrix, which produces many different ion types. Under optimum conditions, the 2 nm TEGOH AuNPs produce \(\text{Au}^+\) as the main interference, and its abundance is low.

![Figure 2.10](image)

\textbf{Figure 2.10}: (a) MALDI mass spectrum of 500 fmol histidine with CHCA as the matrix; * marks matrix ion peaks. (b) SALDI mass spectrum of 500 fmol histidine after inkjet-printing 2 nm TEGOH AuNPs.

\subsection*{2.3 Conclusions}

Monolayer-protected AuNPs can assist the ionization of analytes in LDI-MS, acting in a manner similar to the organic matrix used in MALDI but with fewer low \(m/z\) interferences. If the proper NP surface chemistry and deposition method are chosen, AuNPs can assist ionization of amino acids with great efficiency, reproducibility and with minimal interferences. From our experiments, we find that inkjet printing, as opposed to simple pipette spotting, produces a
homogeneous layer of AuNPs that enables reproducible analyte ionization. Interestingly, we find that NP cluster formation is required to obtain the lowest limits of detection, indicating that there is not only an optimal NP surface density but also that NP clustering plays an important role in analyte ionization mechanism. Future studies will investigate this underlying mechanism and will also explore AuNPs with monolayers that could be used to selectively extract analytes of interest from mixtures, while still allowing efficient ionization.

2.4 Experimental

2.4.1 Chemicals

Arginine, histidine, glycine, glutamic acid, leucine, methionine, phenylalanine, serine, CHCA, glycerol, 1,2-hexanediol and triethanolamine were purchased from Sigma Aldrich. Acetonitrile was purchased from Fisher Scientific. MilliQ deionized water was used in all of the experiments. Aqua regia was prepared using nitric acid and hydrochloric acid, 3:1 (v:v). Aqua regia is highly corrosive and must be handled with caution!

2.4.2 Synthesis of AuNPs

The AuNPs used in these experiments (Figure 1) were prepared using previously published methods: TEGOH, TTMA and TEGCOOH. In short, the Brust-Schiffrin two-phase synthesis method was used to synthesize pentanethiol-coated AuNPs with core diameters around 2 nm. Subsequently, the Murray place-exchange method was used to functionalize TTMA and TEGCOOH. TEGOH was synthesized by the single-phase synthesis method described previously. After synthesis, each AuNP was dialyzed for 72 h against MilliQ water using a Spectra/Por Dialysis Membrane (molecular weight cutoff of 1,000 Da) to separate the free ligands from the AuNPs. The resulting AuNPs were then characterized by transmission electron
microscopy (TEM) to confirm the Au core size and LDI-MS to ensure proper monolayer attachment.\textsuperscript{37,45}

2.4.3 Analyte Sample Preparation

The amino acid solutions were prepared in deionized water. Once the AuNPs were deposited on the surface and dried, 1 μL of an amino acid solution was deposited on top of the AuNP surface. This solution was allowed to dry before MS analysis. For MALDI analyses, approximately 30 mg of CHCA was dissolved in 1 mL of a solution containing 70% acetonitrile and 30% water and then mixed in a 1:1 volume ratio with the amino acid solution of interest. For the LDI-MS analyses, 1 μL of an amino acid solution was deposited directly onto a stainless steel surface.

2.4.4 AuNP Surface Preparation

To prepare the pipette spotted samples, 1 μL of a solution of AuNPs in 100% water was spotted onto a MALDI target and allowed to dry. Then, 1 μL of the amino acid solution in water was spotted directly on top of the AuNP spot and allowed to dry prior to LDI analysis. The inkjet-printed AuNP surfaces were prepared using an Epson Artisan 50 inkjet printer. To prepare the AuNP solution for printing, the AuNPs were added to a printing solution which consisted of 69% water, 20% glycerol, 10% 1,2-hexanediol and 1% triethanolamine. This solvent composition provides the proper viscosity to ensure reproducible printing. The final solution of 2 nm TEGOH AuNPs was at a concentration of 4 μM, the 4 nm TEGOH at 400 nM, the 6 nm TEGOH at 148.5 nM and the 8 nm TEGOH at 62.5 nM to keep the amount of gold consistent. The 13 nm citrate AuNP solution was at a final concentration of 4 μM. The resulting AuNP solution was then added to an empty black inkjet printer cartridge, and a metal slide was placed on the CD printing tray. The NPs were printed in grayscale, going from 100% black (RGB = 0) for the high concentration end to white, or 0% black (RGB = 255), at the low concentration end using the printer software. The slides
were covered with a petri dish top to prevent contamination and allowed to dry. Given the low volume droplets (~ pL) deposited by the printer, the printed slides were microscopically dry within minutes; however, the slides were allowed to dry for 24 h before spotting 1 μL of analyte onto the surface.

2.4.5 Transmission Electron Microscopy

The TEM analyses were done with a JEOL 100S transmission electron microscope. The measurements were done at 200.0 kV and at a magnification of 50,000x or 20,000x. AuNP in printing solutions were printed on copper grids at a specific RGB value and allowed to dry for 24 h before analysis. When analyzing these TEM images, amorphous collections of three or more AuNPs were considered a cluster for the purposes of relating ionization efficiency with cluster formation.

2.4.6 ICP-MS Analysis

Inductively coupled plasma (ICP) MS experiments were done on a Perkin Elmer NEXION 300 X ICP mass spectrometer. The operation RF power was 1.6 kW, and the nebulizer gas flow rate was within a range of 0.9-1 L/min. The plasma gas flow rate and auxiliary gas flow rate were 16.5 L/min and 1.4 L/min, respectively. The analog stage voltage and pulse stage for the detector were -1600 V and 950 V, respectively. The deflector voltage was set to -12 V, and 50 ms was selected for the dwell time during the operation of the ICP-MS. To determine the AuNP amounts in a given printed area, slides were rinsed with 1 mL of an aqua regia solution and diluted to a final volume of 10 mL with MilliQ water. The slides were incubated in the same aqua regia solution for 1 h to dissolve the gold on the slide surface. The slides were then removed, and the remaining solution was analyzed by ICP-MS. Calibration standard solutions of 0, 0.2, 0.5, 1.0, 2.0, 5.0, 10.0 and 20.0 ppb of gold were used to quantify the amount of gold on the slides.

2.4.7 SALDI, MALDI and LDI-MS Analysis
We conducted three basic studies with different experimental substrates for comparison purposes: SALDI, MALDI and LDI. SALDI experiments involved depositing AuNPs onto a stainless steel target and then depositing analyte on top. MALDI experiments were performed according to the typical protocol that involves co-cystallization of a matrix with the analyte. LDI experiments were conducted by depositing analyte onto a stainless steel surface without the addition of AuNPs or matrix. The effect of laser energy, as reported by the instrument software, on the resulting ion signal was investigated to find the optimum laser energy for SALDI, MALDI, and LDI. An example data set can be seen in Figure 2.11. A laser energy of 70% was used for the SALDI and MALDI experiments, whereas a laser energy of 85% was used for the LDI experiments. The optimum laser energy of 70% was independent of the AuNP sizes used in the SALDI experiments.

![Figure 2.11: The effect of laser power on the ion intensity of the protonated arginine peak when ionizing arginine by MALDI (with 30.2 mg/mL CHCA), SALDI (with 2 nm TEGOH AuNPs) and LDI. The laser energy required to ionize the analyte can also be observed.](image)

SALDI, MALDI, and LDI MS experiments were done in positive mode on a reflectron-type time-of-flight (TOF) Bruker Autoflex III Smartbeam mass spectrometer that is equipped with a 355 nm Nd:YAG laser. About 50 laser shots were fired at a frequency of 100 Hz to collect one spectrum with a reflectron voltage of 20.92 kV and an ion source voltage of 18.95 kV. The samples were
prepared on stainless steel targets. In some cases, MS imaging was done on the samples using a raster width of 100 μm. After the imaging analyses, specific m/z values could be selected to generate images that simplified identification of optimal conditions.

2.5 References


4 Kaufmann, R. J. Biotech. 1995, 41, 155–175.


CHAPTER 3

ENHANCED LASER DESORPTION/IONIZATION MASS SPECTROMETRIC DETECTION OF GOLD NANOPARTICLES IN BIOLOGICAL SAMPLES USING THE SYNERGY BETWEEN ADDED MATRIX AND THE GOLD CORE

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3.1 Introduction

Nanoparticles (NPs) are widely used in areas such as sensing, imaging, therapeutics and drug delivery.1-6 There is also growing concern over the fate of NPs in the environment, making their tracking in complex environmental settings important.7-13 Our group has developed a mass spectrometry (MS) based approach that utilizes laser desorption/ionization (LDI) to characterize, detect, and image functionalized NPs in a multiplexed fashion and in a variety of samples, including cells and tissues.14-21 The LDI-MS method relies on laser absorption by the NP core that then enables desorption and ionization of attached monolayers. The result is selective detection of NPs according to the same surface ligands that dictate their biological behavior. We have successfully used this approach to quantify ligands on mixed monolayer NPs,16,20 to understand how NP surface chemistry influences cellular uptake,14 to monitor NP stability in cells,17,19 and to image NPs for security applications and biodistribution studies.18,21 Others have also recently shown that LDI-MS can be extended to other materials, including gold nanorods and carbon nanomaterials.22-25
Although we and others have found that LDI-MS is very effective for detecting a variety of NPs with different core materials and surface ligands, NPs with certain surface ligands (e.g. zwitterionic) are difficult or impossible to detect with this method. This shortcoming limits the scope of materials that can be monitored by LDI-MS. To overcome this limitation, we have investigated the addition of standard matrix-assisted LDI (MALDI) matrices to improve the detectability of gold NPs having surface ligands that are difficult to ionize. From these experiments, we find that matrix concentrations far below those used in typical MALDI experiments can dramatically enhance ligand detection from gold NPs, while maintaining the selective detection capability of the technique. Our results demonstrate a synergistic relationship exists between the NP’s gold core and the matrix that facilitates ligand ionization. This new ability to detect a wider variety of NPs will greatly enhance the utility of LDI-MS for studying nanomaterials in biological systems.

Figure 3.1: Structures of AuNPs and the various monolayer ligands attached that were used.
3.2 Results and Discussion

3.2.1 Improving Detection of NP Ligands

We have used LDI-MS previously to detect and study AuNPs with various ligands attached. While the exact mechanism of ligand ionization from the NPs is not clear, it is presumed to occur via rapid vaporization of the gold core and consequent desorption and ionization of the attached ligands. Although others have attempted to exploit surface plasmons to enhance ionization off AuNPs, the NPs and laser wavelength used in this chapter are not expected to give rise to surface plasmon effects. Small 2 nm core AuNPs do not have appreciable plasmon bands, and the larger AuNPs described later in this chapter have plasmons centered around 520 nm that do not extend to the laser wavelength (i.e. 355 nm) used in our study. The success of LDI-MS for analyzing AuNPs, however, does not extend to all ligands types, as there are some that are difficult or impossible to ionize by LDI. For example, while the ligand ions from TTMA and TEGOH NPs can be readily detected (Figure 3.2a and b), other ligands from NPs such as ZNP1 cannot be observed (Figure 3.2c). As seen in Figure 3.2c, the only NP-related ions observed when doing LDI-MS on such zwitterionic AuNPs are from the gold core. The LDI-MS results in Figure 3.2c and on other NPs with both zwitterionic ligands (data not shown) and amino acid ligands (Figure 3.3) suggest that laser absorption by the gold core is not sufficient to efficiently desorb and ionize these types of ligands.
Figure 3.2: LDI mass spectra of (a) 1 pmol of TTMA AuNPs, (b) 1 pmol of TEGOH AuNPs and (c) 1 pmol of ZNP1 AuNPs. For the TTMA and TEGOH NPs, the peaks indicated by A result from the successive losses of ethylene glycol units from the ligand, while the peaks indicated by B result from the successive losses of methylene groups from the intact ligands.

Figure 3.3: a) LDI mass spectrum of 0.5 pmol Arg NPs. b) LDI mass spectrum of 0.5 pmol Phe NPs. c) MALDI mass spectrum of 0.5 pmol Phe NPs using 2.5 mg/mL CHCA. Black asterisks mark matrix peaks while α and β mark peaks from by-products from the synthesis of the Phe NP ligands. These by-products are ligands that still have a t-butyl group, which is used as a protecting group, attached. Structures can be seen in Figure 3.6.

MALDI matrices improve the detection of compounds that cannot be ionized by LDI alone.\textsuperscript{28-31} Considering this fact, we investigated different matrices and different matrix concentrations to determine if added matrix could enable the detection of ligands from zwitterionic and amino acid-containing AuNPs (e.g. Figure 3.4). Using 1,5-diaminonaphthalene (DAN) or α-cyano-4-hydroxycinnamic acid (CHCA), depending on the AuNP, we find that matrix concentrations as low as 0.5 mg/mL allow for the detection of a variety of zwitterionic or amino-acid containing ligands from AuNPs (Figure 3.5). This matrix concentration is about 20 times lower than the typical DAN concentrations used in MALDI and 60 times lower than the typical CHCA concentration, indicating that only low level matrix is necessary to enable ionization of ligands attached to the gold core. Moreover, the small amount of matrix suppresses the signal from the
gold ions and minimizes the extensive ligand fragmentation that is observed in the LDI spectra of other functionalized AuNPs (e.g. Figure 3.2a and b). Generally, we found that DAN provided better signal for zwitterionic NPs, whereas CHCA provided better signal enhancement for all other AuNPs. To prove that the improved MALDI signal was not due to free ligands that are present in the AuNP solution, samples of every AuNP used in these studies were washed and centrifuged in a molecular cut-off filter prior to analysis. The intact AuNPs are retained, while any free ligands that might be present pass through the filter. In no case are free ligands detected by MALDI in the pass-through solution.

Figure 3.4: MALDI ion intensities at various matrix concentrations for a) 1 pmol of TTMA AuNPs and b) 1 pmol of TEGOH AuNPs using CHCA, and for c) 1 pmol of ZNP2 AuNPs using DAN. In most cases, higher matrix concentrations give rise to higher ion signal, yet lower matrix concentrations could be used to maintain ionization/detection selectivity of the AuNPs in the presence of other molecules.
Figure 3.5: MALDI mass spectra of (a) 1 pmol of ZNP2, (b) 1 pmol of ZNP3 and (c) 0.5 pmol of Arg NPs using 0.5 mg/mL DAN for the ZW AuNPs and 0.5 mg/mL CHCA for the Arg NP. Asterisks mark matrix ions.

The addition of low matrix concentrations not only enables the detection of NP ligands that were previously undetectable but this enhancement also comes without any associated ionization of other compounds that are present with the AuNPs. For example, when AuNPs whose ligands are terminated with Phe and t-butyl Phe (Figure 3.6) are mixed at 1 pmol with 80 pmol of the peptide bradykinin and 0.1 mg/mL of CHCA, the ligands from the AuNP can be detected without any associated ionization of the peptide bradykinin (Figure 3.7). Other AuNPs behave in a similar fashion when low matrix concentrations are used (Figure 3.8). Higher concentrations of matrix further improve the S/N of the NP ligand, but as matrix concentrations exceed around 0.5 mg/mL, peptide ion signals begin to appear. These results are intriguing because they suggest that proper choice of matrix concentration can still allow for the selective detection of NPs by their attached ligands, just as has been observed in our previous LDI-MS studies. As a side note, others have demonstrated that AuNPs themselves can be used to enable peptide ionization in the absence of a traditional organic matrix; however, this ionization of unattached analytes is not observed in our experiments. The reasons for the failure to ionize peptides here may be the types of ligands attached to the AuNPs or the relatively higher concentrations of AuNPs used in this chapter. Previous reports demonstrated peptide ionization using samples with a ratio of peptide concentration to NP concentration of 500:1. When we mix peptides with AuNPs at these ratios, though, we still do not observe peptide signal, suggesting that the chemical nature of the ligand influences peptide ionization.
Figure 3.6: Structures of peaks seen in the MALDI mass spectrum of Phe AuNPs, a) and b) phenylalanine ligand, c) a fragment of the t-butyl derivatized Phe ligand and d) and e) a by-product of the synthesis of phenylalanine ligands with an added t-butyl group, which is used as a protecting group in the synthesis.

Figure 3.7: MALDI mass spectrum of 1 pmol Phe and t-butyl Phe AuNPs together with 80 pmol bradykinin using 0.1 mg/mL CHCA. Only ion signals for the AuNPs are observed, indicating the
selective detection of the NPs at this matrix concentration. An asterisk marks the matrix peak, while a and b indicate the Na\(^+\) and K\(^+\) ions of the Phe AuNP ligand, and c, d, and e indicate ions from the t-butyl Phe AuNPs.

Figure 3.8: Mass spectra of a) a mixture of 1 μM ZNP2 AuNPs and 80 μM bradykinin using 0.5 mg/mL DAN, and b) a mixture of 1 μM TTMA AuNPs and 80 μM bradykinin using 0.1 mg/mL CHCA.

3.2.2 Gold-Matrix Synergy

Given the dramatic enhancement that a small amount of added matrix has on the ionization of ligands attached to AuNPs, we speculated that there may exist a synergistic relationship between the gold core and the matrix when it comes to ligand ionization. To test this idea, we first compared LDI and MALDI of AuNPs with MALDI of the corresponding free ligands (i.e. ligands that are not attached to a gold core) (Figure 3.9). Figure 3.10 summarizes the results for four selected NPs and their corresponding free ligands at different ligand concentrations. The ligand concentrations on the NPs were calculated from the known number of ligands that can bind to AuNPs of varying core sizes (Tables 3.1 and 3.2).\(^{36}\) For the difficult to ionize AuNPs (e.g. ZNP3 – Fig. 3.10a and ZNP2 – Fig. 3.10b), adding matrix to the AuNP significantly improves ligand detection beyond what is possible with MALDI of the free ligand. Indeed, for ZNP3 the ligand is only detected well when MALDI of the AuNPs is performed, indicating that both gold and matrix work together to ionize this ligand. Ligand detection is also improved for the TEGOH ligands when
MALDI is conducted on its AuNP, again suggesting the synergistic role of gold and matrix. The TTMA ligand (Fig. 3.10d), however, does not behave in the same manner. While this ligand is detected more readily off the AuNP when matrix is present than when it is not present, the free ligand is detected even more easily by MALDI indicating that not all ligands benefit from the synergistic relationship between gold and the matrix. It should be noted that the matrix concentrations used for the experiments summarized in Figure 3.10 were chosen based upon the lowest matrix concentrations necessary to give reliable ion signals for the free ligands so that valid comparisons between the three conditions could be made.
Figure 3.9: Mass spectra of 80 μM free ZNP3 ligands analyzed with 10.2 mg/mL DAN (a), 1 μM ZNP3 AuNPs analyzed by LDI (b), 1 μM ZNP3 AuNPs analyzed with 10.2 mg/mL DAN (c), 80 μM
free ZNP2 ligands analyzed with 10.2 mg/mL DAN (d), 1 μM ZNP2 AuNPs analyzed by LDI (e), 1 μM ZNP2 AuNPs analyzed with 10.2 mg/mL DAN (f), 80 μM free TEGOH ligands analyzed with 2.5 mg/mL CHCA (g), 1 μM of 2 nm TEGOH AuNPs analyzed by LDI (h), 1 μM of 2 nm TEGOH AuNPs analyzed with 2.5 mg/mL CHCA (i), 80 μM free TTMA ligands analyzed with 0.25 mg/mL CHCA (j), 1 μM TTMA AuNPs analyzed by LDI (k), 1 μM TTMA AuNPs analyzed by 0.25 mg/mL CHCA (l), 2 μM of 2 nm TEGOH AuNPs analyzed with 2.5 mg/mL CHCA (m), 2 μM of 4 nm TEGOH AuNPs analyzed with 2.5 mg/mL CHCA (n) and 2 μM 8 nm TEGOH AuNPs analyzed with 2.5 mg/mL CHCA (o). The peaks indicated by A result from the successive losses of ethylene glycol units from the ligand, while the peaks indicated by B result from the successive losses of methylene groups from the intact ligands.

**Figure 3.10:** Comparison of ligand ion intensity when doing MALDI of the free ligand, MALDI of the AuNP and LDI of the AuNP for (a) ZNP3 with 10.2 mg/mL DAN for MALDI, (b) ZNP2 with 10.2 mg/mL DAN for MALDI, (c) TEGOH with 2.5 mg/mL CHCA for MALDI and (d) TTMA with 0.25 mg/mL CHCA for MALDI. The matrix concentrations used in each case were chosen based upon
the lowest matrix concentrations necessary to give reliable ion signals for the free ligands so that valid comparisons between the three conditions could be made.

**Table 3.1:** Sample calculations of the concentration of the 2, 4 and 8 nm AuNPs necessary to give a ligand concentration of 80 μM.\(^{36}\)

<table>
<thead>
<tr>
<th>Au Core Size</th>
<th>Calculation</th>
<th>[AuNP]</th>
</tr>
</thead>
</table>
| 2 nm         | \[
\frac{80 \text{ ligands}}{1 \text{ NP}} = \frac{80 \, \mu M}{[2 \, nm \, AuNP]} \\
\]
|              | 1 μM        |
| 4 nm         | \[
\frac{300 \text{ ligands}}{1 \text{ NP}} = \frac{80 \, \mu M}{[4 \, nm \, AuNP]} \\
\]
|              | 267 nM      |
| 8 nm         | \[
\frac{1,200 \text{ ligands}}{1 \text{ NP}} = \frac{80 \, \mu M}{[8 \, nm \, AuNP]} \\
\]
|              | 66.7 nM     |
Table 3.2: Concentrations of different sized AuNPs used in order to obtain the specified ligand concentration.\(^{36}\)

<table>
<thead>
<tr>
<th>Au Core Size</th>
<th>Ligands on NP</th>
<th>[Ligand] (μM)</th>
<th>[AuNP]</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 nm</td>
<td>80 ligands</td>
<td>5</td>
<td>62.5 nM</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
<td>125 nM</td>
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<td>20</td>
<td>250 nM</td>
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<td></td>
<td>40</td>
<td>500 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>80</td>
<td>1 μM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>160</td>
<td>2 μM</td>
</tr>
<tr>
<td>4 nm</td>
<td>300 ligands</td>
<td>5</td>
<td>16.7 nM</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
<td>33.3 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>20</td>
<td>66.7 nM</td>
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<td></td>
<td></td>
<td>40</td>
<td>133 nM</td>
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<td></td>
<td></td>
<td>80</td>
<td>267 nM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>160</td>
<td>533 nM</td>
</tr>
<tr>
<td>8 nm</td>
<td>1,200 ligands</td>
<td>5</td>
<td>4.18 nM</td>
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<tr>
<td></td>
<td></td>
<td>10</td>
<td>8.35 nM</td>
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<td>20</td>
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<td>33.3 nM</td>
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<td>66.7 nM</td>
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<tr>
<td></td>
<td></td>
<td>160</td>
<td>133 nM</td>
</tr>
</tbody>
</table>

To investigate if the synergistic relationship between gold and matrix requires ligand attachment to the gold, zwitterionic AuNPs were mixed with free zwitterionic ligands of a different
structure, and MALDI was performed on the resulting mixture. As an example, upon analyzing ZNP2 AuNPs and ZNP3 free ligands with varying matrix concentrations at the same total ligand amount of 80 pmol, the ZNP2 ligands were more efficiently detected. When the converse experiment was done, so that the ZNP3 ligands were attached to the core and the ZNP2 ligands were free, the ion abundance of the ZNP3 ligands is much higher than the free ZNP2 ligands (Figure 3.11). This more efficient detection of the ligands attached to the NPs instead of the free ligands demonstrates that the ligands have to be attached to the AuNP in order to benefit from the gold/matrix synergy.

![Graph](image)

**Figure 3.11:** MALDI ion intensities at various concentrations of 1,5-diaminonaphthalene (DAN) of a mixture of a) 1 pmol ZNP2 AuNPs and 80 pmol free ZNP3 ligands (not attached to a gold core) and b) 1 pmol ZNP3 AuNPs and 80 pmol free ZNP2 ligands (not attached to a gold core).

Because increased matrix concentrations increase ligand signal, we also speculated that increasing gold amounts should do the same. To test this hypothesis, TEGOH AuNPs with different core sizes were analyzed with 2.5 mg/mL of CHCA present. In these experiments, the different sized AuNPs were separately prepared at concentrations that allowed the indicated ligand concentration to be the same in each case. For example, 0.5 pmol of AuNPs with a 2 nm core size were added to produce a total ligand amount of 40 pmol, whereas 67 fmol of AuNPs with an 8
nm core size were added to produce the same total ligand amount of 40 pmol. In the former case, 2 nm core AuNPs have approximately 80 ligands per NP, while in the latter case the 8 nm core AuNPs have approximately 1,200 ligands per NP. From the graph in Figure 3.12 we can see that as the ligand concentration increases, so does the resulting ion signal; however, as the size of the AuNP core increases, the ligand signal increases to a greater extent. In other words, for a given ligand concentration, higher ligand ion signals are observed when a greater amount of gold is present, further supporting a synergistic relationship between gold and the matrix.

![Graph showing ion intensity against ligand concentration for different AuNP sizes.](image)

**Figure 3.12:** Comparison of MALDI (2.5 mg/mL CHCA) of different sized TEGOH AuNPs at different ligand concentrations. Example spectra from MALDI of each AuNP size can be seen in Figure 3.9.

### 3.2.3 AuNP Analysis in Biological Samples

We have demonstrated that the addition of low matrix concentrations improves ligand ion signals for pure NP samples, but can these NPs be detected in complex mixtures. To test this idea, zwitterionic AuNPs were mixed with HeLa cell lysate and analyzed with and without added matrix. Figure 3.13 illustrates that LDI alone is not enough to ionize the ligands but the use of low DAN concentrations (2.5 mg/mL) enables the selective detection of the ligands from ZNP2 without interfering biomolecular ions. For reasons that are not clear but are reproducible for different NP
types, a slightly higher DAN concentration than found in our initial optimization experiments (i.e. Figure 3.4c) was necessary to detect the NP ligands in such complex samples.

![Figure 3.13: (a) LDI and (b) MALDI using 2.5 mg/mL DAN of cell lysate samples spiked with ZNP2 AuNPs. Region A marks peaks that arise from the surfactant used in the cell lysate, and the black asterisks mark peaks that arise from the matrix.]

3.3 Conclusions

We have shown that the use of MALDI matrix concentrations far lower than in conventional MALDI allows for the selective detection of AuNP ligands that are difficult to detect by LDI-MS. We assert that this ability comes from a synergistic relationship that exists between the gold core of the AuNP and the matrix that is added. These low matrix concentrations not only enable the detection of difficult to ionize NP ligands, but they also enable their detection in the presence of a large background of biological molecules, such as in a cell lysate. This new detection capability will enable the analysis of a wider variety of nanoparticles with greater sensitivity, increasing the utility of the LDI method for tracking nanoparticles in complex biological and environmental matrices.
3.4 Experimental

3.4.1 Chemicals and Materials

α-cyano-4-hydroxycinnamic acid (CHCA) and 1,5-diaminonaphthalene (DAN) were purchased from Sigma Aldrich. Bradykinin was purchased from the American Peptide Company. Acetonitrile, methanol, Dulbecco’s modified Eagle’s medium (DMEM) cell culture media, phosphate-buffered saline (PBS), penicillin and streptomycin were purchased from Fisher Scientific. Lysis buffer was purchased from Genlantis. Fetal bovine serum was purchased from Atlanta Biologicals. Amicon Ultra 3K molecular weight cut-off filters were purchased from Millipore. Deionized water was obtained from a Millipore Simplicity 185 MilliQ system.

3.4.2 Nanoparticle Synthesis

The AuNPs used in these experiments (Figure 3.1) were prepared according to previously published methods. Briefly, 1-pentanethiol protected AuNPs (Au-C5) with core diameters ~2 nm were first synthesized using the Brust-Schiffrin two-phase synthesis method. The surface functionalized AuNPs were prepared using the Murray place-exchange method to generate the NPs of interest. In a typical reaction 10 mg of the Au-C5 was dissolved in 10 mL of distilled dichloromethane. 30 mg of the desired ligand dissolved in methanol was added to the reaction and stirred for 2 days. Solvent was removed and AuNPs were washed with hexane 3 times to remove 1-pentanethiol and excess ligands. AuNPs were then dialyzed for 72 h against MilliQ water using a Spectra/Por Dialysis Membrane (molecular weight cutoff of 1,000 Da), concentrated via lyophilization, and dissolved in MilliQ water. AuNPs with core diameters of 4 and 8 nm were synthesized via heat-induced size evolution, followed by ligand exchange and purified as described above. All AuNPs were characterized by transmission electron microscopy (TEM) to confirm the Au core size and dynamic light scattering (DLS) to determine hydrodynamic radius. The surface
monolayers were characterized using LDI-MS for TTMA and TEGOH, MALDI-MS for Arg and Phe, and NMR for the zwitterionic NPs.\textsuperscript{15,16,38,41,42,44}

3.4.3 Cell Lysate Preparation

HeLa cells were purchased from ATCC (Manassas, VA). The cells were grown on a 24-well plate (30,000 cells/well) in low glucose (1.0 g/L) DMEM supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 IU/mL penicillin and 100 μg/mL streptomycin). Cultures were maintained at 37 °C under constant saturated humidity with 5% CO\textsubscript{2}. After 24 h of plating, the cells were washed one time with 500 μL cold PBS. Then, 250 μL of β-galactosidase lysis buffer (5 times diluted) was added to each well, and the plate was placed on a shaker for 30 min at room temperature.\textsuperscript{19}

3.4.4 Cell Lysate Sample Preparation

For the cell lysate experiments, 10 μL of a 1 μM AuNP solution was spiked into the well containing the HeLa cell lysate solution and shaken for 15 minutes to ensure proper mixing. Once mixing was done, the cell lysate was transferred to a centrifuge tube, and the well was washed 3 times with 180 μL of MilliQ water. These washes were also added to the same centrifuge tube. After these washing steps, the tube was centrifuged at 14,000 rpm for 90 minutes. The resulting pellet was washed five times with 200 μL of 60/40 acetonitrile/water (with subsequent removal of the supernatant). Once the pellet was washed, it was then transferred to the sample target. If LDI-MS was to be performed, no further preparation was needed. If MALDI-MS was to be performed, 1 μL of matrix solution was spotted directly on top of the pellet and allowed to dry before analysis.

3.4.5 Mass Spectrometry Sample Preparation

MALDI-MS experiments were performed according to the typical protocol that involves co-cystallization of a matrix with the analyte, except in the case of the cell lysate experiments as
described in the previous section. Two different matrices were studied. CHCA was prepared at a stock concentration of 30.0 mg/mL in 70% acetonitrile and diluted to lower concentrations as necessary. DAN was prepared at a stock concentration of 10.2 mg/mL in 70% acetonitrile and also diluted to lower concentrations as necessary. For all MALDI experiments, except ones involving cell lysate, 1 μL of matrix solution was mixed with 1 μL of analyte, and then 1 μL of this mixture was deposited onto the target. LDI-MS experiments were conducted by depositing 1 μL of analyte onto a stainless steel surface, except in the case of the cell lysate samples.

3.4.6 Mass Spectrometry Analysis

MALDI and LDI-MS experiments were done in positive mode on a reflectron-type time-of-flight (TOF) Bruker Autoflex III Smartbeam mass spectrometer that is equipped with a 355 nm Nd:YAG laser. Typically, to acquire a single spectrum 50 laser shots were fired at a frequency of 100 Hz at 35-40% of the full laser power for MALDI and 70% of the full laser power for LDI. In all cases, multiple spectra were averaged to obtain the reported data. The reflectron voltage was set to 20.92 kV, and the ion source voltage was set to 18.95 kV. All samples were prepared on stainless steel targets.

3.5 References


30 Kaufmann, R. J. Biotech. 1995, 41, 155–175.


4.1 Introduction

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) has been used to analyze a wide range of biomolecules due to its high sensitivity and minimal sample consumption. Typically an organic matrix is used to facilitate analyte ionization, but some recent work has been devoted to finding alternative matrices to overcome some of the limitations associated with organic matrices, such as low \( m/z \) interferences and spot-to-spot signal heterogeneity. Silicon and platinum nanostructured surfaces, and nanomaterials including gold, silver, platinum, carbon, silicon, iron oxide, titanium dioxide, and zinc oxide nanoparticles have been widely investigated as alternative matrices for MALDI as a way to minimize low \( m/z \) interferences. For example, Chen et al. used carbon nanodots as a substitute for an organic matrix to analyze a wide range of small molecules, including amino acids, peptides, fatty acids and oligosaccharides.

By comparison, relatively little has been done to explore how nanomaterials might improve spot-to-spot signal variability that occurs when using an organic matrix. Liu et al. demonstrated that visible hot spots are formed when using fluorescent quantum dots together with a typical organic matrix, making it easy to focus the laser on areas that were likely to give the best signal, but the quantum dots themselves did not remove the heterogeneity. Wen et al. noted that with proper choice of size and preparation conditions, silicon NPs produced more homogenous spots than typical organic matrices, giving better reproducibility. Wu et al. also
found that certain spotting procedures could improve shot-to-shot reproducibility when using AuNPs as matrices.\textsuperscript{37,38} Taking this one step further, Kawasaki et al. concluded that depositing AuNPs in a thin film using a layer-by-layer approach also resulted in a more homogenous sample spots.\textsuperscript{39}

We wanted to explore another aspect of how NPs dry on surfaces as a means of improving signal homogeneity in the traditional dried droplet approach to MALDI sample preparation. It is well understood that nanomaterials will accumulate in the outer ring of a spot when deposited onto a surface via a phenomenon known as the “coffee-ring” effect. This effect occurs as solute and suspended particles are carried to the outer edge of the spot due to capillary flow in the drying sample.\textsuperscript{40-42} These flows, known as Marangoni flows, are typically the cause of the heterogeneity observed in traditional MALDI spots, and several creative approaches have been explored to overcome the problem.\textsuperscript{6,43} Because the coffee-ring effect occurs so readily when nanoparticles are present in solution, we were interested to see if this effect could be exploited to improve the detection of biomolecules in MALDI. One previous study had found that PtNPs did not overcome the heterogeneity associated with sample deposition, but this previous work did not consider soluble NPs used together with traditional matrices.\textsuperscript{44} Here, we describe the effect of using soluble NPs to enhance the formation of coffee-rings as a way of concentrating analytes into a smaller area. We find that NPs not only readily concentrate analyte molecules because of the coffee-ring effect, thereby enhancing analyte signal, they also make the analyte rich zones readily apparent and improve the analysis of higher m/z ions. Overall, this chapter represents a new way in which nanomaterials can be exploited to improve biomolecule analysis.
4.2 Results and Discussion

We first set out to establish matrix and AuNP concentrations that could be used together with analytes to form a good coffee ring. We found that coffee rings are best formed from solutions containing low matrix concentrations (< 5 mg/mL) and low pmol amounts of AuNPs (Figure 4.2) in water. When such conditions are used together with µM analyte concentrations, a visible ring is formed (Figure 4.3a). MALDI-MS imaging of these samples reveals that analyte, matrix, and NPs are all observed at the edge of the dried sample rather than in the middle of the spot (Figure 4.3c, d, and e) in contrast to a typical MALDI spot without NPs (Figure 4.3f). These observations not only show that a coffee-ring is formed when NPs are present but also surprisingly show that biomolecules and matrix co-deposit with the AuNPs into a thin layer at the outer edge of the drying sample.
Figure 4.2: Optical images of spots of 2 pmol AuNPs with 1 pmol bradykinin and varying CHCA concentrations; a) 0.5 mg/mL CHCA, b) 1 mg/mL CHCA, c) 2.5 mg/mL CHCA, d) 5 mg/mL CHCA, e) 15 mg/mL CHCA and f) 30 mg/mL CHCA.
Figure 4.3: a) Optical image of a spot of 1 pmol of the peptide bradykinin deposited with 2 pmol of TEGOH AuNPs and 1 mg/mL of CHCA, b) Optical image of a spot of 1 pmol of the peptide bradykinin deposited with 30 mg/mL of CHCA, c) Distribution of the (M+H)^+ signal from the spot seen in (a), d) Distribution of the (M+H)^+ TEGOH ligand signal from the same spot in (a), e) Distribution of the proton-bound dimer of CHCA (i.e.\([2M+H]^+\)) from the same spot in (a), f) Distribution of the (M+H)^+ signal from the spot seen in (b). Sample spectra from (c) and (f) can be seen in Figure 4.4.
Figure 4.4: a) 1 pmol bradykinin with 2 pmol TEGOH and 1 mg/mL CHCA, spectrum taken on the edge of the visible coffee ring formed as the sample droplet dried, b) 1 pmol bradykinin with 2 pmol TEGOH and 1 mg/mL CHCA, spectrum taken inside the coffee ring formed.

We next set out to investigate whether this method of deposition led to analyte signal enhancement via a concentration effect since the analyte collects in a narrow ring. We find that this deposition method results in ion signals that are higher than those obtained from conventional MALDI-MS. As an example, the protonated peptide signals acquired from the 1 pmol samples of bradykinin deposited in the presence of AuNPs and 1 mg/mL CHCA are typically 10-fold and 100-fold higher than the protonated peptide signals acquired when performing conventional MALDI (using 30 mg/mL CHCA) or MALDI with 1 mg/mL CHCA, respectively (Figure 4.5a). Similar signal enhancements are observed with other peptides (Figure 4.6) and lipids such as phosphatidylcholine and phosphatidylglycerol (Figure 4.5c). The presence of both intact AuNPs and matrix are necessary to observe the improved signal. Low concentrations of matrix alone without AuNPs (bottom trace in Figure 4.5a) or AuNPs alone without matrix (data not shown) provide little to no ion signal for peptides and lipids at low pmol amounts. To estimate the analyte and matrix concentrations in the outer edge of the spots, the areas covered by the total spot without AuNPs (e.g. Figure 4.3f) and with AuNPs (e.g. Figure 4.3c) were measured. The ratio of these two areas was found to be approximately 30, suggesting the analyte signal enhancement is primarily due to its concentration in the coffee ring that is formed as the sample dries, rather than via some synergistic effect between the AuNPs and matrix as observed previously. Analyte concentration via the coffee-ring effect suggests a new mode by which NPs facilitate the detection of biomolecules.
Figure 4.5: a) Comparison of 1 pmol bradykinin with TEGOH AuNPs (2 pmol) and 1 mg/mL CHCA (top); 30 mg/mL CHCA and no NPs (middle) and 1 mg/mL CHCA and no NPs (bottom); b) 5 pmol phosphatidylcholine (PC) and 5 pmol phosphatidyglycerol (PG) with 20 mg/mL DHB; c) 5 pmol phosphatidylcholine and 5 pmol phosphatidyglycerol with TEGOH AuNPs (1 pmol) and 1 mg/mL DHB; black asterisks mark matrix peaks, red asterisks mark AuNP peaks and blue asterisks mark lipid fragments.
Figure 4.6: a) 1 pmol kinetensin with 30 mg/mL CHCA, b) 1 pmol kinetensin with 200 nM TEGOH and 1 mg/mL CHCA, c) 1 pmol preproenkephalin with 30 mg/mL CHCA, d) 1 pmol preproenkephalin with 200 nM TEGOH and 1 mg/mL CHCA, e) 1 pmol spinorphin with 30 mg/mL CHCA, f) 1 pmol spinorphin with 200 nM TEGOH and 1 mg/mL CHCA.
To confirm the importance of coffee ring formation on peptide detection, samples were dried on the target at low temperatures, which is known to minimize coffee-ring formation.\textsuperscript{6,42,44,45} Both visual inspection and mass spectrometry imaging indicate that the coffee ring effect is diminished when compared to samples that are allowed to dry at room temperature (Figure 4.7). Although some sample ‘hot spots’ are formed when the sample is prepared at low temperature, the signal for the protonated peptide is typically 5 – 10 times lower than when a coffee ring is formed. This observation supports the idea that the coffee ring accounts for the signal enhancement observed for peptides.

**Figure 4.7:** Minimizing coffee-ring formation leads to lower peptide ion signals. a) Distribution of the \((M+H)^+\) signal from 1 pmol of the peptide bradykinin deposited with 2 pmol of TEGOH AuNPs and 1 mg/mL of CHCA that was dried at room temperature; b) Sample mass spectrum from the
outer ring of the room temperature spot; c) Distribution of the (M+H)^+ signal from the same sample in (a) that was dried at 4°C; d) Example mass spectrum from inside the spot that was allowed to dry at 4°C.

We also investigated the effect of matrix concentration and AuNP surface chemistry on analyte ionization efficiency. Signal enhancement factors were determined for a range of matrix concentrations and for AuNPs with three different ligands (structures shown in Figure 4.1). The signal enhancement factor is calculated by dividing the ion abundance of the protonated analyte (e.g. [M+H]^+ of bradykinin) when adding AuNPs by the ion abundance of the protonated analyte from conventional MALDI (i.e. 30 mg/mL). CHCA concentrations ranging from 0.01 to 30 mg/mL CHCA were investigated, and concentrations from 0.25 to 5.0 mg/mL CHCA were found to provide the highest signal enhancement factors (Figure 4.8) for two of the three AuNPs studied. Better ion signals with the lower matrix concentrations are consistent with our observations that better coffee-rings are formed in the presence of lower matrix concentrations. When comparing the effect of AuNP surface chemistry, we find that the TEGOH AuNPs gave the highest signal enhancement factors, which is consistent with Chapter 2 in which these AuNPs were used as stand-alone matrices.28
Figure 4.8: A comparison of signal enhancement factors for the peptide bradykinin with different AuNPs present and at varying CHCA concentrations. The signal enhancement factor is calculated by dividing the ion abundance of the protonated analyte (e.g. [M+H]⁺ of bradykinin) when adding AuNPs by the ion abundance of the protonated analyte from conventional MALDI (i.e. 30 mg/mL).

The concentration of peptides into the tight coffee ring caused by the presence of the AuNPs also allows for the improved detection of peptide mixtures (Figure 4.9). When a BSA digest is analyzed with AuNPs and low CHCA concentrations, not only are the ion abundances greater for almost all the peptides, but the sequence coverage is improved from 25% to greater than 40% as compared to conventional MALDI. Much of the improved sequence coverage comes from the detection of higher m/z peptides that are not detectable by conventional MALDI. It may be that
these higher m/z peptides experience a greater enhancement in ionization efficiency when the AuNPs are present, which is an observation that we intend to investigate more in the future. Detection limits for protein digests are found to be in the low-to-sub fmol range when using the AuNPs and low CHCA concentrations.

**Figure 4.9**: a) 100 fmol of BSA digest analyzed using 30 mg/mL CHCA, b) 100 fmol of BSA digest analyzed using 200 fmol AuNPs and 1 mg/mL CHCA.

A consistent observation in the mass spectra from the coffee-ring forming samples is the relatively low ion signals from the matrix itself, despite it being concentrated in the outer edge of the samples. These low matrix ion signals reduce spectral congestion in the low m/z range and thus might facilitate the analysis of low molecular weight analytes. To test this idea, we analyzed amino acids and other small molecules with and without AuNPs (Figure 4.10). As can be seen in Figure 4.10, the spectra with AuNPs added are less congested than the spectra without AuNPs added, even though the analyte signal is higher when conventional MALDI is performed. To get these low molecular weight compounds to deposit in the coffee ring, though, we had to use relatively high analyte concentrations (> 100 µM), which is consistent with what is known about the effect of molecular weight on coffee ring formation.44 This result with the low molecular weight compounds and the higher enhancements observed for larger m/z ions (Figure 6) suggest
that there is a molecular weight dependence associated with the coffee-ring signal enhancement effect.

Figure 4.10: a) 1 nmol histidine with 30 mg/mL CHCA, b) 1 nmol histidine with 2 pmol TEGOH and 1 mg/mL CHCA, c) 1 nmol adenosine-5’-diphosphate (ADP) with 30 mg/mL CHCA, d) 1 nmol adenosine-5’-diphosphate (ADP) with 2 pmol TEGOH and 1 mg/mL CHCA, e) 1 nmol arginine with
30 mg/mL CHCA, f) 1 nmol arginine with 2 pmol TEGOH and 1 mg/mL CHCA; black asterisks mark matrix peaks and red asterisks mark peaks from the TEGOH AuNPs.

While the presence of AuNPs causes analytes to concentrate into a ring at the edge of the dried sample, we surmised that salts, such as NaCl, might deposit in a different manner than in normal MALDI dried droplets. If correct, then the coffee-ring effect might be a means of minimizing ionization suppression by salts during the analysis. To test this idea, we analyzed 1 pmol peptide solutions having 150 mM NaCl with and without AuNPs. The peptide ion signals from samples with the AuNPs present (Figure 4.11a) are routinely higher than the peptide ion signals from samples without the AuNPs (Figure 4.11b), indicating that the presence of the AuNPs overcomes some of the signal suppression that occurs with such salty samples. It can also be seen from the optical images of both sample spots that the salt is distributed in a very different manner and is typically not associated with the coffee ring (Figure 4.12).

**Figure 4.11**: a) Mass spectra of samples with 1 pmol bradykinin, 2 pmol TEGOH AuNPs, 1 mg/mL CHCA, and 150 mM NaCl, b) Mass spectra of samples with 1 pmol bradykinin, 30 mg/mL CHCA and 150 mM salt; asterisks mark matrix peaks in both spectra.
Figure 4.12: a) Optical image of a sample spot of 1 pmol bradykinin, 2 pmol TEGOH AuNPs, 1 mg/mL CHCA, and 150 mM NaCl, b) Optical image of a sample spot of 1 pmol bradykinin, 30 mg/mL CHCA and 150 mM salt.

4.3 Conclusions

The addition of NPs to samples containing matrix and analyte force the mixtures to dry with a visible coffee ring. When probed by MALDI, these coffee rings are found to be enriched in analyte, thereby providing enhanced ion signals for a range of biomolecules. The fact that the analytes are deposited in the outer ring generates clearly visible sample regions, allowing the analyte to be easily located and sampled by the laser. The coffee rings are most readily formed when using low (< 5 mg/mL) matrix concentrations and pmol levels of NPs. Analyte molecular weight also appears to influence ion signals as higher molecular weight compounds are enhanced to a greater extent than lower molecular weight compounds. This molecular weight dependency also allows samples with high ionic strengths to be analyzed with less analyte suppression, as the salts are not concentrated in the outer ring formed upon drying in the presence of the NPs. Overall, the coffee-ring effect represents a new mode by which nanomaterials can be used to enhance the MALDI-based detection of biomolecules.
4.4 Experimental

4.4.1 Chemicals and Materials

α-cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), phosphatidylcholine, phosphatidylglycerol, histidine, arginine and adenosine-5’-diphosphate were purchased from Sigma Aldrich (St. Louis, MO). Bradykinin, preproenkephalin, kinetensin and spinorphin were purchased from the American Peptide Company (Sunnyvale, CA). A bovine serum albumin (BSA) digest standard was obtained from Protea Biosciences (Morgantown, WV). Acetonitrile and methanol were purchased from Fisher Scientific. Deionized water was obtained from a Millipore Simplicity 185 MilliQ system.

4.4.2 Nanoparticle Synthesis

The AuNPs used in these experiments (Figure 4.1) were prepared according to previously published methods. Briefly, 1-pentanethiol protected AuNPs (Au-C5) with core diameters of approximately 2 nm were first synthesized using the Brust-Schiffrin two-phase synthesis method. The surface functionalized AuNPs were prepared using the Murray place-exchange method to functionalize the TTMA and TEGCOOH AuNPs. The TEGOH AuNPs were synthesized by the single-phase synthesis method described previously. In a typical reaction, 10 mg of the Au-C5 was dissolved in 10 mL of distilled dichloromethane. 30 mg of the desired ligand dissolved in methanol was added to the reaction and stirred for 2 days. Solvent was removed and AuNPs were washed with hexane 3 times to remove 1-pentanethiol and excess ligands. AuNPs were then dialyzed for 72 h against MilliQ water using a Spectra/Por Dialysis Membrane (molecular weight cutoff of 1,000 Da), concentrated via lyophilization, and dissolved in MilliQ water. AuNPs with core diameters of 4 and 8 nm were synthesized via heat-induced size evolution, followed by ligand exchange and purified as described above. All AuNPs were characterized by transmission electron microscopy (TEM) to confirm the Au core sizes and
dynamic light scattering (DLS) to determine hydrodynamic radii. The surface monolayers were characterized using LDI-MS.\textsuperscript{53,54}

4.4.3 Mass Spectrometry Sample Preparation

MALDI-MS experiments were performed according to the typical protocol that involves co-cystallization of an organic matrix with the NP-analyte solution. CHCA was prepared at a stock concentration of 30.0 mg/mL in 70% acetonitrile and diluted to lower concentrations as necessary. DHB was prepared at a stock concentration of 20.0 mg/mL in 70% acetonitrile and diluted to lower concentrations as necessary. To prepare the NP-analyte solution, NPs were first mixed with the analyte(s) of interest at the desired ratio. Then, 5 μL of this NP-analyte solution was mixed with 5 μL of matrix solution, and 1 μL of this mixture was deposited onto the target. LDI-MS experiments were conducted by depositing 1 μL of the NP-analyte mixture onto a stainless steel surface.

To minimize the coffee ring effect, a stainless steel slide was first stored at -20 °C for one hour before spotting samples on it. The samples were prepared according to the procedure described above. Once the samples were ready to spot, the slide was removed from the freezer, and the samples were immediately spotted at room temperature. After spotting, the samples were then stored at 4 °C until they were completely dry.

4.4.4 Mass Spectrometry Analysis

MALDI and LDI-MS experiments were done in positive mode on a Bruker Autoflex III reflectron-type time-of-flight (TOF) mass spectrometer that is equipped with a 355 nm Nd:YAG laser that is part of the Smartbeam system. The reflectron voltage was set to 20.92 kV, and the ion source voltage was set to 18.95 kV. Typically, to acquire a single spectrum 200 laser shots were fired at a frequency of 100 Hz at 25% of the full laser power for MALDI and 70% of the full laser power for LDI. In all cases, multiple spectra were averaged to obtain the reported data. All samples
were prepared on stainless steel targets. In some cases, MS imaging was done on the samples using a raster width of 50 μm. After the imaging analyses, specific m/z values could be selected to generate images that simplified identification of optimal conditions.

4.5 References


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CHAPTER 5

IONIZATION ENERGETICS OF GOLD NANOPARTICLE ASSISTED LASER DESORPTION/IONIZATION

MASS SPECTROMETRY

5.1 Introduction

LDI-MS and MALDI-MS have both been used to analyze a wide variety of compounds.\textsuperscript{1-7} LDI is limited, though, to compounds that absorb the laser energy. This limitation is remedied by adding a small organic compound (i.e. a matrix) that absorbs the laser energy and transfers it to the analyte. In an attempt to overcome some of the limitations of using an organic matrix,\textsuperscript{3,4} researchers have investigated the use of nanomaterials to assist in ionization. Various inorganic and noble metal nanomaterials have been investigated as alternative matrices for use with a wide range of biomolecules.\textsuperscript{8-15}

While it is well understood that using these nanomaterials enhances the ionization of various compounds, the understanding of the mechanism of ionization is limited. The MALDI ionization mechanism is hypothesized to be different than the ionization mechanism that exists when using nanomaterials.\textsuperscript{16,17} Investigating the extent of energy transfer during these nanomaterial-assisted processes would provide some insight into the ionization mechanism when nanomaterials are used. One approach to studying the energetics of the ionization process is to use thermometer ions, or compounds that fragment in a known fashion and with known energetics. These thermometer ions report on ionization energetics via the relative intensities of the parent and fragment ions.\textsuperscript{18} One of the more popular thermometer ions that has been used to investigate ionization mechanisms is a benzylpyridinium salt.\textsuperscript{19-23} Tang et al. recently used this
compound to investigate the internal energy transfer using various carbon-based mass spectrometry analyses in order to better design these nanomaterials to assist in ionization.\textsuperscript{19} Lai et al. have used these ions to look into the various phase transition stages during the desorption and ionization process when using gold nanoparticles, including melting, vaporization and phase explosion of the AuNPs.\textsuperscript{22}

We have previously used gold nanoparticles to assist in the ionization of both small and large biomolecules (see chapters 2 and 4).\textsuperscript{15,24} However, little is known about why these processes result in higher analyte ion intensities. In this study, we used a benzylpyridinium salt to investigate and compare energy transfer in LDI, conventional MALDI, and AuNP assisted MALDI. We also explored the effect that matrix concentration, AuNP surface chemistry and size, and AuNP concentration have on the ionization energetics. We find that adding AuNPs results in similar desorption efficiencies as conventional MALDI but often with slightly higher survival yields of the parent ion. These results indicate that the ionization process that occurs when using AuNPs is gentler than the conventional MALDI process.
5.2 Results and Discussion

We first set out to compare conventional MALDI analysis with AuNP assisted low matrix MALDI analysis using the thermometer ion benzylpyridinium salt. Figure 5.2a is a typical spectrum of benzylpyridinium salt with 15 mg/mL CHCA (conventional MALDI), and Figure 5.2b is a spectrum of benzylpyridinium salt with TEGOH AuNPs and 0.5 mg/mL CHCA. The parent ion is observed at m/z 170 while the main fragment can be seen at m/z 91 (see Figure 5.1b for structures). It can be seen from these spectra that using AuNPs and low concentrations of matrix to assist in analyte ionization is a comparable ionization process to conventional MALDI given the similar ion abundances for the parent and fragment ions.
Using the intensities of both the parent ion and the fragment ion obtained from spectra like those seen in Figure 5.2, both the desorption efficiency and the survival yield can be calculated (see experimental section). These values provide information about the ability of the process to produce analyte ions and can be used to compare the ionization energies of the various processes. The fragmentation of the benzylpyridinium salt was used to compare the ionization processes involved in LDI and MALDI both with and without AuNPs at different laser fluences (Figure 5.3). As can be expected, as the laser fluence is increased, the desorption efficiency and the energy of the ionization process both increase due to the increased energy from the laser. As can be seen from Figure 5.3a, conventional MALDI results in higher desorption efficiencies than LDI both with and without AuNPs, meaning MALDI is more efficient at producing analyte ions than LDI. At first, examining the survival yield with these methods in Figure 5.3b does not reveal any definitive trends. However, when comparing just LDI with and without AuNPs, it can be seen that adding AuNPs results in a higher energy ionization processes, as indicated by the increased fragmentation of the benzylpyridinium ion (Figure 5.3c). The addition of AuNPs with low concentrations of matrix, however, results in very similar survival yields at most laser fluences when compared to conventional MALDI (Figure 5.3d). These results demonstrate that adding AuNPs when doing
MALDI with low concentrations of matrix is comparable to conventional MALDI in terms of efficiency of the process in producing ions at various laser fluences.

**Figure 5.3:** Investigation of laser fluence on the desorption efficiency and survival yield of various different ionization processes; a) Desorption efficiency at different laser fluences for both LDI and MALDI with and without 1 pmol of TEGOH AuNPs, b) Survival yield of the parent ion at different laser fluences for both LDI and MALDI with and without 1 pmol of TEGOH AuNPs, c) Survival yield of the parent ion at different laser fluences for LDI both with and without 1 pmol TEGOH AuNPs, d) Survival yield of the parent ion at different laser fluences for MALDI both with and without 1 pmol TEGOH AuNPs.

We also investigated the ionization energetics associated when using different matrix concentrations. AuNPs and the benzylpyridinium salt were mixed with varying concentrations of
CHCA and analyzed by MALDI. When comparing both the desorption efficiencies and survival yields (Figure 5.4), it is evident that increasing the concentration of the matrix also increases the amount of the analyte ionized. However, using AuNPs with 0.5 mg/mL CHCA results in a higher survival yield of the parent ion than when using just traditional concentrations of CHCA, i.e. 15 mg/mL (Figure 5.4b). Although the survival yield is increased with AuNPs present as compared to conventional MALDI, there is no difference when compared to using low concentrations of CHCA without AuNPs. There is, though, an increase in the desorption efficiency when AuNPs are present with low matrix concentrations, as compared to low matrix concentrations alone. These results indicate that there is a trade-off when using low matrix concentrations and AuNPs to assist in ionization. Analyte ions can be produced more efficiently in the presence of AuNPs, but the survival yields are not necessarily better. Overall, these observations are consistent with our observations in chapter 4 that using AuNPs and low CHCA concentrations can improve detection efficiency as compared to conventional MALDI. These results are also consistent with the matrix concentrations identified in chapter 4 as the survival yields with both AuNPs and CHCA are higher at 0.5 mg/mL CHCA concentrations than at 15 mg/mL CHCA concentrations.24

Figure 5.4: Investigation of varying CHCA concentration of the ionization process; a) Desorption efficiency at different CHCA concentrations both with and without 1 pmol TEGOH AuNPs, b)
Survival yield of the parent ion at different CHCA concentrations both with and without 1 pmol TEGOH AuNPs.

We also demonstrated in chapters 2 and 4 that some AuNP surface chemistries are more effective at facilitating biomolecule ionization than others.\textsuperscript{15,24} To better understand why these specific surface chemistries provide the greatest ionization efficiencies and enhancement, we determined the desorption efficiencies and survival yields of benzylpyridinium with three different NPs (Figures 5.5). As can be seen from Figure 5.5, AuNPs with a TEGOH ligand result in both the highest desorption efficiency and survival yield of the parent ion when compared to AuNPs with TTMA and TEGCOOH ligands (see Figure 5.1a for the structure of these ligands). These results indicate that the TEGOH ligands provide a relatively low energy ionization process while also being the most efficient at desorbing the analyte.

\textbf{Figure 5.5}: a) Desorption efficiency and b) survival yield of the parent ion for AuNPs with varying surface chemistries.

When the concentration of TEGOH AuNPs used with MALDI analysis was investigated, we found that increasing the concentration of the AuNPs increases the desorption efficiency but there were no significant changes in the survival yield of the parent ion over the AuNP concentration range used in this study (Figure 5.6). From this, we concluded that more AuNPs
leads to a more efficient analyte ionization but has little effect on the amount of energy involved in the ionization process.

**Figure 5.6**: Desorption efficiency and survival yield of the parent ion at different concentrations of TEGOH AuNPs.

We also set out to investigate the energy involved in the ionization processes that occur on the inkjet printed surfaces we previously used to assist in ionization. As can be seen in Figure 5.7, the desorption efficiency decreases as the amount of AuNPs that are deposited on the surface also decreases across the printed AuNP gradient. This is consistent with our previous observation that lowering the concentration of AuNPs used to assist in ionization results in a less efficient ionization process. However, the survival yields of the parent ion remain constant across the inkjet printed AuNP gradient, even at the optimal printed region that was determined in chapter 2. This
leads to the conclusion that the energy involved in the ionization processes using different concentration ranges of the printed AuNPs is similar regardless of the amount of AuNPs on the surface.

Figure 5.7: a) Desorption efficiency and b) survival yield of the parent ion at different amounts of AuNPs on an inkjet printed surface.

Finally, considering that the amount of gold present might affect the energy involved in the ionization process, we investigated the effect that increasing the AuNP core size would have on the fragmentation of the benzylpyridinium salt. Figure 5.8 depicts both the desorption efficiencies and survival yields of the parent ion with AuNPs of varying core sizes. Increasing the core size, and therefore the amount of gold, decreases both the desorption efficiency and survival yield of the parent ion. This indicates that larger AuNPs result in a less efficient, higher energy ionization process, and therefore more fragmentation of the analyte. Considering this observation, TEGOH AuNPs with a smaller gold core size (i.e. 2 nm) would be the optimal AuNPs to use to assist in ionization because they are most efficient at producing intact analyte ions. This conclusion is consistent with our previous findings from chapters 2 and 4 when using AuNPs to enhance signal from various biomolecules.  

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Based on the investigation of the fragmentation of benzylpyridinium salt with various ionization processes, it was determined that using AuNPs with low matrix concentrations is comparable to conventional MALDI in terms of both the ionization efficiency of the analyte and the survival yield of the parent ion. This indicates that both the ability of the method with AuNPs to produce analyte ions and the ionization energy involved in the process is similar to MALDI. This helps to explain our previous observations that using both AuNPs and low matrix concentrations can assist in the ionization of analytes. The energy of these processes was also investigated with different matrix concentrations, AuNPs with different surface chemistries and core sizes, and different AuNP concentrations. It was concluded that using 2 nm TEGOH AuNPs at low μM concentrations with 0.5 mg/mL CHCA resulted in a higher survival yield of the parent ion and the most efficient ionization process, which is consistent with our previous findings.
5.4 Experimental

5.4.1 Chemicals and Materials

\(\alpha\)-cyano-hydroxycinnamic acid (CHCA) was purchased from Sigma Aldrich. Acetonitrile was purchased from Fisher Scientific. Deionized water was obtained from a Millipore Simplicity 185 MilliQ system. The benzylpyridinium salt was synthesized using the previously established method.19

5.4.2 Nanoparticle Synthesis

The AuNPs used in these experiments (Figure 5.1a) were prepared using previously published methods: TEGOH25, TTMA25,26 and TEGCOOH.27 In short, the Brust-Schiffrin two-phase synthesis method was used to synthesize pentanethiol-coated AuNPs with core diameters around 2 nm.28 Subsequently, the Murray place-exchange method was used to functionalize TTMA and TEGCOOH. TEGOH was synthesized by the single-phase synthesis method described previously.29,30 After synthesis, each AuNP was dialyzed for 72 h against MilliQ water using a Spectra/Por Dialysis Membrane (molecular weight cutoff of 1,000 Da) to separate the free ligands from the AuNPs.1,31 AuNPs with core diameters of 4 and 8 nm were synthesized via heat-induced size evolution, followed by ligand exchange and purified as described above.32 The resulting AuNPs were then characterized by transmission electron microscopy (TEM) to confirm the Au core size and LDI-MS to ensure proper monolayer attachment.1,31

5.4.3 Sample Preparation

A stock solution of \(\alpha\)-cyano-hydroxycinnamic acid (CHCA) was prepared by dissolving 30 mg of CHCA in 70% acetonitrile. Dilutions of this stock were prepared when necessary. For the MALDI and LDI experiments, 5 \(\mu\)L of a solution of 2 mM benzylpyridinium salt20 was mixed with either 5 \(\mu\)L of a solution of CHCA or water; 1 \(\mu\)L of this mixture was then spotted on the target and allowed to dry prior to analysis. For the experiments in which AuNPs were used, a solution with a
final concentration of 2 mM benzylpyridinium salt and 2 μM AuNPs was prepared. 5 μL of this solution was then either mixed with 5 μL of a solution of CHCA for MALDI analysis or mixed with water for LDI analysis.

5.4.4 Mass Spectrometry Analysis

MALDI and LDI-MS experiments were done in positive mode on a reflectron-type time-of-flight (TOF) Bruker Autoflex III Smartbeam mass spectrometer that is equipped with a 355 nm Nd:YAG laser. Typically, to acquire a single spectrum, 200 laser shots were fired at a frequency of 100 Hz with an offset of 60%. To change the laser fluence, both the range and the laser energy were changed. In all cases, multiple spectra were averaged to obtain the reported data. The reflectron voltage was set to 20.92 kV, and the ion source voltage was set to 18.95 kV. All samples were prepared on stainless steel targets.

5.4.5 Data Analysis

The intensities of both the parent ion and fragment ion of the benzylpyridinium salt were obtained from the mass spectra. These values were then used to calculate both the desorption efficiency and the survival yield. The desorption efficiency (DE) is calculated by summing the intensities of both the parent and fragment ion (Eq. 5.1). The survival yield (SY) is calculated by determining the percentage of parent ion that exists in the total ion yield (Eq. 5.2).19,21,23,33

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\text{Eq. (5.1): } \text{D.E.} = \text{Int. MW Peak} + \text{Int. Fragment Peak}
\]

\[
\text{Eq. (5.2): } \text{S.Y.} = \frac{\text{Int. MW Peak}}{\text{Int. MW Peak} + \text{Int. Fragment Peak}} \times 100\%
\]

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SUMMARY AND FUTURE WORK

6.1 Dissertation Summary

In this dissertation, the use of AuNPs with LDI-MS was investigated. They were used in place of an organic matrix to enhance signal from low molecular weight biomolecules. This was done using a novel method to deposit the AuNPs on the surface. It was also discovered that a synergy exists between the gold core of the AuNPs and a small amount of added matrix, which was used to ionize ligands attached to the AuNPs that were previously not detected with LDI-MS. This method of using both AuNPs and low concentrations of matrix was also used to enhance signal from biomolecules due to increased concentration of these three compounds in the outer ring of the dried spot due to the coffee-ring effect that controls how NPs dry. In addition, thermometer ions were used to investigate the energy involved in each of the aforementioned ionization processes.

First, AuNPs were used to enhance signal from low molecular weight biomolecules like amino acids. The AuNPs minimized the amount of interferences in the low m/z range by removing the matrix typically used to assist in ionization. During these experiments, it was determined that using an inkjet printer to deposit the AuNPs resulted in a more homogenous distribution of AuNPs than typical pipette spotting. An optimal amount of AuNPs on the surface was determined and it was discovered that this optimal amount resulted in clustering of the NPs on the surface, leading to the greatest enhancement of analytes. This suggests that the proximity of the AuNPs to one another is important when they are used to assist in ionization. This could be due to a more efficient or greater transfer of energy from the clustered AuNPs to the analyte that arises from an interaction of the clustered AuNPs when the laser energy is introduced to the sample and the
subsequent energy transfer occurs to the analyte. It is known as the laser energy interacts with
the NPs, it will either result in an increase in temperature of the NPs or induce an electric field
around the NPs, which then influences the ionization of analytes. For example, Obara et. al
irradiated AuNPs around 40-100 nm in size with a laser and measured both the temperature and
strength of the electric field around the AuNPs. They noted that both an increase in temperature
and the formation of an electric field around the AuNPs occurs upon irradiation. Given this
previous observation and our results, it is possible that the aggregation of the 2 nm AuNPs into
the appropriate sizes allows for the proper amount of laser energy to be transferred to desorb
and ionize analytes by increasing the effective temperature and producing an electric field. These
enhanced energy transfers could explain why the clustering of NPs influences the ionization
process more than individual NPs. These new findings suggest a possible explanation for the
enhancement of the wide variety of analytes that have been ionized with the assistance of AuNPs.

Second, a small amount of matrix was added to AuNPs to ionize ligands that were
previously not detected by LDI-MS. It is evident that the presence of both the gold core of the
AuNP and the matrix is necessary to effectively absorb and transfer the laser energy to the AuNP
ligands to allow for their subsequent ionization and detection. One possible explanation is that
the small amount of added matrix allows for an increased amount of laser energy that is absorbed
by the entire sample, which can be used to overcome an ionization energy barrier that could not
be reached when the gold core of the AuNP was solely used to desorb and ionize the attached
difficult to ionize ligands. As stated earlier, it is known that NPs either heat up or generate an
electric field when irradiated by a laser and these processes allow for the transfer of energy to
ionize analytes present. Knochenmuss has extensively investigated and reviewed the ionization
mechanism involved in MALDI and it is understood that the matrix absorbs the laser energy and
converts it to heat, which then allows for analyte ionization. The addition of a matrix to a sample
containing AuNPs could result in a more rapid increase in temperature or an enhanced electric field around the NPs due to the ability of the matrix to also convert the laser energy to heat. These enhanced energy transfer processes could explain why both the NPs and the matrix are necessary in order to ionize certain ligands attached to the NPs. This suggests that these components are working synergistically to transfer the laser energy to the attached ligands. This synergy not only allowed us to detect AuNPs in complex biological systems that we could not detect using LDI-MS but it also provided a selective process in which biomolecules in the sample were not ionized or detected. This method provides a way to monitor a wider range of AuNPs in complex biological systems, which would further our understanding of the effect AuNPs have when introduced to biological systems.

Third, the manner in which AuNPs dry (coffee-ring effect) was taken advantage of to enhance signal from biomolecules like peptides and lipids. Mixing AuNPs, a small amount of matrix and the analyte results in the concentration of the three components to the outer ring of the dried spot. This concentration effect results in an enhancement of the analyte and the deposition of the AuNPs closer to one another. As concluded earlier, the proximity of AuNPs to one another is important in order to allow for an efficient transfer of the laser energy to the analyte to occur. However, the fact that these AuNPs deposit in a small area not only allows for a minimized distance between them but it also creates a dark ring that is visible to the naked eye. This ring that is formed by the AuNPs actually points out exactly where the AuNPs, matrix and analyte are due to the concentration of the three components to this ring. While nanomaterials have been used to improve the laser-based analysis of various analytes by minimizing low m/z interferences\textsuperscript{7-9}, allowing for a more efficient transfer of energy\textsuperscript{7,9-11} and increasing both selectivity and sensitivity\textsuperscript{7-9,11-13}, the exploitation of the coffee-ring effect is a new mode by which analyte
ionization can be improved using nanomaterials. This method provides a simple way to visualize the location of the analyte and enhances the analyte signal, resulting in lower limits of detection.

Finally, thermometer ions were used to investigate the energy involved in each of the ionization processes studied. The fragmentation of benzylpyridinium salt was monitored and used to calculate both the desorption efficiencies and survival yields of the processes investigated. These calculations provided insight into how efficient each process is at producing analyte ions and the ionization energy associated with each process. These results indicate that conventional MALDI and the use of AuNPs with small amounts of matrix are comparable ionization processes in terms of analyte desorption efficiency and the ionization energy of the process. They also indicate, however, that the use of the correct matrix concentration and AuNP in regards to size and surface chemistry are crucial to allowing for the most efficient ionization process. This suggests that each detail of the AuNPs used must be considered before using it to assist in laser-based mass spectrometry techniques and provides some insight into how the laser energy may be transferred from the gold core of the AuNP to the attached ligands and to analytes present in the sample as well. For example, AuNPs with smaller gold cores and a neutral surface chemistry allowed for the greatest enhancement throughout this work. Russell et al. used AuNPs of varying sizes to enhance peptides and concluded that larger AuNPs involve a mostly thermally driven energy transfer while smaller AuNPs (around 2 nm) involve a predominantly electronic driven energy transfer process.14 Perhaps this largely electronic energy transfer process allows for a more localized transfer of energy to the analyte than a thermally driven process and the neutral surface chemistry results in fewer interferences due to minimal interactions with other components. Future work will allow for further investigation into the ionization mechanism that occurs when AuNPs are used to assist in the ionization of various analytes and will provide more insight into why these specific aspects of the AuNPs are crucial to analyte ionization.
6.2 Future Directions

In the following sections, possible new uses of AuNPs with LDI-MS and variations of this mass spectrometry method will be described.

6.2.1 Electro spray-assisted laser desorption/ionization mass spectrometry (ELDI-MS) for AuNP analysis

While LDI-MS analysis of AuNPs can provide information regarding the ligands attached to the NPs, and ICP-MS can be used to obtain information about the core of the NPs, one technique that could potentially provide information about the entire NP would be beneficial. One possible method that could be used is electro spray-assisted laser desorption/ionization mass spectrometry (ELDI-MS). This technique incorporates two modes of ionization, which increases sensitivity due to the ionization of neutrals that are desorbed in the first ionization process are subsequently ionized by the second process. ELDI has been used to detect various biomolecules like peptides and proteins.\textsuperscript{15,16} This method could be applied to AuNP analysis to provide more information about them. When AuNPs are analyzed by LDI, only the ligands are ionized. However, there are many products that are formed when the laser ablates the NPs and these products are neutral. Introducing a second form of ionization could allow for the detection of gold atoms with ligands still attached, providing further information about the biodistribution of AuNPs in biological systems.

6.2.2 Tailored AuNPs for capturing biomolecules with subsequent ionization

Previous work has been done in which the surface chemistries of AuNPs have been altered in order to capture certain biomolecules that can then be ionized by MALDI to selectively extract
and enhance them.\textsuperscript{17,18} However, these studies require separations after the extraction because conventional MALDI is used and will ionize peptides that are not captured by the AuNP. We have enhanced both ligands attached to the AuNP and biomolecules utilizing both the synergy between the gold core of the AuNPs and a small amount of added matrix, and the coffee ring effect that concentrated the analyte, AuNP and matrix to a visible area. Either or both of these methods could be used with AuNPs with attached ligands that can selectively capture specific biomolecules. This technique would still extract and enhance the desired biomolecules just by synthesizing a ligand that is selective, but would also remove the separation step previously required because only analytes bound to the AuNP would be ionized. This would allow it to be used to improve sensitivity in complex biological samples due to minimal sample loss; one possibility would be screening for disease biomarkers. One other possibility would be to synthesize mixed monolayer AuNPs with either varying selective properties or with a selective capture ligand and another ligand that would enhance ionization.

\textbf{6.2.3 AuNP assisted imaging of biomolecules in tissues injected with AuNPs}

We have previously studied the biodistribution of AuNPs in mice by using mass spectrometric imaging to locate the AuNPs in their tissues.\textsuperscript{19} Knowing that AuNPs can assist in the ionization of low molecular weight biomolecules, and larger biomolecules with the addition of low concentrations of matrix, they could be used to enhance signal from nearby biomolecules in these tissues. Seeing as the mice have been injected with AuNPs and these AuNPs are already present in the tissues imaged, they could enhance nearby biomolecules. This would provide information about the effect AuNPs have on biomolecule distribution in tissues once they have entered. However, it would be selective to biomolecules that are close to the AuNPs because the low matrix concentrations used would not ionize every biomolecule in the tissue. This would
further our understanding of the effect AuNPs have on important biomolecules when they enter a biological system.

6.2.4 Investigating the ionization process involved when using AuNPs with LDI-MS

While the mechanism of both LDI and MALDI ionization is still unknown, there are plenty of theories that exist. Considering we use LDI-MS to analyze and detect AuNPs in complex biological systems, a better understanding of the mechanism of ionization would be beneficial. It is understood that the AuNPs transfer the laser energy to the attached ligands or nearby molecules, which are subsequently desorbed and ionized. However, further insight into this process could lead to improvements in sensitivity and selectivity. Pyridine-functionalized AuNPs were previously synthesized for on-surface host-guest chemistry. If these AuNPs were functionalized with a benzylpyridinium group instead, the fragmentation of this group could provide insight into the energy involved in the ligand ionization process. Varying the chain length of the ligand could also provide some information about the distance necessary for efficient energy transfer. Furthermore, ligands we previously used to enhance biomolecules could be used with varying chain lengths to determine how close these biomolecules have to be in order for the energy transfer to occur.

6.3 References


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