Studies of atmospheric pressure visible-wavelength MALDI-MS

Zhen Sun
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A Thesis

entitled

Studies of Atmospheric Pressure Visible-Wavelength MALDI-MS

by

Zhen Sun

Submitted to the Graduate Faculty as partial fulfillment of the requirements for the

Master of Science Degree in Chemistry

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Dr. Dragan Isailovic, Committee Chair

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The University of Toledo

May 2012
An Abstract of

Studies of Atmospheric Pressure Visible-Wavelength MALDI-MS

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Matrix-assisted laser desorption/ionization technique (MALDI) is well proven as a
soft and efficient ionization technique used in mass spectrometry (MS) for the analysis of
biomolecules (such as sugars, peptides, DNAs, and proteins), and large organic
molecules (such as synthetic polymers). While the technique has been developed in
1980s, the improvements in MALDI-MS instrumentation and search for new matrices
have continued.

In the first project of this thesis, an atmospheric pressure (AP) visible-wavelength
MALDI source was developed and coupled to a quadrupole time-of-flight (Q-TOF) mass
spectrometer. This instrument employed a pulsed laser emitting light at a wavelength of
532 nm to desorb and ionize samples such as dyes, oligosaccharides, peptides, and
synthetic polymers at atmospheric pressure. Ions formed were analyzed by the Q-TOF in
both MS and tandem MS (MS/MS) modes. Several visible-wavelength absorbing dyes
were successfully utilized and studied as matrices. Among these dyes, nuclear fast red
(NFR), rhodamine B isothiocyanate (RITC), and 5(6)-carboxytetramethyl-rhodamine
N-succinimidyl ester (TAMRA, SE) are reported as effective visible-wavelength
MALDI-MS matrices for the first time. This project demonstrates applicability of
visible-wavelength AP MALDI-MS and AP MALDI-MS/MS to the detection and structural analysis of dye molecules, biomolecules such as sugars and peptides, and synthetic polymers.

In the second project, the model protein lysozyme was modified with fluorescein isothiocyanate (FITC). 64% of lysozyme was conjugated with FITC. Crystallization of pure lysozyme and FITC-conjugated lysozyme were performed after protein purification and pre-concentration. Pure lysozyme samples formed very good crystals, but there was no crystal observed for FITC-conjugated lysozyme. These two samples were then analyzed by different sample preparation methods using MALDI-MS. However, only lysozyme crystal with UV MALDI matrix spotted on top of it worked well. By performing a series of experiments, we confirmed that: it is crucial to have a matrix for the analysis of a single lysozyme crystal and the lysozyme crystal has different crystal structure than the matrix-analyte co-crystal. Secondly, it has not been possible to form crystal with 64% of lysozyme being conjugated with FITC dye.

In summary, we developed an AP visible-wavelength MALDI-MS methodology, which enabled the analysis of biomolecules and synthetic polymers. Visible-wavelength AP MALDI-MS and MS/MS analyses of a variety of samples at atmospheric pressure were presented for the first time. Several visible-wavelength absorbing dyes were successfully utilized as matrices, and three dyes were reported as novel visible-wavelength MALDI matrices. Additional experiments were performed to covalently attach FITC to lysozyme and crystallize fluorochrome-conjugated protein. MALDI-MS was employed to study crystals of unlabeled lysozyme, while crystallization of FITC-conjugated lysozyme needs to be optimized further.
This thesis is dedicated to my mother. Thank you for always being there for me.
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Additionally, I would like to thank Walter Berger Jr. for machining parts for instrument, Shu Xu for help with crystallization of lysozyme and Alan Caporali and Samuel Dapore-Schwartz for useful advices. PEG samples, glutathione, and protein crystallization supplies were generous gifts from laboratories of Drs. Ronald Viola, Kenneth Hensley and Max Funk, respectively. Thank you to the past and present members of Dr. Dragan’s Group: Nidhi Jaiswal, Suraj Saraswat, Yang Xu, Rachel Marvin, Raymond West, and all others. Thank you all for sharing your time and expertise with me. I had a good time working with all of you. Last but not least, I would like to thank everyone in the Instrumentation Center for generous help in the past two years. Finally, I would like to thank the UT Chemistry department for giving me the opportunity to study here.

To my family far away in China, I appreciate all the love and understanding.
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List of Abbreviations

5(6)-TAMRA, SE ......5(6)-Carboxytetramethyl-rhodamine N-succinimidyl ester
ACTH 1-17 ............Adrenocorticotropic hormone fragment
AgTFA ................Silver trifluoroacetylacetone
AP-MALDI ............Atmospheric pressure matrix-assisted laser desorption ionization
collision induced dissociation
ESI-MS ................Electrospray Ionization Mass Spectrometry
FITC ....................Fluorescein isothiocyanate
Glufib ...................[Glu^1]-Fibrinopeptide B
LOD .....................Limit of detection
ITO .....................Indium-tin oxide
MALDI ..................Matrix-assisted laser desorption/ionization
UV-MALDI ............MALDI with ultraviolet laser wavelengths
IR-MALDI ............MALDI with infrared laser wavelengths
AP-MALDI ............MALDI at Atmospheric Pressure
MS .....................Mass Spectrometry
MS/MS .................Tandem mass spectrometry
NFR ....................Nuclear fast red
NR .....................Neutral red
PEG .....................Polyethylene glycol
PEG, mme .............Polyethylene glycol monomethyl ether
R6G .....................Rhodamine 6G
RB .....................Rhodamine B
RITC ...................Rhodamine B isothiocyanate
SDS-PAGE .............Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis
TFA .....................Trifluoroacetic acid
TOF .....................Time-Of-Flight
TOF-MS ................Time-Of-Flight Mass Spectrometer
UV-MALDI-MS ........Ultraviolet Matrix-assisted laser desorption/ionization
UV-VIS ................Ultraviolet-Visible wavelengths
Chapter 1:

Introduction

1.1 Mass Spectrometry

Mass spectrometry (MS) is an analytical method, which measures the mass-to-charge ratios of the charged samples. The applications of MS are broad due to the useful information on mass and structure of the analytes that this technique provides. All MS instruments consist of three major parts: an ion source, a mass analyzer, and a detector. Generally, the role of the ion source is to convert the sample from solid or liquid phase to gas phase and ionize it, and then to transfer into the mass analyzer usually by applying voltage. The mass analyzer is designed to separate the sample ions apart according to their mass-to-charge ratios in a vacuum chamber in several steps including accelerating and focusing of ions by applying electromagnetic fields. Some mass analyzers can also be used to select a particular mass of ions or to filter a particular range of masses of ions. Finally, the ion detector measures the mass-to-charge ratios of ions and transfers the signal to an output computer. As the mass analyzer and detector are designed mainly to sort and detect the analyte ions, their selections are based on the type of MS experiments performed, the mass of the analytes, and mass resolution and accuracy needed.
However, the ion source performance is mainly sample-dependent. So far, several different ionization techniques have been developed based on the physical and chemical properties of the samples of interest. For example, electron ionization (EI) and chemical ionization (CI) are techniques that can be chosen for the analysis of volatile samples, while fast atom bombardment (FAB), thermospray, matrix assisted laser desorption ionization (MALDI), electrospray ionization (ESI), and atmospheric pressure chemical ionization (APCI) [1] can be adapted to the analysis of non-volatile samples. Among those ionization techniques, MALDI and ESI are the most popular and powerful techniques that are used for the analyses of large biomolecules.

1.2 Matrix-Assisted Laser Desorption/Ionization

Matrix-assisted laser desorption/ionization technique (MALDI) is well proven as a soft and efficient ionization technique used in mass spectrometry (MS) for the analysis of biomolecules and large organic molecules since its invention in 1988 by Karas, Hillenkamp, and Tanaka et al [2, 3]. MALDI, along with ESI [4], has became the most popular and powerful technique utilized in the studies of macromolecules, which were considered “fragile” earlier in the studies using other MS ionization techniques.

As the name of MALDI indicates, the laser is used to achieve desorption and ionization of the sample of interest. To do this, a specific matrix is chosen to assist this process. Generally, the MALDI process is broken into three steps (Figure 1-1) [5]. First of all, a laser of a specific wavelength is utilized to ablate and ionize the analyte/matrix mixture; secondly, the matrix chosen should strongly absorb the laser energy and pass some of that energy to the analyte; finally, the charged analyte ions are formed and
moved to the mass analyzer with the charged ions forced by electric field applied to the sample plate.

![Diagram of MALDI process](image)

Figure 1-1: Matrix-assisted laser desorption and ionization process.

The mechanism of ion formation in MALDI process is still unclear. The MALDI process is a complicated process, and there is no single mechanism that can explain the origin of all ions observed in a MALDI spectrum. A large number of possible mechanisms for MALDI process have been postulated [6]. However, it is proved by all of the studies that the matrix chosen must absorb very well at the laser wavelength used for desorption and ionization [5, 7, 8].

1.2.1 MALDI at Different Wavelengths

Since the development of MALDI, UV lasers such as the nitrogen laser (337 nm) and the frequency-tripled Nd:YAG laser (355 nm) have been largely used for MALDI-MS analysis in combination with aromatic matrices which have strong electronic
transitions in the UV region [5]. Very powerful lasers with repetition rates of up to 1 kHz have been built at UV wavelengths. Therefore, it is not surprising that most of the commercially available MALDI-MS instruments have been equipped with UV lasers. And that most of the research involving MALDI-MS has been done by utilizing commercially available UV-MALDI-MS instrumentation.

Recently, more and more IR and visible-wavelength lasers have been used for desorption and ionization of a variety of samples. First report utilizing infrared laser was published by Overberg et al [9]. A mechanically Q-switched Erbium-YAG laser with a pulse width of ~200 ns was used for the experiments in which carboxylic acids, glycerol and urea served as matrices. In 1998, Niu et al. showed that MALDI mass spectra obtained with UV and IR laser wavelengths look similar for the analysis of proteins [10]. However, IR-MALDI requires higher pulse energy due to lower matrix absorption, which results in higher sample consumption per pulse compared to UV-MALDI [10, 11]. In the meantime, IR-MALDI-MS has shown to be “softer” than UV-MALDI-MS, causing less ion fragmentation and adduct formation [7, 10-12]. Similarly, visible-wavelength MALDI-MS may result in less fragmentation and photolytic damage of the analytes than UV-MALDI-MS since it is “softer” than the latter technique and macromolecules are transparent in the visible region of the spectrum.

1.2.2 Matrix Selection

The matrix plays an important role in MALDI-MS. The main requirements when selecting a matrix for the analysis of a particular sample are [1]:

i. The matrix chosen must be able to absorb most of the laser energy via electronic excitation and pass some of the energy to the analyte.
ii. The matrix can co-crystallize with the analyte molecules in order to isolate the analyte molecules and prevent analyte aggregation.

Based on these criteria, α-cyano-4-hydroxycinnamic acid (CHCA) [13] and 2,5-dihydroxybenzoic acid (DHB) [14] were shown to be suitable and effective for the analysis of peptides and oligosaccharides by mass spectrometers that employ ultraviolet (UV) lasers for desorption and ionization. Sinapinic acid was shown to be a very effective matrix for the analysis of large proteins, even though it often produces the protein-SA adduct peaks [15]. Juhasz et al introduced HABA as a matrix for peptides, proteins, and glycoproteins with the mass of up to 250 kDa [16] and Wu et al found that 3-HPA is a good matrix for the analysis of oligonucleotides [17]. Those matrices all have strong electronic absorption at UV region and are able to donate protons to analyte ions, as well as co-crystallize with the analytes. Many UV matrices have been reported working well in the infrared region of the spectrum by Overberg et al [9]. The most commonly used UV-MALDI-MS matrices and their structures are shown in Table 1.1.

Unfortunately, because the mechanism of matrix-analyte energy transfer is still unclear, there is no standard guideline for the selection of matrices for a particular kind of analytes. Most of matrices commonly used so far were selected after a time-consuming period of empirical screening and testing of a large number of possible candidates.

However, the search for new matrices and the studies about the mechanism of MALDI-MS have not stopped. Various compounds have been screened as potential MALDI matrices for the analysis at different wavelengths [8, 18, 19]. Also, special sample plate surfaces, which can serve as a buffer between the laser power and the analytes ions have also been designed and used to assist the process of desorption and ionization of biomolecules [20].
Table 1.1: Names, abbreviations, structures, and applications of commonly used matrices.

<table>
<thead>
<tr>
<th>Name</th>
<th>Abbreviation</th>
<th>Structure</th>
<th>Application</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Cyano-4-hydroxycinnamic acid</td>
<td>α-CHCA</td>
<td><img src="image" alt="Structure" /></td>
<td>Peptide/protein Mass &lt; 10 kDa</td>
</tr>
<tr>
<td>3,5-dimethoxy-4-hydroxy-4-hydroxycinnamic acid</td>
<td>SA</td>
<td><img src="image" alt="Structure" /></td>
<td>Peptide/protein Mass &gt; 10 kDa Dendrimers;</td>
</tr>
<tr>
<td>2,5-Dihydroxybenzoic acid</td>
<td>DHB</td>
<td><img src="image" alt="Structure" /></td>
<td>Synthetic polymers Carbohydrates</td>
</tr>
<tr>
<td>2-(4-Hydroxyphenylazo)benzoic acid</td>
<td>HABA</td>
<td><img src="image" alt="Structure" /></td>
<td>Peptide/protein Mass &gt; 10 kDa</td>
</tr>
<tr>
<td>Glycerol</td>
<td>---</td>
<td><img src="image" alt="Structure" /></td>
<td>Peptide/protein Liquid matrix</td>
</tr>
<tr>
<td>2,4,6-Trihydroxyacetophenone</td>
<td>THAP</td>
<td><img src="image" alt="Structure" /></td>
<td>Oligonucleotide Mass &lt; 3.5 kDa</td>
</tr>
<tr>
<td>3-Hydroxypicolinic acid</td>
<td>3-HPA</td>
<td><img src="image" alt="Structure" /></td>
<td>Oligonucleotide Mass &gt; 3.5 kDa</td>
</tr>
<tr>
<td>Dithranol</td>
<td>DIT</td>
<td><img src="image" alt="Structure" /></td>
<td>Lipids</td>
</tr>
</tbody>
</table>
1.3 Atmospheric Pressure MALDI-MS

Because most of the commercial MALDI-MS instruments are expensive, the development of inexpensive “homemade” atmospheric pressure MALDI ion sources has become more popular. Unlike vacuum MALDI sources, AP MALDI sources can be built based on the researchers’ demand and easily interchanged with ESI sources of most commercially available quadrupole time-of-flight and ion trap MS instruments. Current, different types of commercial AP MALDI ion sources were designed for specific type of mass spectrometers and they commonly use a UV laser for desorption and ionization [21]. Some studies of AP MALDI-MS were done utilizing Q-TOF and ion trap mass analyzers [22-24]. It was shown that AP MALDI-MS can produce comparable results to conventional vacuum MALDI-MS in terms of ionization and sensitivity [24], and both MS and tandem MS data can be obtained. Structural studies of biomolecules such as oligosaccharides and sulfonic acid derivatized tryptic peptides and synthetic polymers have been performed using AP MALDI-MS utilizing mainly UV lasers for desorption and ionization. AP MALDI utilized IR lasers for desorption/ionization of biomolecules have also been studied as well as imaging analysis using an infrared laser [18, 21-27].

1.4 Quadrupole Time-of-Flight (Q-TOF) Mass Spectrometer

Q-TOF is the mass spectrometer that we used to perform visible-wavelength MALDI-MS experiments described in this thesis. It is a hybrid mass spectrometer that consists of a combination of the quadrupole analyzer and the orthogonal time-of-flight analyzer.

The quadrupole analyzer consists of four parallel circular rods with each opposing rods connected together electrically. A radio frequency (RF) voltage is applied between
one pair of rods and a direct current voltage is then superimposed on the RF voltage. While ions travel through the analyzer between the four rods, only those of a certain mass-to-charge ($m/z$) ratio will pass along the middle of the quadrupole for a given ratio of voltages. The other ions, which have unstable trajectories, will collide with the rods. This allows the operator to scan for a certain range of $m/z$ values by continuously varying the applied voltage and also to filter ions with specific $m/z$ values by applying a particular value of voltage [28].

Between the quadrupole analyzer and the orthogonal time-of-flight analyzer, there is the hexapole collision cell and the hexapole transfer lens. The hexapole collision cell has a gas inlet port to introduce a buffer gas (argon), which can be used for collision-induced dissociation (CID). The hexapole transfer lens is used for ion transfer with less or no loss of ions. After leaving the quadrupole analyzer, the ions of interest are transferred and focused into the pusher. The pusher then will accelerate a section of the ion beam along the axis perpendicular to their initial direction of motion towards the reflectron time-of-flight analyzer, which will then reflect ions back to reach the detector. The pusher has a pulse repetition rate of up to 30 kHz, which provides a full spectrum every 33 microseconds [29].

While ions travel from the pusher to the reflectron then to the detector, they are separated by the time-of-flight analyzer based on their mass-to-charge ratios [30]. This can be derived from the equations below:

\[ E_p = z \times U \]  
\[ E_k = \frac{1}{2}mv^2 \]
Equation 1.1 reveals that the potential energy \((E_p)\) of a charged ion in the electric field is related to charge of the ions and the voltage applied \((U)\). Equation 1.2 shows the kinetic energy of an ion, where \(E_k\) is the kinetic energy, \(m\) is mass of the ion, and \(v\) is the velocity of the ion.

When an ion of a certain mass-to-charge ratio is accelerated into the time-of-flight tube by applying voltage \(U\), its potential energy is converted to its kinetic energy. So, \(E_p\) equals \(E_k\) in this case. The velocity of the ion can also be determined by the length of the time-of-flight tube \((L)\) divided by the time of the flight of the ion \((t)\). Therefore, above equations can be written as:

\[
z \times U = \frac{1}{2} m \cdot \left(\frac{L}{t}\right)^2 \quad \text{Equation 1.3}
\]

By rearranging Equation 1.3, the mass-to-charge ratio of ion can be expressed by the other factors:

\[
\frac{m}{z} = \frac{2U}{L^2 \cdot t^2} \quad \text{Equation 1.4}
\]

The Equation 1.4 reveals that the mass-to-charge ratio of an ion is proportional to the time-of-flight square. Based on this equation, the ions can be separated according to their flight times, with ions of the smaller mass-to-charge ratios arriving first.

In our studies, we coupled a visible-wavelength AP MALDI ion source to a Q-TOF mass spectrometer. We performed MS and MS/MS analyses at atmospheric pressure using visible-wavelength matrices. With AP-MALDI-Q-TOF instrument setup, six visible-wavelength absorbing dyes were successfully utilized and studied as matrices. Dye molecules, sugars, peptides, and synthetic polymers were analyzed, and fragmentation of sugars and peptides has been studied. Limit of detection and sample
morphology in our AP visible-wavelength MALDI-MS technique were also investigated. Additionally, crystallization of proteins in the presence of fluorochrome was studied as a tool that may improve detection of proteins by MALDI-MS. Detailed information about the experiments performed and results obtained are shown in the following chapters.
Chapter 2

Visible-wavelength MALDI MS

2.1 Introduction

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been used for the analysis of biomolecules and synthetic polymers since its development [2, 3]. Soon after the invention of MALDI, it was shown that matrices such as α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) are suitable and effective for the analysis of peptides and oligosaccharides by mass spectrometers that employ ultraviolet (UV) lasers for desorption and ionization [13, 14]. UV lasers such as the nitrogen laser (337 nm) and the frequency-tripled Nd:YAG laser (355 nm) often show better MALDI-MS performance than lasers at other wavelengths. Therefore, it is not surprising that most of the commercially available MALDI-MS instruments have been equipped with UV lasers.

However, the improvements in MALDI-MS instrumentation and search for new matrices have continued. Various compounds have been screened as potential MALDI matrices, while IR and visible-wavelength lasers have been used for desorption and ionization of a variety of samples. IR-MALDI-MS has shown to be “softer” than UV-MALDI-MS, resulting in less ion fragmentation, but it requires higher pulse energy [7, 10-12]. Similarly, visible-wavelength MALDI-MS may result in less fragmentation...
and photolytic damage of the analytes than UV-MALDI-MS since it is “softer” than the latter technique and most analytes do not absorb visible light.

While there have been a few studies published using visible-wavelength MALDI-MS, the full potential of this methodology is not completely explored. Earlier studies have used rhodamine 6G [31-33], rhodamine B [33], neutral red [34], 2-amino-3-nitrophenol [35] and coumarin dyes [8] as matrices for visible-wavelength MALDI-MS. Also, binary matrix systems have been introduced by mixing strong visible-wavelength absorbers with organic components, which helped the ionization of peptides and proteins [19, 32, 36]. In one report, a gold film was used to assist the ionization of peptides in the presence of the matrix [20]. This system functioned because the surface plasmon absorption band of gold nanoparticles formed upon ablation of the gold film resonated well with emission wavelength of the laser. All of these studies were carried out in vacuum using either readily-available frequency-doubled Nd: YAG lasers operated at 532 nm or tunable visible-wavelength lasers. However, in order to encourage a wider application of visible-wavelength MALDI-MS, there is a need for a detailed evaluation of the technique and the discovery of more efficient visible-wavelength MALDI matrices.

One way to enhance the utilization of visible-wavelength MALDI-MS is to study its performance under atmospheric pressure (AP) conditions. While most UV, IR, and visible-wavelength MALDI-MS studies have been performed in vacuum, reports of AP MALDI-MS have been made utilizing Q-TOF and ion trap mass analyzers [22-24]. AP MALDI-MS produced results comparable to conventional vacuum MALDI-MS in terms of ionization and sensitivity [24]. However, unlike vacuum MALDI sources, AP MALDI sources can be readily built and are convenient for operation at atmospheric pressure.
Furthermore, it appears that any type of mass spectrometer that was originally designed to operate under atmospheric pressure can be coupled to an AP MALDI source. Current, commercially available AP MALDI instruments utilize UV lasers for desorption and ionization [21]. Structural and imaging studies of biomolecules and synthetic polymers have been performed using AP MALDI-MS utilizing mainly UV or IR lasers for desorption and ionization [18, 21-27]. For further exploration and utilization of MALDI-MS, it would be useful to perform MALDI-MS analyses under ambient pressure conditions using visible-wavelength lasers.

In this report, we coupled an AP visible-wavelength MALDI source to a quadrupole time-of-flight (Q-TOF) mass spectrometer. Mass spectra were recorded by utilizing a Nd:YAG laser operating at 532 nm. Six visible-wavelength absorbers were evaluated as matrices, including three dyes (NFR, RITC, and TAMRA, SE) that have not been explored previously as visible-wavelength matrices. Several types of analytes were tested, including dyes, oligosaccharides, peptides, and synthetic polymers, and data in both MS and MS/MS modes were obtained. Ion source design and instrument settings were optimized in order to increase the efficiency of the ion transfer from the AP source into the vacuum of the mass analyzer. Additionally, sample spots were imaged by light microscopy to investigate the influence of their morphology onto MALDI-MS signals.

2.2 Experimental

2.2.1 Materials and Reagents

Indium-tin oxide (ITO)-coated glass slides, CHCA, DHB, and the peptide calibration standard mixture were purchased from Bruker Daltonics (Bremen, Germany). Acetonitrile (CH$_3$CN, HPLC grade), water (HPLC grade), and MRFA (Met-Arg-Phe-Ala)
were from Fisher Scientific (Pittsburgh, PA). 7-diethylaminocoumarin-3-carboxylic acid, succinimidyl ester was purchased from Invitrogen (Carlsbad, CA). Glutathione was from Alfa Aesar (Ward Hill, MA). All other peptides as well as dyes, oligosaccharides, and polymers were purchased from Sigma (St. Louis, MO). All chemicals were used without further purification. Marker dyes originated from Sharpie markers (Newell Rubbermaid Office Products, Oak Brook, IL).

2.2.2 Sample Preparation

Initial studies utilized the ink from Sharpie permanent marker pens. Red and blue marker dyes were drawn onto an ITO-coated glass slide and analyzed without addition of any matrices. Six visible-wavelength MALDI matrices: neutral red (NR, purity > 90 %), nuclear fast red (NFR), rhodamine B (RB), rhodamine 6G (R6G), rhodamine B isothiocyanate (RITC) and 5(6)-carboxytetramethyl-rhodamine N-succinimidyl ester (5(6)-TAMRA, SE, purity ~80 % ), were analyzed after dissolving them in 1:1 (v:v) H₂O:CH₃CN solution containing 0.1 % trifluoroacetic acid (TFA, purity >98 %). Structures of the six matrices are shown in Figure 2-1. The concentration of matrix solutions was 10 mg/ml except for NFR, which saturated at a lower concentration. 2-amino-3-nitrophenol was also used as a visible wavelength matrix. It was dissolved in acetone at concentration of 10 mg/ml. CHCA was used as matrix for ultraviolet (UV)-MALDI-MS analyses of peptides and was saturated in the same solvent as visible-wavelength matrices. DHB served as the matrix for UV-MALDI-MS analysis of oligosaccharides and was dissolved in 1 mM solution of sodium acetate at concentration of 10 mg/ml.
Figure 2-1: Structures of the matrices tested: neutral red (NR); nuclear fast red (NFR); rhodamine B (RB); rhodamine 6G (R6G); rhodamine B isothiocyanate (RITC), and 5(6)-Carboxytetramethyl-rhodamine N-succinimidyl ester (5(6) TAMRA,SE).

Coumarin labeled peptides were prepared by mixing 10 μL of 10 mg/mL peptide with 1 μL of 10 mg/mL coumarin dye, and then incubated in the dark for 1 hour. D-(+)-raffinose pentahydrate (purity ≥ 99%), L-glutathione (reduced, 97%), angiotensin II (purity ≥93%), bradykinin (purity ≥ 98%), [Glu1]-Fibrinopeptide B (purity ≥90%), adrenocorticotropic hormone fragment (ACTH 1-17, purity ≥97%), coumarin-labeled peptides, polyethylene glycol (PEG 1000, average MW 1000), and PEG methyl ether (PEG 2000 mme, average MW 2000) were analyzed after mixing them with visible-wavelength MALDI matrices. Samples analyzed in positive ion mode were dissolved in 1:1 (v:v) H₂O:CH₃CN solution containing 0.1 % TFA, whereas samples analyzed in negative ion mode were prepared in 1:1 (v:v) H₂O: CH₃CN solution.
Matrix-to-analyte molar ratio was varied from 2:1 to ~300:1. PEG samples were also analyzed after mixing with silver trifluoroacetate (AgTFA) and 2-amino-3-nitrophenol matrix [35].

Sample spots analyzed by visible-wavelength MALDI-MS were prepared by mixing 2 μL of a sample with 2 μL of the matrix and dried under vacuum in a desiccator. UV-MALDI MS sample preparation was done using the dried-droplet method [2]. Briefly, 1 μL of each sample was mixed with the same volume of appropriate matrix and air dried.

2.2.3. UV-VIS and Fluorescence Spectroscopy

Absorption spectra of pure matrices were measured using a photodiode array (PDA) UV-Vis spectrophotometer HP8452 (Hewlett Packard, Palo Alto, CA). Absorption spectra of matrices in solution were measured in quartz cuvettes in the wavelength range from 200 nm to 800 nm. Absorption spectra of MALDI spots were measured on ITO-coated glass slides in the wavelength range from 400 nm to 800 nm.

Emission fluorescence spectra of matrices were measured using a LS 50B luminescence spectrometer from PerkinElmer (Beaconsfield, Buckinghamshire, England). Fluorescence spectra of matrices in solution were measured between 550 and 700 nm using an excitation wavelength of 532 nm. Fluorescence spectra of solid matrices deposited on a glass slide were measured under similar excitation and emission conditions using a front surface accessory (PerkinElmer).
2.2.4. AP Visible-Wavelength MALDI-TOF MS Instrument

The AP-LDI-MS instrument (Figure 2-2) was built by coupling a frequency-doubled Nd:YAG laser (Continuum, Santa Clara, CA), which emits pulsed light at 532 nm with repetition rate up to 15 Hz, with a commercial electrospray ionization (ESI) Q-TOF mass spectrometer (Micromass Q-TOF Micro, Waters, Milford, MA). Lens tubes, broadband dielectric mirror (400-750 nm), long-travel focusing tube for 1/2” spherical optics, and micro prism were purchased from Thorlabs (Newton, NJ). The turning prism and spherical objectives were purchased from Edmund Optics (Barrington, NJ).

Figure 2-2: Schematic of the AP visible-wavelength MALDI-MS instrument.

Prior to installing the visible wavelength MALDI source, the instrument was calibrated in the m/z range from ~100-1300 using ESI-MS/MS analysis of doubly-charged [Glu] - Fibrinopeptide B ion (m/z = 785.0). The NanoLockSpray source
and stage platform purchased with the instrument, were modified and used as part of the AP-LDI source assembly. An in-house designed target slide holder replaced the nanoflow sprayer and its platform. The target slide holder was designed with a flange for alignment and was placed onto the sliding stage platform. Target alignment was adjustable in all three axes. The slide holder was designed so that it was easy to slide it back and forth for MALDI spot positioning reserving the stage motion controller for fine adjustment.

MALDI sample spots were deposited on an ITO-coated glass slide. This target slide was placed into the holder and locked into position by gently tightening a metal screw, which pushed the ITO coated slide securely against the holder. Capillary voltage was connected to the holder that was carrying ITO-coated glass slide. The laser was set on a platform above the mass spectrometer, and its green light was reflected, focused, and refracted onto a sample spot on the glass slide using a broadband dielectric mirror, a spherical lens in a focusing mount in one of the tubes, and a micro prism, respectively. A 3-D micrometer stage allowed for the manual movement of the target slide to a fresh sample spot after the sample was depleted, and was used for optimization of signal based upon the distance between slide and spectrometer inlet.

2.2.5 Operation and Data Acquisition

The MS instrument was operated either in positive or negative ion mode (depending on the structure of a sample analyzed) using MassLynx (Waters) software. Operating parameters used for the Q-TOF mass spectrometer during MS and MS/MS data acquisition are shown in Table 2.1. All experiments were carried out with a laser repetition rate of 10 Hz. The pulse width of the laser pulses was measured using a 2467B 400 MHz oscilloscope (Tektronix, Beaverton, OR) and was determined to be ~4.8 ns (+/-
0.2 ns). The laser beam was elliptically shaped, occupying an area of ~1 mm\(^2\). The laser pulse energy was measured using a laser power meter (Scientech, Boulder, CO) and the fluence per shot was ~1.3 mJ/mm\(^2\). MS scan interval was set at 1 s, while mass spectra presented were accumulated from couple of minutes to several minutes depending on the analyte ionization efficiency.

**Table 2.1**: Settings of the Q-TOF mass spectrometer for visible-wavelength AP-MALDI-MS.

<table>
<thead>
<tr>
<th>Q-TOF mass spectrometer settings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collision Gas</td>
</tr>
<tr>
<td>Capillary (V)</td>
</tr>
<tr>
<td>Sample Cone (V)</td>
</tr>
<tr>
<td>Extraction Cone (V)</td>
</tr>
<tr>
<td>Source Temp (°C)</td>
</tr>
<tr>
<td>Collision Energy (V)</td>
</tr>
<tr>
<td>MCP (V)</td>
</tr>
<tr>
<td>Pusher Pulse Width</td>
</tr>
</tbody>
</table>

2.2.6. UV-MALDI-MS

Samples that were analyzed by visible-wavelength MALDI-MS were also analyzed by UV-MALDI-MS. The samples were spotted onto a MTP 384 ground steel target plate (Bruker Daltonics) besides a spot containing peptide standard mixture for mass calibration. Mass spectra were acquired in positive ion mode by an UltrafleXtreme MALDI-MS instrument (Bruker Daltonics). This instrument uses a pulsed Smartbeam II laser emitting light at 355 nm and operating at a repetition rate of 1 kHz. The MS spectra
were acquired in the \( m/z \) range from \(~400 \) to \( 3500 \) using reflectron mode and FlexControl software. The signal obtained from several hundred laser shots was summed up for each spectrum collected.

2.2.7. Light Microscopy

MALDI spots were imaged using an Eclipse 80i (Nikon, Melville, NY) upright microscope equipped for work in brightfield, differential interference contrast (DIC), and epi-fluorescence modes. A 10X Plan Fluor objective (numerical aperture, \( NA = 0.3 \)) was used for imaging. Optics for DIC microscopy included a DIC prism, a polarizer, and an analyzer. For fluorescence imaging, TRITC filter cube (excitation range 530-560 nm; emission range 560-620 nm) was used. A halogen lamp was used for illumination of samples by brightfield and DIC microscopy, while an X-Cite 120 illuminator (EXFO Life Sciences, Mississauga, Canada) coupled to a liquid light guide was used for illumination of samples for fluorescence microscopy. Images of spots were acquired using a Photometrics CoolSNAP ES2 CCD camera (Tucson, AZ). The camera contained an imaging array consisting of 1392 x 1040 pixels, and the size of each pixel was 6.45 \( \mu m \) x 6.45 \( \mu m \).

2.3 Results and Discussion

2.3.1 Instrument Optimization and Matrix Selection

In this study, we successfully coupled an AP-MALDI source equipped with a 532-nm visible-wavelength laser to a Q-TOF mass spectrometer (Figure 2-2). As the AP-MALDI source is a weaker ion source compared to a vacuum MALDI source, with less sensitivity caused by the lower ion transfer efficiency [22]. Thus, it is crucial to
optimize the experimental conditions to increase the ion transfer efficiency from the atmospheric pressure environment to vacuum inside the mass analyzer. The laser beam spot size was optimized by changing laser power while recording data, the best laser power was selected with less sample consumption and promising signal. The distance between the target slide and the sample cone were optimized in order to increase ion transmission by moving the slide holder while recording signal. It was found that the best ion signal was obtained if the target slide was located ~4 mm away from the sample cone. Q-TOF instrument settings have also been optimized to increase the ion transfer efficiency and improve the instrument sensitivity (Table 2.1). The instrument was operated both in MS and MS/MS modes, and some of its performance characteristics were similar to the conventionally used ESI-MS mode. For example, mass resolution of this reflectron-based Q-TOF is ~5000 (FWHM) for singly-protonated leucin enkephalin ion (m/z = 556.3). The mass range of the Q-TOF is restricted by the transmission range of the quadrupole, which is factory set to m/z of ~ 4,000.

Because we had only one visible wavelength at our disposal, compounds that efficiently absorb green light were selected for evaluation as matrices (Figure 2-1). Their absorption spectra were measured in both the liquid (Figure 2-3a) and solid state (Figure 2-3b) using UV-Vis spectroscopy. The absorption spectra of matrices were broader in solid state, and they overlapped well with emission wavelength of the laser (Figure 2-3b).

While NR and NFR are low-fluorescence dyes, fluorescence of the rhodamine-based dyes (RB, R6G, RITC, and TAMRA) was significantly decreased in the solid state in comparison with their fluorescence in solution. Additionally, 2-amino-3-nitrophenol was used as a matrix since it worked well as a visible-wavelength matrix [35]. The absorption
maximum of this compound is located at 428 nm, and its absorption at 532 nm may be due to multi-photon excitation into UV absorption bands [35].

Figure 2-3: Absorption spectra of visible-wavelength MALDI-MS matrices in (a) solution and (b) solid states.

As suggested previously [36], the mechanism of visible-wavelength desorption and ionization probably involves absorption of light by the matrix followed by the energy transfer to the analyte. This is supported by the fact that matrices used for conventional UV-MALDI-MS such as CHCA and DHB did not work as visible-wavelength matrices in our experiments. We found that many of the dyes studied in this work functioned as
UV-MALDI-MS matrices, but were not as effective as CHCA and DHB (data not shown). This may be attributed to their low absorption values at the UV laser wavelength (data not shown).

2.3.2 AP LDI-MS Analysis of Dyes and Matrices

Six visible-wavelength dyes (Figure 2-1) as well as red and blue marker dyes of unknown composition were readily analyzed by visible-wavelength LDI-MS instrument without addition of any matrix. For example, the MS spectrum of rhodamine B in positive ion mode (Figure 2-4a) shows a rhodamine cation peak at $m/z = 443.0$ which is formed from rhodamine B (MW = 479.0 Da) by the loss of a chlorine anion (Cl$^-$). The AP LDI-MS analysis of red marker generated a mass spectrum (Figure 2-4b) that was similar to the spectrum of rhodamine B indicating that rhodamine is one of the components of red marker. The fragment ion at $m/z = 399.1$ likely corresponds to a cation obtained upon the rearrangement and loss of $C_3H_8$ from rhodamine cation [37]. It was noticed that the level of rhodamine B fragmentation can be controlled by the incident laser power as reported earlier [38].
Figure 2-4: Mass spectra of (a) rhodamine B and (b) red marker.
Mass spectra of other matrices and dyes were also analyzed (Table 2.2). R6G (MW = 479.0 Da) shows a similar mass spectrum as rhodamine B due to similarity of structures of these compounds [38]. RITC (MW = 536.1 Da) showed an intense fragment peak at \( m/z \) of 458.4, while TAMRA, SE (MW = 527.5 Da) showed as a base peak a fragment with \( m/z \) of 430.7 due to the loss of N-succinimidyl group. Neutral red (MW=288.8) shows a peak at \( m/z \) of 252.9 due to the loss of Cl\textsuperscript{-} anion [34]. Nuclear fast red (MW=357.27) shows the base peak at \( m/z \) of 254.3 in negative ion mode presumably due to the loss of sodium cation and SO\textsubscript{3}. The blue marker produces an intense peak at \( m/z = 478.1 \), which most probably corresponds to a dye called basic blue [39].

**Table 2.2: Base peaks observed by visible-wavelength MALDI-MS of matrices.**

<table>
<thead>
<tr>
<th>Matrix</th>
<th>NR</th>
<th>NFR</th>
<th>RB</th>
<th>R6G</th>
<th>RITC</th>
<th>5(6)TAMRA, SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base Peak ( m/z )</td>
<td>252.9</td>
<td>254.3</td>
<td>443.0</td>
<td>443.0</td>
<td>458.4</td>
<td>430.7</td>
</tr>
<tr>
<td>Ion Mode</td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
</tr>
</tbody>
</table>

NFR was the most efficient matrix for the analysis of raffinose (MW = 504.4), while neutral red also worked well for ionization of this oligosaccharide (Table 2.3). MS and MS/MS spectra of raffinose obtained in positive ion mode using NFR as the matrix are shown in Figure 2-5. The MS spectrum is dominated by a sodium adduct of raffinose (\( m/z = 527.5 \)), but it also shows a few raffinose fragments (Figure 2-5a). MS/MS spectrum obtained upon collision-induced dissociation (CID) of sodiated raffinose ion (Figure 2-5b) clearly shows glycosidic bond and cross-ring cleavage fragments, which were assigned using the nomenclature of Domon and Costello [40]. The analysis of oligosaccharides (and other compounds) using NFR as the matrix in positive ion mode is
accompanied with low or no matrix interfere peaks since NFR ionizes well only in negative ion mode.

2.3.3 AP Visible-Wavelength MALDI-MS of Oligosaccharides

NFR was the most efficient matrix for the analysis of raffinose (MW = 504.4), while neutral red also worked well for ionization of this oligosaccharide (Table 2.3). MS and MS/MS spectra of raffinose obtained in positive ion mode using NFR as the matrix are shown in Figure 2-5. The MS spectrum is dominated by a sodium adduct of raffinose (m/z = 527.5), but it also shows a few raffinose fragments (Figure 2-5a). MS/MS spectrum obtained upon collision-induced dissociation (CID) of sodiated raffinose ion (Figure 2-5b) clearly shows glycosidic bond and cross-ring cleavage fragments, which were assigned using the nomenclature of Domon and Costello [40]. The analysis of oligosaccharides (and other compounds) using NFR as the matrix in positive ion mode is accompanied with low intensity of matrix peaks since NFR ionizes well only in negative ion mode.
Figure 2-5: (a) Mass spectrum and (b) MS/MS spectrum of raffinose using NFR as matrix.
2.3.4 AP Visible-Wavelength MALDI-MS of Peptides

Peptides were detected by visible-wavelength MALDI-MS using either positive or negative ion mode. For example, MRFA (MW = 523.3) was detected in positive ion mode and its spectrum (Figure 2-6a) is dominated by protonated (m/z =524.6), sodiated (analyte-sodium adduct peak) (m/z =546.5), and doubly-sodiated singly-charged ions (m/z =568.5). MS/MS spectrum of protonated MRFA ion shows formation of a, b, y, and z ions, immonium ions, as well as ions obtained upon loss of ammonia or addition of water (Figure 2-6b). Unlabeled angiotensin II and bradykinin showed singly-protonated peaks with m/z values of 1046.1 and 1060.9, respectively, while coumarin-labeled peptides showed respective peaks at 1248.0 and 1262.6 (Figures 2-7a and 2-7b). Therefore, the mass increment after labeling of peptides with coumarin was ~202 [41]. Glutathione (MW= 307.3) was detected in negative ion mode upon loss of a proton (m/z =306.2) using R6G as the matrix (Figure 2-8). ACTH (MW= 2093.4) showed a singly charged protonated ion at m/z = 2094.8 (Figure 2-9).
Figure 2-6: Mass spectrum (a) of MRFA in positive ion mode using NFR as matrix; (b) MS/MS spectrum of MRFA in positive ion mode using NFR as matrix.
Figure 2-7: (a) Mass spectrum of angiotensin II and coumarin labeled angiotensin II mixture in positive ion mode using NR as matrix; (b) Mass spectrum of bradykinin and coumarin labeled bradykinin mixture in positive ion mode using NR as matrix.
Figure 2-8: Mass spectrum of glutathione in negative ion mode using RB as matrix.

Figure 2-9: Mass spectrum of ACTH (1-17) in positive ion mode using NFR as matrix.
Most matrices worked well for the analysis of peptides in positive ion mode (Table 2.3). Rhodamine based matrices (RB, R6G and RITC) performed well during the analysis of glutathione in negative ion mode, while NFR did not. Detection of ACTH, which was the largest peptide analyzed, was done in the presence of NFR and RITC. Several visible-wavelength matrices have been used previously for the analysis of larger polypeptides and proteins [10-13,15] with different instrumentation. Such analyses have not been feasible in the present experiments due to a limited mass range for the detection of singly charged ions by our quadrupole-reflector TOF instrument.

Since most of the matrices used in this study are ionic compounds (Figure 2-1), the formation of adduct ions was noticed during visible-wavelength MALDI-MS analysis of peptides (Figure 2-6~2-9). Although the formation of adduct ions facilitates MALDI-MS analysis of oligosaccharides and synthetic polymers, it often increases background noise and complicates interpretation of MALDI-MS spectra of peptides.

2.3.5 AP Visible-Wavelength MALDI-MS of Synthetic Polymers

Synthetic polymers were analyzed by the described visible-wavelength MALDI-MS instrument in positive ion mode. Figure 2-10 shows mass spectrum of PEG 1000 recorded in the presence of NFR as the matrix. The spectrum is dominated with PEG ions that were formed by addition of potassium, which is present in PEG samples [42]. UV-MALDI-MS analysis of PEG using CHCA as the matrix confirmed the presence of potassium adducts. A distribution of sodiated PEG ions with a lower intensity was also observed in this sample (Figure 2-10). When 2-amino-3-nitrophenol with AgTFA was used for PEG analysis, the mass spectrum was dominated by silver adducts of PEG, while sodium adducts were detected in the presence of other matrices such as NR and R6G. The
analysis of PEG 2000 mme was also successful, but signal-to-noise ratio of these measurements was lower than for PEG 1000 due to lower sensitivity of the instrument for singly-charged ions that have masses higher than ~2000.

Figure 2-10: AP visible-wavelength MALDI-MS spectrum of PEG1000 using NFR as matrix. Structure of PEG is shown in the inset.
Table 2.3: An overview of samples and matrices analyzed by AP visible-wavelength MALDI-MS.

<table>
<thead>
<tr>
<th>Matrices</th>
<th>Samples</th>
<th>Raffinose</th>
<th>Glutathione</th>
<th>MRFA</th>
<th>Angiotensin II</th>
<th>Bradykinin</th>
<th>coumarin labeled Angiotensin II</th>
<th>coumarin labeled Bradykinin</th>
<th>ACTH</th>
<th>PEG 1000</th>
</tr>
</thead>
<tbody>
<tr>
<td>NR</td>
<td>√ a</td>
<td>x</td>
<td>√</td>
<td>√*</td>
<td>√*</td>
<td>√*</td>
<td>√*</td>
<td>x</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>NFR</td>
<td>√* b</td>
<td>x</td>
<td>√*</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√*</td>
<td>√*</td>
<td>√</td>
<td>√*</td>
</tr>
<tr>
<td>RB</td>
<td>x c</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>x</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>R6G</td>
<td>x</td>
<td>√*</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>x</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>RITC</td>
<td>x</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>x</td>
</tr>
<tr>
<td>TAMRA, SE</td>
<td>x</td>
<td>x</td>
<td>√</td>
<td>√*</td>
<td>√*</td>
<td>√*</td>
<td>√*</td>
<td>x</td>
<td>√</td>
<td></td>
</tr>
<tr>
<td>2-amino-3-nitrophenol</td>
<td>x</td>
<td>x</td>
<td>x</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>√</td>
<td>x</td>
<td>√* e</td>
<td></td>
</tr>
</tbody>
</table>

a √ shows that particular matrix worked well for visible-wavelength MALDI-MS of a sample
b ∗ represents the most efficient matrix for visible-wavelength MALDI-MS of particular sample
c x shows that a matrix didn’t work for visible-wavelength MALDI-MS of particular sample
d glutathione was analyzed in negative ion mode; other samples were analyzed in positive ion mode
e this sample was analyzed in the presence of Ag TFA.

2.3.6 Limit of Detection (LOD)

Limit of detection is a term used to describe the smallest concentration or amount that can be reliably measured with signal-to-noise ratio of at least 3:1. As with other MS techniques, the LOD of visible-wavelength MALDI-MS is both sample and instrument dependent. The LOD of the instrument was determined in positive ion mode using raffinose and PEG 1000 in the presence of nuclear fast red as the matrix. Two sets of experiments have been done to determine the LOD. In the first trial, we fixed the concentration of the analyte solution but kept decreasing the concentration of the matrix.
In this way, the intensities of corresponding analyte peaks versus the molar ratio between analyte and matrix were studied. In the second set of experiments, a mixture of analyte and matrix with fixed molar ratio was prepared, and its concentration was sequentially decreased by dilution. These samples have been deposited at ITO-coated glass slides and further analyzed to determine the LOD. Sample spots for both sets of experiments were prepared freshly using the same sample preparation methods as described in Section 2.2.2 and analyzed with the MS instrument and laser settings as described in Section 2.2.5. Spectra were summed and compared after each sample spot was completely ablated.

It was found that amounts of ablated materials corresponding to ~100 pmol of raffinose and ~50 pmol of PEG can be readily detected with a signal-to-noise ratio of over 3:1. Matrix-to-analyte ratios for these measurements were ~140 and 280 to one, respectively. Lower amounts of both samples were analyzed with the same or higher matrix-to-analyte ratios, but no spectrum was obtained with signal-to-noise of 3:1.

Studies performed previously have shown that picomole and even femtomole amounts of analytes can be detected by visible-wavelength MALDI-MS in vacuum [8, 19, 33, 35]. Femtomole amounts of analytes can be also detected by AP UV-MALDI [22, 23]. Such detection limits may be possible on the present instrument if nitrogen gas is used to pneumatically assist ion transfer from the atmospheric pressure sampling area to vacuum of the mass spectrometer [22] or if pulsed delay focusing (PDF) is used [43]. Higher laser powers can also lead to a higher ion signal, but also potentially increase the extent of fragmentation and intensities of peaks due to the matrix [34]. The sample consumption was higher by visible-wavelength AP MALDI-MS than by UV-MALDI-MS due to ion losses at atmospheric pressure and ~10 fold difference in the diameters of laser beams used in our visible-wavelength and UV-MALDI-MS instruments (1 mm and 100 µm,
respectively). Future experiments will address the influence of laser beam diameter on LOD and process of visible-wavelength MALDI ionization at atmospheric pressure.

Sample preparation and matrix selection are also important aspects of instrument sensitivity. Several visible-wavelength matrices (NR, RB, R6G, 2-amino-3-nitrophenol) used in this study have been used before [31-35]. We evaluated those along with expanding the set of matrices studied with NFR, RITC, and TAMRA, SE. Table 2.3 presents a qualitative comparison of the performances of various matrices for types of samples analyzed in this work. This table can be useful as a guide that enables suitable choice of visible-wavelength matrices. Visible-wavelength MALDI-MS analyses of the samples presented in the Table 2.3 were not possible in the absence of a matrix, confirming that the matrices studied facilitate desorption and ionization of the analytes.

Among newly used matrices, NFR performed very well for all samples analyzed in positive ion mode, while it was less useful for analysis in negative ion mode because it forms negatively-charged matrix peaks (as mentioned in subsection 3.2.). TAMRA-SE worked especially well for MALDI-MS analyses of peptides, while RITC was also effective for analyses of peptides. While both RITC and TAMRA-SE could make covalent bonds with amines of peptides at basic pH, such chemical reactions were not favored under the sample preparation conditions used. Matrices selected in this study performed very well for analysis of biopolymers and synthetic polymers and provided good signal-to-noise for the samples analyzed. Novel matrices that improve the ion signal and decrease background chemical noise will certainly provide a wider venue for applications of visible-wavelength MALDI-MS [19].

Previously, it was shown that AP UV-MALDI-MS sample preparation protocols were similar as those used for vacuum UV-MALDI-MS [23]. Matrix-to-analyte ratios
used were similar [8, 19] or lower [34] for visible-wavelength MALDI-MS in comparison to UV-MALDI-MS. In our experiments, the matrix-to-analyte molar ratios used were ranging from 2:1 to ~300:1, and were approaching those used for UV-MALDI-MS, which are typically 100:1 to 1000:1. While crystal formation is important (although not a must) for vacuum MALDI-MS analysis of polypeptides [14], it is important to note that in this study samples were desorbed and ionized by AP visible-wavelength MALDI without crystal formation. For example, Figure 2-11a shows a MALDI spot in which raffinose was mixed with NFR and no crystals were formed. By comparison, crystals were formed when CHCA was used as the UV-MALDI-MS matrix for the analysis of peptides (Figure 2-11b). Therefore, the ability to analyze non-crystalline samples at atmospheric pressure is a potential advantage of visible-wavelength MALDI-MS.

Figure 2-11: Brightfield microscopy images of (a) a spot containing raffinose in the presence of NFR as the matrix, and (b) a spot containing angiotensin II in the presence of CHCA as the matrix.
2.4 Conclusions

An AP visible-wavelength MALDI-MS instrument was successfully developed. In addition to analysis of biopolymers and synthetic polymers by MS, the instrument was used to demonstrate visible-wavelength MALDI MS/MS analyses of biomolecules for the first time. While marker dyes were analyzed in the absence of a matrix, MS and MS/MS analyses of samples were performed by this instrument in the presence of several visible-wavelength matrices. These matrices were used for visible-wavelength MALDI-MS analysis of different types of peptides, oligosaccharides, and PEG samples. Besides previously studied matrices (NR, RB, and R6G), three new dyes (NFR, RITC, and TAMRA, SE) were determined to be viable visible wavelength matrices, with the dye NFR being the most effective among them. Matrices studied did not need to co-crystalize with the sample for this methodology to work opening possibility for the analysis of noncrystalline samples by visible-wavelength AP MALDI-MS. Instrument settings have been optimized to increase the ion transfer efficiency and detection of picomole amounts of analytes was achieved.

Present visible-wavelength MALDI-MS methodology provides opportunities for the convenient analysis of various types of samples that absorb green light. Future studies will be aimed toward improving sensitivity of the present methodology. Additionally, the mechanism of ionization in visible region of the spectrum and the energy transfer between dye matrices and analytes will be explored. The instrument will also be employed for further structural and imaging analysis of complex samples.
Chapter 3

Fluorochrome-Modified Lysozyme Crystallization for MALDI-MS Analysis

3.1 Introduction

The matrix plays an important role for the analysis of biomolecules using MALDI-MS. When selecting a matrix for a particular kind of analyte, it is often critical that the matrix molecule can co-crystallize with the analyte molecule. After forming the matrix-analyte co-crystal, the matrix can serve as a buffer between the powerful laser beam and the fragile analyte molecules to reduce the degradation of the analyte [13]. The co-crystal formation can also isolate the analyte molecules further from each other in order to prevent the analyte-analyte interactions and analyte aggregation. However, as most of the proteins can crystallize itself under certain conditions, it is natural to consider the possibility of modifying these kinds of proteins with laser energy absorbers prior to performing crystallization. If laser absorbers would bind to protein molecules and embed in the protein crystals, this may assist desorption and ionization of protein molecules in MALDI-MS analysis.

The conjugation of fluorochrome with proteins has been generated and established as a useful tool with broad applications. A number of studies involving conjugation of fluorochrome to a protein were carried out to identify the conjugation sites in order to achieve a better understanding of protein function [44, 45]. Trace fluorescent covalent
labeling of protein molecules prior to crystallization has already been studied and proved to be a powerful aid for finding crystals in screening experiments [46]. Model proteins such as insulin, ribonuclease, lysozyme and thaumatin were labeled with the fluorescent dye carboxyrhodamine and successfully crystallized in those experiments. It is important to test and analyze those fluorochrome-modified protein crystals directly by MALDI-MS without adding any matrix. This can serve as an alternate sample preparation method for the analysis of proteins and may be helpful for solving the MALDI mechanism puzzle.

In this study, we modified the model protein lysozyme with fluorescein isothiocyanate (FITC) prior to performing crystallization. This isothiocyanate dye can react with N-terminus and amine groups of protein to yield FITC-conjugated protein. Pure lysozyme crystallization had also been performed as a control experiment. Both samples after crystallization were observed using microscope and then analyzed using MALDI-MS. MALDI-MS analyses were performed using different sample preparation methods, including: i) no added chemicals, ii) the addition of 0.1% TFA on top of the sample spot, and iii) the addition of sinapinic acid on top of the sample spot.

3.2 Experimental

3.2.1 Reagents and Materials

Hen egg white lysozyme (lysozyme; MW = 14,307) and fluorescein isothiocyanate isomer I (FITC; MW = 389.0) were purchased from Sigma-Aldrich (St. Louis, MO). Distilled water was obtained from a Thermo Scientific Barnstead B-Pure laboratory grade water system (Dubuque, IA). Slide-A-Lyzer dialysis cassettes (10K MWCO) were obtained from Thermo Fisher (Vernon Hills, IL). Linbro plates and siliconized 22 mm
cover slides were generously provided by the research group of Prof. Max Funk (University of Toledo). Sephadex G-25 medium and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

3.2.2 Sample Preparation

3.2.2.1 Fluorochrome Modification of Lysozyme

The labeling of protein with FITC was performed following a standard protocol [42]. Briefly, 10 mg of lysozyme was dissolved in 1 mL of freshly prepared 0.1 M sodium carbonate buffer (pH 9.0), while 5 mg of FITC was dissolved in 1 mL of anhydrous DMSO. For each 1 mL of lysozyme solution, 50 μL of FITC solution was added very slowly in 5 μL aliquots, while gently and continuously stirring the protein solution. After all the required amount of FITC solution was added, the reaction was incubated at room temperature in the dark for 6 hours. After that, NH₄Cl was added to the final concentration of 50 mM and reaction mixture was incubated for 2 hours at room temperature in the dark.

3.2.2.2 Crystallization of Lysozyme

To perform crystallization of pure protein, 10 mg of pure lysozyme was dissolved in 1 mL of DI water and followed by protein dialysis using a Slide-A-Lyzer dialysis cassette. Briefly, lysozyme sample was first injected into the cassette by inserting the syringe needle through the gasket via one of the corner ports of the cassette and the excess of air was removed after protein sample loading. The cassette was then attached to a float buoy and dialyzed in a beaker full of DI water. The dialyzed solution was incubated at 4°C with continuous stirring. The DI water was changed twice each after 5 hours, and then
kept overnight. After dialysis, the dialyzed lysozyme sample solution was withdrawn carefully.

The protein solution was pre-concentrated before crystallization using an Amicon ultra-0.5 centrifugal tube. First, 500 μL of the protein solution was added to the filter device and capped. The filter device was then inserted into a microcentrifuge tube. Two capped microcentrifuge tubes were placed and balanced into the centrifuge rotor with the cap strap toward the center of the rotor. Then the centrifuge rotor was spun at ~14000 RPM for 15 min. Finally, the concentrated lysozyme solution was removed from the microcentrifuge tubes using a pipette.

Protein concentration was checked by using UV spectrometer (Section 3.2.3) prior to performing protein crystallization. Hanging drop technique [42] was used to perform protein crystallization. The reservoir solutions were prepared freshly and added into a linbro plate following Table 3.1.

Table 3.1: Reservoir solution recipe.

<table>
<thead>
<tr>
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<tbody>
<tr>
<td>A</td>
<td>0.1 M NaOAc pH 4.2 0.6 M NaCl</td>
<td>0.1 M NaOAc pH 4.2 0.7 M NaCl</td>
<td>0.1 M NaOAc pH 4.2 0.8 M NaCl</td>
<td>0.1 M NaOAc pH 4.2 0.9 M NaCl</td>
<td>0.1 M NaOAc pH 4.2 1.1 M NaCl</td>
<td>0.1 M NaOAc pH 4.2 1.2 M NaCl</td>
</tr>
<tr>
<td>B</td>
<td>0.1 M NaOAc pH 4.4 0.6 M NaCl</td>
<td>0.1 M NaOAc pH 4.4 0.7 M NaCl</td>
<td>0.1 M NaOAc pH 4.4 0.8 M NaCl</td>
<td>0.1 M NaOAc pH 4.4 0.9 M NaCl</td>
<td>0.1 M NaOAc pH 4.4 1.1 M NaCl</td>
<td>0.1 M NaOAc pH 4.4 1.2 M NaCl</td>
</tr>
<tr>
<td>C</td>
<td>0.1 M NaOAc pH 4.6 0.6 M NaCl</td>
<td>0.1 M NaOAc pH 4.6 0.7 M NaCl</td>
<td>0.1 M NaOAc pH 4.6 0.8 M NaCl</td>
<td>0.1 M NaOAc pH 4.6 0.9 M NaCl</td>
<td>0.1 M NaOAc pH 4.6 1.1 M NaCl</td>
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<td>D</td>
<td>0.1 M NaOAc pH 4.8 0.6 M NaCl</td>
<td>0.1 M NaOAc pH 4.8 0.7 M NaCl</td>
<td>0.1 M NaOAc pH 4.8 0.8 M NaCl</td>
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<td>0.1 M NaOAc pH 4.8 1.1 M NaCl</td>
<td>0.1 M NaOAc pH 4.8 1.2 M NaCl</td>
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Then twenty four 22 mm siliconized glass slides were deposited with 2 µL of pre-concentrated lysozyme solution and 2 µL of corresponding reservoir solution, and then slides were flipped over to seal the reservoir wells.

Because the drop contains a lower reagent concentration than the reservoir, the water vapor will leave the drop and go into the reservoir to achieve equilibrium. As water leave the drop, both the protein sample and the reagent undergo an increase in concentrations. Equilibration is reached when the reagent concentration in the drop is approximately the same as that in the reservoir. Crystals were formed within 2 days.

3.2.2.3 Crystallization of FITC-Conjugated Lysozyme

After the conjugation of FITC to lysozyme, the un-reacted free FITC left in solution was then separated from the FITC-conjugated lysozyme. Separation was performed using a Sephadex G-25 medium size-exclusion column. The fractions with FITC-conjugated lysozyme were collected and then purified and pre-concentrated following the same protocol used for the pure lysozyme solution.

Crystallization was performed in an identical linbro plate with the same reservoir solution recipe as for the pure lysozyme crystallization. (Page 42)

3.2.3 UV-Vis Spectrophotometer

After the removal of excess dye from the sample by size exclusion chromatography and dialysis, the nonconjugated FITC should be completely removed from the solution for accurate determination of the dye/protein ratio using a UV-Vis spectrophotometer. As the concentration of lysozyme for crystallization should be increased to perform
crystallization [46], the absorbance of the pre-concentrated lysozyme solution at 280 nm should also be measured before performing crystallization.

The purified and pre-concentrated lysozyme and FITC conjugated lysozyme solutions (5 μL) were extracted, and then diluted 400 times with DI water. The absorbances of pure lysozyme solution and FITC conjugated lysozyme solution were measured at 280 nm using a cuvette that has a 1 cm path length. The absorbance of FITC conjugated lysozyme solution at 494 nm (λ_{max} for FITC) was also obtained. Each measurement had been performed twice to get the average absorbance.

All absorption spectra were measured in a quartz cuvette using a photodiode array (PDA) UV-Vis spectrophotometer HP 8452 (Hewlett Packard, Palo Alto, CA) in the wavelength range from 200 nm to 800 nm. DI water was used as blank.

3.2.4 Light Microscopy

Lysozyme crystals were imaged using an Eclipse 80i (Nikon, Melville, NY) upright microscope equipped for work in brightfield and differential interference contrast (DIC) modes. A 20X Plan Fluor objective (numerical aperture, NA = 0.3) was used for imaging. Optics for DIC microscopy included a DIC prism, a polarizer, and an analyzer. A halogen lamp was used for illumination of samples by brightfield and DIC microscopy. Images of spots were acquired using a Photometrics CoolSNAP ES2 CCD camera (Tucson, AZ). The camera contained an imaging array consisting of 1392 x 1040 pixels, and the size of each pixel was 6.45 μm x 6.45 μm.
3.2.5 MALDI-TOF MS Analysis

Lysozyme crystal and FITC-conjugated lysozyme sample were carefully transferred using a loop onto an MTP 384 ground steel target plate provided by Bruker Daltonics, and then analyzed without adding of any matrices. Those samples were also analyzed with 1 μL of 0.1% TFA solution spotted on top of the sample to improve the protonation of the proteins. Lysozyme crystals were also analyzed with 1 μL of sinapinic acid (SA) solution spotted on top. FITC conjugated lysozyme sample was analyzed by dissolving in 1:1 (v:v) H$_2$O:CH$_3$CN solution containing 0.1 % TFA, and then it was mixed with SA. Sample was then spotted utilizing the dried-droplet method [1]. Briefly, 1 μL of each sample was mixed and deposited with the same volume of appropriate matrix and air dried. Protein calibration standard mixture was prepared using the same method described above. SA and protein calibration standard mixture were all dissolved in 1:1 (v:v) H$_2$O:CH$_3$CN solution containing 0.1 % TFA.

Mass spectra were acquired in positive ion mode by an UltrafleXtreme MALDI-MS instrument (Bruker Daltonics). This instrument uses a pulsed Smartbeam II laser emitting light at 355 nm and operating at a repetition rate of 1 kHz. The MS spectra were acquired using FlexControl software of the m/z range from ~4000 to 20000 Da in linear mode. Each spectrum was obtained using 500 laser pulses for each shot and then summed up to a single spectrum with a total of 1000-5000 laser pulses.
3.3 Results and Discussion

3.3.1 Determination of the Concentration of the Pre-concentrated Lysozyme and FITC-Conjugated Lysozyme before Crystallization

Concentrations of lysozyme and FITC conjugated lysozyme must be calculated by measuring the UV absorbance at 280 nm and 494 nm before performing protein crystallization, as 40 mg/mL of protein concentration is needed to grow the crystal.

For pure protein solution, the concentration of protein can be determined by Equation 3.1 as shown below:

$$\frac{A_{280}}{\varepsilon_{280} b} \times \text{dilution factor} \quad \text{Equation 3.1}$$

In this equation, $A_{280}$ represents absorbance (A) of a dye solution measured at the 280 nm). $\varepsilon_{280}$ is protein extinction coefficient at 280 nm (2.64 ml·mg$^{-1}$·cm$^{-1}$ for lysozyme), b is the pathlength of the quartz cuvette (1 cm). Dilution factor represents the extent to which the protein:/dye sample was diluted for absorbance measurement (400, in this case). The average absorbance of the diluted lysozyme solution at 280 nm ($A_{280}$) measured using UV-Vis spectrometer was 0.636.

The concentration of pure lysozyme solution protein can be calculated by inputting the numbers above into equation 3.1, so concentration of the lysozyme solution is 96.4 mg/mL. It is concentrated enough to perform crystallization.

For FITC-conjugated protein solution, the concentration of conjugated protein can be calculated as below:

$$\frac{A_{280} - (A_{\text{max}} \times \text{CF})}{\varepsilon_{280} b} \times \text{dilution factor} \quad \text{Equation 3.2}$$
Where the $A_{\text{max}}$ is the absorbance of a dye solution measured at the wavelength maximum ($\lambda_{\text{max}}$) for the dye molecule, it is at 494 nm for FITC. CF is Correction factor used to adjust the amount of absorbance at 280 nm caused by the dye, the correction factor for FITC is 0.3000. The average absorbance measured using UV-Vis spectrometer for the diluted conjugate at 280 nm is 0.594, whereas the average absorbance of the diluted conjugate at 494 nm ($\lambda_{\text{max}}$ of FITC) is 0.511.

So the concentration of FITC conjugated lysozyme solution calculated based on Equation 3.2 is 67.1 mg/mL.

The calculations above show that, the concentration of pure lysozyme is 96.4 mg/mL and of FITC conjugated lysozyme is 67.1 mg/mL. Both are high enough to perform the crystallization experiment.

3.3.2 Determination of Degree of Protein Labeling

The molar ratio of dye-to-protein can be determined by following equation:

$$\frac{A_{\text{max}} \text{ of the labeled protein}}{\varepsilon' \times \text{protein concentration (M)} \times \text{dilution factor}}$$  \hspace{1cm} \text{Equation 3.3}

Where $\varepsilon'$ is the molar extinction coefficient of the fluorescent dye, which is 68000 M$^{-1}$·cm$^{-1}$ at 494 nm at pH 8.0 for FITC. As mentioned above, the concentration of FITC-conjugated protein is 67.1 mg/mL, the average absorbance at 494 nm for FITC is 0.511, and dilution factor is 400 in this case. So by substitution of all the values in Equation 3.3, the number of moles of dye per mole protein equal 0.64. Hence, the calculation above shows that 64% of lysozyme was successfully conjugated with FITC dye.
3.3.3 Light Microscopy Images

Pure lysozyme crystals formed in two days and were big enough to be observed by naked eye. Crystals were then carefully removed from the hanging droplet solution, and transferred onto a microscope glass slide. Lysozyme crystals were then imaged using an Eclipse 80i upright microscope in brightfield mode. A 20X Plan Fluor objective was used for imaging. A lamp was used for illumination of samples from the side, because the crystals were transparent. Typical tetragonal lysozyme crystals were formed in our case (Figure 3-1).

![Figure 3-1: Brightfield microscopy images of tetragonal Lysozyme crystals.](image)

As shown in Figure 3-1, the size of lysozyme tetragonal crystals is about 300 μm, It is much bigger than the normally matrix-analyte co-crystal which is less than 50 μm.

After incubation of FITC-conjugated lysozyme for 2 days, no clear crystals were observed by naked eye. The hanging droplet solution was not a clear colorless solution as for pure lysozyme but a yellow opaque solution. The yellow color is due to the yellow FITC dye itself. The opaque look of the solution makes it impossible to see through the solution and difficult to search for crystal formation using a microscope.
3.3.4 MALDI-TOF MS Analysis

Lysozyme crystal and FITC conjugated lysozyme samples were carefully deposited onto the MALDI target plate after two washings in the mother liquor and then analyzed without adding any matrices. Linear mode method was utilized and the laser power used to perform the MALDI analysis was adjusted from low (30%) to high (80%) of full power. But, no signal was obtained even with 80% laser power for both pure lysozyme crystal and FITC conjugated lysozyme samples.

Those samples were then analyzed with 1 μL of 0.1% TFA solution spotted on top to improve the protonation of the lysozyme molecule. But the lysozyme crystal dissolved and lost the crystal shape after spotted with the 0.1% TFA, while nothing obvious happened to the yellow FITC conjugated lysozyme sample. Laser power was varied from 30% to 80% again, but still no signal was obtained for both samples.

Figure 3-2: Image of the “melted” lysozyme crystal using MALDI camera.
Pure lysozyme crystal was then analyzed with 1 μL of sinapinic acid solution spotted on top. The shape of the crystal remained the same, but the edge of the crystal “melted” after mixing with SA matrix (Figure 3-2). MALDI-MS analysis shows that the surface of the lysozyme crystal can give off lysozyme peak under this condition, but only for the first 1-2 shots.

Figure 3-3a shows the spectrum obtained with the sinapinic acid on top of the pure lysozyme crystal. 59 % of laser power was applied and 5000 shots were summed. As shown in the spectrum, singly charged (m/z= 14304.865) and doubly charged (m/z= 7152.418) lysozyme peaks were obtained with high intensity. The “shoulder” peaks on the right side of each 1+ and 2+ lysozyme peaks are due to the lysozyme-SA adduct formation. Sinapinic acid has been reported as tending to form a photodehydration adduct with a mass of 206 Da higher than the singly-charged protonated protein peak [15], which matches very well our results (m/z= 14512.337).

FITC-conjugated lysozyme sample was also analyzed with 1 μL of sinapinic acid solution spotted on top. A broad peak was observed at around 14000 Da, but the signal was too noisy to read an accurate protein mass.

FITC conjugated lysozyme sample was then analyzed by dissolving it in 1:1 (v:v) H2O:CH3CN solution containing 0.1 % TFA (TA solution), and then mixed with sinapinic acid. Sample spot was deposited utilizing dried-droplet method. Figure 3-3b shows the spectrum obtained. The peak with the mass of 14695.129 is the peak of lysozyme (m/z = 14307) conjugated with FITC with a mass of 389.0 Da. It proves that we did successfully conjugate lysozyme with FITC and we can detect it using standard MALDI sample preparation method, but this FITC conjugation cannot serve as a matrix to assist the process of desorption and ionization.
Figure 3-3: (a) Mass spectrum of the surface of the “melted” lysozyme crystal, and (b) Mass spectrum of FITC conjugated lysozyme dissolved in TA solution with SA as matrix.
Based on the calculations, the degree of labeling is 64% which means the molar ratio of FITC to lysozyme is 0.64:1. However, recommended matrix-to-analyte ratio in sample preparation process for MALDI analysis is much larger, generally from 100:1 to 1000:1. Thus, in order to increase the energy transfer, we need to increase the labeling concentration of the conjugated sample. Unfortunately, N-terminal amine-labeled lysozyme crystals cannot be formed if the labeling percentage of protein is higher than 10% [46].

The fact that no matter how strong the laser power is, no spectrum can be obtained by LDI analysis of the crystal without adding any matrix proves, that it is difficult to desorb and ionize the protein molecule in crystal form. After the sinapinic acid was completely ablated for the first 1-2 laser shots (500-1000 laser pulses), the lysozyme crystal cannot be further desorbed and ionized without the assisting of a matrix. Therefore, the presence of a matrix, in our case, sinapinic acid, is crucial to the analysis of lysozyme crystal.

3.4 Conclusion

In this study, the model protein lysozyme was modified with fluorescein isothiocyanate (FITC), and 64% of lysozyme was conjugated with FITC. Pure lysozyme crystals were obtained and further analyzed with and without the assistance of matrix using MALDI-MS. FITC conjugated lysozyme was purified and then attempted to perform crystallization following the same protocol used for pure lysozyme crystallization. The product was then tested with and without the assisting of matrix using MALDI MS.
After crystallization both samples were observed using a light microscope. Pure lysozyme samples formed very good crystals. Due to the FITC-conjugated lysozyme sample is yellow and the sample solution became opaque after crystallization. Therefore, it was hard to tell whether there was crystal formed based on the light microscope observation.

These two samples were then analyzed using MALDI-MS by different sample preparation methods, including: i) no added chemicals, ii) the addition of 0.1% TFA on top of the sample spot, and iii) the addition of sinapinic acid on top of the sample spot. The first two methods didn’t work out for both samples, while the third method worked well for pure lysozyme protein but not for FITC conjugated protein. So the FITC conjugated protein sample has been further analyzed after standard MALDI sample preparation method, and the spectrum shows that the FITC molecule did bind to lysozyme successfully.

By performing this series of experiments, we confirmed that: it is crucial to have a matrix for the analysis of a single lysozyme crystal. Lysozyme crystal has different crystal structure than the matrix-analyte cocrystal, and couldn’t assist the process of desorption and ionization. Secondly, it is hard to form protein crystal with 64% of lysozyme being conjugated with FITC. Future experiments will be focused on increase of the FITC labeling concentration, the conjugation of fluorochromes that have high absorbance in UV and visible regions of the spectrum, and crystallization of fluorochrome-conjugated protein samples.
Chapter 4

Conclusions

In the first project, we successfully built our own AP visible-wavelength MALDI ion source, and then coupled it to a quadrupole time-of-flight (Q-TOF) mass spectrometer. This ion source proved to be straightforward to build and quick to exchange with the ESI ion source. A Nd:YAG laser operating at 532 nm was utilized to provide the visible-wavelength laser light. Six visible-wavelength absorbers were tested and evaluated as matrices, including three dyes (NFR, RITC, and TAMRA, SE) that have not been explored previously as visible-wavelength matrices. Different types of analytes were tested, including dyes, oligosaccharides, peptides, and synthetic polymers. Data in both MS and MS/MS modes were obtained. In order to increase the ion transfer efficiency from the atmospheric pressure ion source into the vacuum chamber of the mass analyzer, the design of our ion source and the Q-TOF instrument settings were optimized. Additionally, sample spots were imaged by light microscopy to investigate the influence of their morphology onto MALDI-MS signals.

In the second project, the model protein lysozyme was modified with fluorescein isothiocyanate (FITC). 64% of lysozyme has been conjugated with FITC. Crystallization of pure lysozyme and FITC conjugated lysozyme were performed after protein
purification and pre-concentration. Pure lysozyme samples formed very good crystals, while it is hard to tell whether there was crystal formed for FITC conjugated lysozyme. These two samples were then analyzed using MALDI MS by different sample preparation methods, but only lysozyme crystal with UV MALDI matrix spotted on top worked well. By performing this series of experiments, we confirmed that: it is crucial to have a matrix for the analysis of a single lysozyme crystal and the lysozyme crystal has different crystal structure with the matrix-analyte co-crystal. Secondly, it is hard to form crystal with 64% of lysozyme being conjugated with FITC dye.

In the future, more experiments need to be carried out for this project focusing on increasing the FITC labeling concentration, performing crystallization of this conjugated sample, and try conjugating the lysozyme sample with other fluorochromes, which have high absorbance in UV and visible regions of electromagnetic spectrum.
References


Appendix A

Identification of detergent-extracted membrane proteins using MALDI-MS and MALDI-MS/MS

Introduction

Membrane proteins are the last major frontier for protein structural investigation. These proteins include the cell surface receptors, signal transducers, metabolite transporters and membrane channels that are the targets for the majority of the drugs currently on the market, as well as for many of those in the developmental pipeline. The successful development of these drugs has been accomplished despite the paucity of detailed structural information about their biological targets. The development of improved methods that lower the barriers to membrane protein extraction, stabilization, crystallization and structural determination will accelerate structure-guided approaches to support the production of new drugs. Extraction of membrane proteins from the phospholipid bilayer environment is a critical first step in their purification and structural characterization. It requires disruption of the bilayer structure to affect their removal, a task that must be accomplished without also irreversibly disrupting the protein structure. Detergents have been most widely used to accomplish this task, and ever-widening arrays
of molecules with detergent-like properties are being specifically synthesized for this purpose.

In this study, Buenafe T. Arachea from Dr. Viola’s group employed eight different detergents and determined their extraction efficiencies against a model protein, KcsA, as well as constitutive membrane proteins isolated from the membrane fraction of *Escherichia coli* cells. Identities of extracted membrane protein were verified using MALDI-MS and MALDI-MS/MS analyses by me with Ena’s help for sample preparation. All work related to cell membrane protein exaction and purification, and detergents’ efficiency determinations were done by her. All tables and figures related to MALDI protein identification shown below were finished by her while discussed and checked by us and our advisors.

Experimental

Mass Spectrometry Sample Preparation and Protein Identification

Bands corresponding to constitutive membrane proteins were examined by MALDI mass spectrometry to identify individual proteins. Initial trials were performed using the total membranes isolated from XL-1 Blue cells to determine the optimal concentration needed for protein identification in MS analysis. Protein bands were excised from the gels, destained and digested overnight with trypsin following the method of Shevchenko et al. [1]. Briefly, gel bands were initially incubated and destained with acetonitrile. Samples were reduced by the addition of freshly prepared dithiothreitol (10 mM DTT in 100 mM ammonium bicarbonate) and then alkylated with iodoacetamide (55 mM iodoacetamide in 100 mM ammonium bicarbonate). In-gel digestion was performed by incubating each of the gel pieces with 13 ng/μL of sequence grade trypsin dissolved in
100 mM ammonium bicarbonate/10% (v/v) acetonitrile solution overnight at 37°C. The resulting solutions containing the digested peptides were concentrated in a vacuum concentrator for 2 h.

The samples were prepared for mass spectrometric analysis using the dried droplet method. Briefly, 1 μl of each sample was mixed with an equal volume of saturated α-cyano-4-hydroxycinnamic acid dissolved in 1:1 (v/v) acetonitrile:water containing 0.1% TFA. The samples were spotted onto a MTP 384 ground steel target plate along with a peptides standard mixture for mass calibration, prepared using the same method described above.

MALDI-MS Analysis

Mass spectra were acquired by an UltrafleXtreme MALDI TOF/TOF instrument (Bruker Daltonics) in positive ion mode using a Smartbeam II laser operating at a repetition rate of 1 kHz. The MS spectra were acquired in the m/z range from 800 to 3500 using reflection mode and flexControl software. The signal obtained from a total of 1000 acquisitions was summed for each peptide mass fingerprinting (PMF) search. For the MS/MS analysis, the LIFT TOF/TOF method [2] was used to analyze fragments formed by laser induced dissociation (LID). The MS/MS spectra were collected using 1000-2000 acquisitions for each sample with laser power settings higher than those used for the PMF experiments. Data analysis and PMF and MS/MS searches were carried out using the flexAnalysis (Bruker Daltonics) and Mascot (Matrix Sciences), respectively. Protein identities and classifications were verified by using the UniProt database [3].

Results for Protein Identification
The bacterial and yeast membrane preparations (total membrane and outer membrane) solubilized in various test detergents were loaded onto an SDS-PAGE gel to separate the constitutive proteins. Initial trials were performed using the total membranes isolated from XL-1 Blue cells to determine the optimal concentration needed for protein identification in MS analysis. Our trials indicated that ~50 μg of total protein loaded on each lane is an optimal concentration that showed intense and well-resolved bands that could be separately excised from the gel for MS sample preparation (Figure A-1).

Figure A-1: Optimization of protein loading concentrations for protein band isolation.

To further increase the amount of protein needed to improve the signal-to-noise ratio during protein identification, triplicate gels were run. When necessary, protein bands migrating at similar positions in each of the gel were pooled and considered as one
protein entity. Samples were then processed as previously described (Experimental) and protein identification was carried out using MALDI-TOF MS and MS/MS analyses coupled with database searching following the workflow shown in Figure A-2.

The acquired experimental peptide masses were compared with the theoretical peptide masses from protein database using the Mascot search engine. This search generates a list of candidate proteins with corresponding peptide matches. Each candidate protein from the PMF search was then scored and this value was compared with the threshold score for the taxonomy indicated in the search. This threshold score is the minimum score required to obtain positive protein identification within a high confidence level (i.e., greater than 95% certainty) [4]. If the PMF score did not exceed the allowed threshold, verification of the peptide matches was carried out by MS/MS fragmentation. The MS/MS spectrum generates fragment ion masses to give information on the complete or partial amino acid sequence of the peptide. The experimental fragment ion masses are then compared with those present in the database. Identification of a positive peptide match from the MS/MS fragmentation follows similar scoring function as described in the PMF search.
Figure A-2: Protein identification workflow.
To optimize the search parameters for the database searching, we utilized the band corresponding to the KcsA protein at ~ 64 kDa region (Figure A-3). This protein migrates as a stable tetramer [4] in standard SDS-sample buffer solution used for electrophoresis. We also have previously worked on this protein and we have confirmed this stable tetramer conformation by subjecting purified KcsA samples to Western blot analysis, thus allowing us to have a good estimate of the position of the KcsA in the SDS-PAGE gels.

Protein bands that correspond to the tetrameric form of KcsA from each detergent lane and buffer control were analyzed. Using an initial setting of maximum of 5 missed cleavages, significant PMF scores for KcsA were obtained from samples solubilized in OG, Cyma5 and Fos-choline (Table A.1). In addition to KcsA, we have also observed other candidate proteins (tnaA, atpB) in this band with higher PMF scores than KcsA in the detergent samples. Since we have not purified proteins, and we are using 1D gels with limited resolving power as compared to 2D gels, it is likely that these two proteins (tnaA, atpB) comigrate in a similar position with KcsA in the gel.

Based on our analysis, an allowed missed cleavage of 1 appears to give reliable results with high score. Using the same cleavage setting in the MS/MS analysis further confirmed the identity of the constitutive proteins. All protein identification using MALDI-TOF MS and MS/MS were carried out following the set parameters and methods described in Experimental section.
Figure A-3: Constitutive membrane proteins extracted by test detergents from the total cell membrane of E. coli.
We have been able to identify a total of 35 constitutive proteins from the total membrane fraction isolated from bacterial membranes (XL-1 Blue) using peptide mass fingerprint and MS/MS analysis, in addition to the recombinantly expressed model alpha-helical membrane protein, KcsA. Eleven proteins were classified as belonging to the inner membrane (IM), whereas three were found in the periplasmic space, and another three were designated as outer membrane (OM) proteins (Table A.2). It is likely that these proteins are associated with the membrane or with integral membrane proteins and they were not fully eliminated during the washing step done for the membrane pellet prior to detergent solubilization.

From the outer membrane fraction, we identified 11 constitutive OMPs which function either in ion or lipid transport, or in assembly of outer membranes in the cell such as in maintaining the bacterial envelope integrity (Table A.3). An additional six IMPs are also found in the outer membrane samples, some of which (ATP synthase subunits, NADH-quinone oxidoreductase) have been previously found in the total membrane fraction. Ribosomal and chaperone also proteins are present in the buffer control (Table A.4).

For both cell membrane preparations, a total of 14 (including KcsA) unique inner membrane proteins were extracted in varying combinations by the test detergents (Table A.5) and positively identified by mass spectrometric experiments. Thirteen outer membrane proteins and three periplasmic proteins were also isolated. The different classes of membrane proteins, their extraction efficiencies and the behavior of the protein-detergent complexes will be discussed in the following sections. Our results from the constitutive protein identification from the total and outer cell membrane were
combined to give a concise and clear summary of the different protein types extracted by various detergents (Table A.5).
Table A.1: Peptide mass fingerprint analysis for KcsA.

<table>
<thead>
<tr>
<th>Sample Code</th>
<th>Accession Number</th>
<th>Mass (Da)</th>
<th>Score</th>
<th>Expect</th>
<th>Matches</th>
<th>Protein Name</th>
</tr>
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<tbody>
<tr>
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<td>UGPC_ROSD6 (1)</td>
<td>39751</td>
<td>55</td>
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<td>5</td>
<td>Sn-glycerol-3-phosphate import ATB-binding protein R.denitrificans</td>
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<tr>
<td></td>
<td>RS19_MYCS5 (2)</td>
<td>10280</td>
<td>49</td>
<td>6.4</td>
<td>4</td>
<td>30S ribosomal protein S19 M.synomiae</td>
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<tr>
<td></td>
<td>COXAM_YEAS1 (3)</td>
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<td>25</td>
<td>4</td>
<td>COX assembly mitochondrial protein S.cerevisiae</td>
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<td>SDS</td>
<td>ATPA_DESMR (1)</td>
<td>54645</td>
<td>51</td>
<td>2.6</td>
<td>6</td>
<td>ATP synthase subunit alaph D.magnetcus</td>
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<tr>
<td></td>
<td>RL9_NEIMA (2)</td>
<td>15752</td>
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<td>22</td>
<td>4</td>
<td>50S Ribosomal protein L9 N.meningitis serogroup A</td>
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<tr>
<td></td>
<td>MUTH_HAEIA (3)</td>
<td>24914</td>
<td>41</td>
<td>24</td>
<td>4</td>
<td>DNA mismatch repair protein mutH H.influenza</td>
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<tr>
<td></td>
<td>RIMM_ALISL (1)</td>
<td>20085</td>
<td>69</td>
<td>0.045</td>
<td>5</td>
<td>Ribosome maturation factor rimM A.salmonicida</td>
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<td>Triton</td>
<td>KCSA_STRCO (6)</td>
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<td>52</td>
<td>1.9</td>
<td>5</td>
<td>Voltage-gated potassium channel S.coelicolor</td>
</tr>
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<td></td>
<td>KCSA_STRLI (7)</td>
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<td>1.9</td>
<td>5</td>
<td>Voltage-gated potassium channel S.lividans</td>
</tr>
<tr>
<td>4-142-5</td>
<td>KCSA_STRCO (1)</td>
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<td>84</td>
<td>0.0013</td>
<td>8</td>
<td>Voltage-gated potassium channel S.coelicolor</td>
</tr>
<tr>
<td></td>
<td>KCSA_STRLI (1)</td>
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<td>84</td>
<td>0.0013</td>
<td>8</td>
<td>Voltage-gated potassium channel S.lividans</td>
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<td></td>
<td>RUVB_PROM (2)</td>
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<td></td>
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<td>90</td>
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<td>Voltage-gated potassium channel S.lividans</td>
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<td>Voltage-gated potassium channel S.lividans</td>
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<tr>
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<td>DNAA_PROMS (2)</td>
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<td>1.1</td>
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<tr>
<td></td>
<td>QUEC_RICK (1)</td>
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<td>120</td>
<td>3</td>
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<tr>
<td>Mega9</td>
<td>DDL_RICTY (2)</td>
<td>36089</td>
<td>30</td>
<td>300</td>
<td>3</td>
<td>D-alanine ligase R.typhi</td>
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<td></td>
<td>YCIN_ECO57 (3)</td>
<td>9380</td>
<td>28</td>
<td>490</td>
<td>2</td>
<td>Protein ycin E.coli J157:H7</td>
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<tr>
<td></td>
<td>RS12_NOP7 (1)</td>
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<td></td>
<td>LDAO_RS12_ANASP (2)</td>
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<td>6</td>
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<tr>
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<td>0.12</td>
<td>12</td>
<td>DNA directed RNA polymerase subunit beta C.perfringens</td>
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<tr>
<td>Fos</td>
<td>KCSA_STRCO (4)</td>
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<td>0.12</td>
<td>6</td>
<td>Voltage-gated potassium channel S.coelicolor</td>
</tr>
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<td></td>
<td>KCSA_STRLI (5)</td>
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Table A.2: Summary of identified membrane proteins from total membrane fractions.

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<thead>
<tr>
<th>Protein ID</th>
<th>Protein Name</th>
<th>Mr (kDa)</th>
<th>Localization</th>
<th>Function</th>
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<tbody>
<tr>
<td>atpA</td>
<td>ATP synthase subunit alpha</td>
<td>55420</td>
<td>Inner Membrane</td>
<td>ATP synthesis coupled proton transport</td>
</tr>
<tr>
<td>atpB</td>
<td>ATP synthase subunit beta</td>
<td>50352</td>
<td>Inner Membrane</td>
<td>ATP synthesis coupled proton transport</td>
</tr>
<tr>
<td>atpF</td>
<td>ATP synthase F(0) sector subunit b</td>
<td>17264</td>
<td>Inner Membrane</td>
<td>ATP synthesis coupled proton transport</td>
</tr>
<tr>
<td>atpG</td>
<td>ATP synthase gamma chain</td>
<td>31673</td>
<td>Inner Membrane</td>
<td>ATP synthesis coupled proton transport</td>
</tr>
<tr>
<td>dacC</td>
<td>D-alanyl-D-alanine-carboxypeptidase</td>
<td>43639</td>
<td>Inner Membrane</td>
<td>Cell wall biogenesis</td>
</tr>
<tr>
<td>dhsA</td>
<td>Succinate dehydrogenase flavoprotein subunit</td>
<td>65019</td>
<td>Inner Membrane</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>dhsB</td>
<td>Succinate dehydrogenase iron-sulfur subunit</td>
<td>27390</td>
<td>Inner Membrane</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>hflK</td>
<td>Modulator of FtsH protease HflK</td>
<td>45517</td>
<td>Inner Membrane</td>
<td>Inner membrane biogenesis</td>
</tr>
<tr>
<td>kcsA</td>
<td>Voltage-gated potassium channel</td>
<td>17694</td>
<td>Inner Membrane</td>
<td>Ion transport</td>
</tr>
<tr>
<td>nuoG</td>
<td>NADH-quinone oxidoreductase subunit G</td>
<td>101226</td>
<td>Inner Membrane</td>
<td>Electron transport</td>
</tr>
<tr>
<td>nuoCD</td>
<td>NADH-quinone oxidoreductase subunit C/D</td>
<td>68425</td>
<td>Inner Membrane</td>
<td>Electron transport</td>
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<tr>
<td>ppiD</td>
<td>Peptidyl-prolyl cis-trans isomerase D</td>
<td>68108</td>
<td>Inner Membrane</td>
<td>Folding of OM proteins</td>
</tr>
<tr>
<td>fdoG</td>
<td>Formate dehydrogenase-O major subunit</td>
<td>113291</td>
<td>Periplasm</td>
<td>Anaerobic respiration</td>
</tr>
<tr>
<td>oppA</td>
<td>Periplasmic oligopeptide-binding protein</td>
<td>60977</td>
<td>Periplasm</td>
<td>Peptide/Protein transport</td>
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<tr>
<td>rbsB</td>
<td>D-ribose-binding periplasmic protein</td>
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<td>Periplasm</td>
<td>Sugar transport</td>
</tr>
<tr>
<td>nlpB</td>
<td>Lipoprotein 34</td>
<td>36842</td>
<td>Outer Membrane</td>
<td>Outer membrane assembly</td>
</tr>
<tr>
<td>ompP</td>
<td>Outer membrane protease ompP</td>
<td>35477</td>
<td>Outer Membrane</td>
<td>Protease</td>
</tr>
<tr>
<td>yaeT</td>
<td>Outer membrane protein assembly factor</td>
<td>90612</td>
<td>Outer Membrane</td>
<td>Outer membrane assembly</td>
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</table>
Table A.3: Summary of identified membrane proteins from outer membrane fractions.

<table>
<thead>
<tr>
<th>Protein ID</th>
<th>Protein Name</th>
<th>Mr (kDa)</th>
<th>Localization</th>
<th>Function</th>
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</thead>
<tbody>
<tr>
<td>acrA</td>
<td>Acriflavine resistance protein A</td>
<td>42229</td>
<td>Inner Membrane</td>
<td>Drug efflux</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lipid-anchor</td>
<td></td>
</tr>
<tr>
<td>atpA*</td>
<td>ATP synthase subunit alpha</td>
<td>55420</td>
<td>Inner Membrane</td>
<td>ATP synthesis coupled proton transport</td>
</tr>
<tr>
<td>atpB*</td>
<td>ATP synthase subunit beta</td>
<td>50352</td>
<td>Inner Membrane</td>
<td>ATP synthesis coupled proton transport</td>
</tr>
<tr>
<td>atpG*</td>
<td>ATP synthase gamma chain</td>
<td>31673</td>
<td>Inner Membrane</td>
<td>ATP synthesis coupled proton transport</td>
</tr>
<tr>
<td>ftsH</td>
<td>ATP-dependent zinc metalloprotease</td>
<td>70763</td>
<td>Inner Membrane</td>
<td>Protein catabolic process</td>
</tr>
<tr>
<td>nuoG*</td>
<td>NADH-quinone oxidoreductase subunit G</td>
<td>101226</td>
<td>Inner Membrane</td>
<td>Electron transport</td>
</tr>
<tr>
<td>fadL</td>
<td>Long-chain fatty acid transport protein</td>
<td>48512</td>
<td>Outer Membrane</td>
<td>Lipid transport</td>
</tr>
<tr>
<td>lptD</td>
<td>LPS-assembly protein</td>
<td>89836</td>
<td>Outer Membrane</td>
<td>Outer membrane assembly</td>
</tr>
<tr>
<td>nlpB*</td>
<td>Lipoprotein 34</td>
<td>36842</td>
<td>Outer Membrane</td>
<td>Outer membrane assembly</td>
</tr>
<tr>
<td>ompA</td>
<td>Outer membrane protein A</td>
<td>37294</td>
<td>Outer Membrane</td>
<td>Porin, Ion transport</td>
</tr>
<tr>
<td>ompF</td>
<td>Outer membrane protein F</td>
<td>39309</td>
<td>Outer Membrane</td>
<td>Porin, Ion transport</td>
</tr>
<tr>
<td>ompX</td>
<td>Outer membrane protein X</td>
<td>18649</td>
<td>Outer Membrane</td>
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</tr>
<tr>
<td>pal</td>
<td>Peptidoglycan-associated lipoprotein</td>
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<td>Outer Membrane</td>
<td>Bacterial envelope integrity</td>
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<tr>
<td>toiC</td>
<td>Outer membrane protein</td>
<td>53708</td>
<td>Outer Membrane</td>
<td>Transport</td>
</tr>
<tr>
<td>tsx</td>
<td>Nucleoside-specific channel-forming protein</td>
<td>33568</td>
<td>Outer Membrane</td>
<td>Transport</td>
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<td>yfIO</td>
<td>UPF0169 lipoprotein</td>
<td>27870</td>
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<td>Outer membrane assembly</td>
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<td>ybjP</td>
<td>Uncharacterized lipoprotein</td>
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<td>Outer Membrane</td>
<td>Lipid-anchor</td>
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* Also identified in the total membrane extractions
Table A.4: Summary of cytoplasmic proteins detected from total and outer membrane* fractions.

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<th>Protein ID</th>
<th>Protein Name</th>
<th>Mr (kDa)</th>
<th>Localization</th>
<th>Function</th>
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<tbody>
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<td>Alkyl hydroperoxide reductase subunit C</td>
<td>20864</td>
<td>Cytoplasm</td>
<td>Redox reactions</td>
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<td>bgal</td>
<td>Beta-galactosidase</td>
<td>117366</td>
<td>Cytoplasm</td>
<td>Lactose catabolic process</td>
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<tr>
<td>chs01*</td>
<td>60 kDa chaperonin</td>
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<td>Cytoplasm</td>
<td>Protein folding</td>
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<td>cysK</td>
<td>Cysteine synthase</td>
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<td>Cytoplasm</td>
<td>Cysteine biosynthesis</td>
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<tr>
<td>dnaK</td>
<td>Chaperone protein</td>
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<td>Cytoplasm</td>
<td>Protein folding</td>
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<td>DNA protection during starvation protein</td>
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<td>efg</td>
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<td>efts</td>
<td>Elongation factor Ts</td>
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<td>Cytoplasm</td>
<td>Protein biosynthesis</td>
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<td>eftu1*</td>
<td>Elongation factor Tu 1</td>
<td>43430</td>
<td>Cytoplasm</td>
<td>Protein biosynthesis</td>
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<td>glpD</td>
<td>Aerobic glycerol-3-phosphate dehydrogenase</td>
<td>56889</td>
<td>Cytoplasm</td>
<td>Glycolysis</td>
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<tr>
<td>hso</td>
<td>33 kDa chaperonin</td>
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<td>Cytoplasm</td>
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<td>Small heat shock protein</td>
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<td>Cytoplasm</td>
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<td>Pyruvate dehydrogenase E1 component</td>
<td>99954</td>
<td>Cytoplasm</td>
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<tr>
<td>pckA</td>
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<td>Cytoplasm</td>
<td>Carbohydrate biosynthesis</td>
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<td>me</td>
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<td>Cytoplasm</td>
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<td>15769</td>
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Table A.5: Summary of constitutive membrane proteins extracted by test detergents from total and outer membranes.

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<tr>
<th>Protein Types</th>
<th>Buffer</th>
<th>SDS</th>
<th>DM</th>
<th>OG</th>
<th>CmvA5</th>
<th>Mega9</th>
<th>LDAO</th>
<th>Fos</th>
<th>Tween</th>
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<tr>
<td>Model Protein (KcsA)</td>
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<td>kcsA</td>
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Conclusions

MALDI-MS and MS/MS analyses were used to identify constitutive membrane protein components starts with the treatment of each sample with trypsin. After treatment and sample preparation, the digested samples are then loaded onto the sample plate with matrix. Tryptic digest peptide peaks were obtained and searched against a database. Database searching protocol was optimized using a model protein. Finally, a total of 35 different constitutive proteins were identified from the detergent extracts of the *E. coli* total membrane by using peptide mass fingerprinting and MS/MS analysis, with 14 assigned as membrane proteins, 3 periplasmic proteins and 18 classified as cytosolic or soluble proteins. Fractionation of the outer membrane of the *E. coli* C41 strain followed by detergent extractions yielded 13 OM proteins, included one previously identified from total membrane extractions, 8 IM proteins, including 4 not observed from the total membrane, 1 additional periplasmic protein, 2 ribosomal proteins and 5 additional soluble/cytosolic proteins. Thus, the different detergent extractions from both *E. coli* membrane fractions yielded a total of 60 unique proteins, including 36 integral, periplasmic and ribosomal proteins identified from these bacterial membranes. An initial protein-detergent extraction profiles that could be expanded to facilitate the selection of detergents for optimal extraction of different target membrane proteins was established.
References


