Infrared laser desorption/ionization mass spectrometry: fundamental and applications

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INFRARED LASER DESORPTION/IONIZATION MASS SPECTROMETRY: FUNDAMENTALS AND APPLICATIONS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of
Doctor of Philosophy

in

The Department of Chemistry

by

Mark Little
B.S., Wake Forest University, 1998
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The popular quote, "L'enfer, c'est les autres" from an existential play by Jean-Paul Sartre is translated “Hell is other people”. I chose this quote because if it were not for all the “other people” in my life, I would be sitting on a beach right now sipping margaritas but I would not have my Ph.D. Therefore, I raise my salt covered glass to all my friends, family and colleagues for without their support this program of study would never been completed. These honorary people are in no particular order:

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ABSTRACT

This dissertation on infrared laser desorption/ionization mass spectrometry encompasses fundamental studies of the desorption/ionization process and direct-from-gel laser desorption/ionization applications. Understanding of the fundamentals behind desorption and ionization can lead to improvements in the technique and to new applications. Experiments aimed at advancing this goal are wavelength studies and two-laser infrared/ultraviolet matrix-assisted laser desorption/ionization experiments. The direct-from-gel laser desorption/ionization application in which infrared laser desorption/ionization of analytes directly ionized after gel electrophoretic separation will also be presented. Protein and peptide standards were used as test analytes and the infrared lasers used were a 10.6 μm CO₂ laser and a mid-IR optical parametric oscillator. In the wavelength experiments, the optical parametric oscillator was tuned from 2.8 to 3.6 μm and the minimum laser fluence to produce a detectable ion signal (threshold fluence) was recorded. Comparison of the threshold fluence to the infrared absorption of the sample indicates that the analyte is absorbing the laser light. Scanning electron microscopy images of the sample after laser irradiation show melting and indications of explosive boiling. It is concluded from these results that ionization occurs through the sacrifice of some of the protein molecules that absorb the laser energy and act as an intrinsic matrix. In the two-laser experiments, a mixture of analyte with a laser light-absorbing matrix was deposited on the sample target and irradiated with an infrared laser, followed, after an adjustable delay, by an ultraviolet nitrogen laser. Laser fluences were attenuated below the one-laser ionization threshold and two-laser signal was obtained at delays up to several hundred microseconds. The results can be explained by infrared laser heating of the sample that leads to an enhancement of ultraviolet matrix-assisted laser desorption/ionization. Direct-from-gel laser desorption/ionization experiments used the optical
parametric oscillator to ionize electrophoretically separated biomolecules directly from conventional gel slabs and capillary gels in plastic microfluidic chips. An increase in sensitivity was found when moving to the microfluidic chip design from analyses using gel slabs. This technique shows promise for the identification of both parent and fragment masses of proteins contained in gels.
CHAPTER 1. INTRODUCTION

1.1. Proteomics and Mass Spectrometry

The field of proteomics is defined as “the study of the set of proteins expressed by an organism, its relationship to the genes coding for them, and to physiological and pathological processes.”\(^1\) The entire collection of an organism’s proteins, called it’s proteome, is expressed from the genes of the organism, or it’s genome. With the exception of evolution, the genome of an organism remains unchanged, however, the number and type of proteins expressed from this genetic material is very dependent on the organism’s physiological state.\(^2\) Knowledge of what proteins are expressed in an organism under a certain set of conditions, such as temperature, pH, shock trauma, etc., provide useful information for drug discovery and biological functionality. In order to learn about the proteome of a given organism, proteins must be identified and their structure determined. The number of proteins expressed in an organism can range in the thousands; therefore, a fast and accurate method of analysis is necessary to carry out protein identification and structural determination.

Mass spectrometry (MS) has become the primary analysis method in proteomics because of its high throughput, sensitivity and high mass accuracy. MS involves the ionization of molecules using various ionization sources, such as electrons or photons and the separation of the molecules based on their mass-to-charge \((m/z)\) ratio in a mass analyzer. Until the introduction of soft ionization methods, mass spectrometric analysis of molecules was limited to volatile compounds with small molecular weights of a few hundred Daltons (Da). Proteins are nonvolatile and have molecular weights that range from thousands to several hundred thousand Da. Soft ionization methods, such as, matrix-assisted laser desorption/ionization (MALDI)\(^3,4\) and
electrospray ionization (ESI)\textsuperscript{5,6} are suitable for achieving the goal of identification and structural determination of proteins.

1.2. Soft Laser Desorption and Ionization

Soft laser desorption/ionization (LDI) involves the removal of material from a solid or liquid sample using a pulsed laser and detecting the resulting ions in a mass spectrometer as intact species rather than as fragments. For a successful soft LDI analyses to occur, the proper laser energy and wavelength, such as UV or IR, as well as sample preparation must be chosen to ensure no fragmentation occurs. With regards to sample preparations, the sample and/or sample target can absorb the laser light or a matrix can be added to absorb the laser light in the case of MALDI MS (described below).

Some of the first experiments using soft LDI were performed using 10.6 µm CO\textsubscript{2} lasers and low molecular weight, nonvolatile molecules.\textsuperscript{7-12} These studies were characterized by the detection of intact ions where thermionic emission of neutrals upon laser desorption was followed by ionization in the gas-phase by cation attachment. Later, the soft LDI technique was extended to ultraviolet lasers.\textsuperscript{13-15} In one study by Karas et. al., 266 nm and 355 nm wavelengths generated by the 3rd and 4th harmonics of an Nd:YAG laser were used to ionize amino acids and dipeptides.\textsuperscript{13} Control of desorption could be achieved by selecting the proper wavelength and soft ionization was possible by adding additional analyte to absorb the laser energy.

An added light-absorbing matrix and UV lasers were later used to ionize proteins.\textsuperscript{3,4,16} Most of the work was accomplished using a crystalline, organic acid matrix and an Nd:YAG or N\textsubscript{2} laser. This technique, now referred to as MALDI, or matrix-assisted laser desorption/ionization, requires the proper dispersion of the protein molecule in a laser energy-absorbing matrix, followed by desorption and ionization at an appropriate laser energy and
wavelength. Absorption of the UV laser light by these crystalline matrices occurs through electronic transitions.

The MALDI technique has also been demonstrated using IR lasers.\textsuperscript{17-20} With the exception of the free electron laser (FEL), which is tunable from 2 to 9 \( \mu \text{m} \), and the 10 \( \mu \text{m} \) CO\(_2\) laser, the wavelengths of these IR lasers are in the mid-IR region around 3 \( \mu \text{m} \). Similar to the UV MALDI process, the matrix absorbs the laser light, however, absorption occurs through vibrational energy absorption. Since both the matrix and analyte can have the same functional groups, IR laser light can be absorbed by both sample components. Also, residual solvent not removed by the vacuum of the mass spectrometer and interactions of the solvent with the analyte (e.g. waters of hydration) can also absorb the IR laser light. Some studies have used frozen solvent as the matrix.\textsuperscript{21,22} Hillenkamp and coworkers successfully demonstrated IR MALDI using 2.94 \( \mu \text{m} \) Er:YAG laser and the O-H stretching vibration of water ice as the light-absorbing matrix.\textsuperscript{21} Sheffer et. al. performed a similar study using 2.9 \( \mu \text{m} \) IR laser light from an optical parametric oscillator (OPO) and the O-H stretching vibration of frozen alcohol matrices.\textsuperscript{22} A study employing the FEL at Vanderbilt University was carried out by selecting wavelengths based on the stretching vibration of the matrix.\textsuperscript{19} Succinic acid, fumaric acid and nicotinic acid were evaluated as potential matrices over 2.8 to 4.0 \( \mu \text{m} \) and 5.5 to 5.6 \( \mu \text{m} \) representing the O-H and C=O stretching vibrations, respectively. Using a tunable laser source such as the FEL, the study concluded that MALDI ionization mechanism studies could be performed by studying the ion signal or laser fluence over a range of wavelengths.

Soft LDI can also be applied to small peptides and low molecular weight proteins using sample preparations with no added matrix and IR laser light sources. Ullom et. al. used a custom
built optical parametric oscillator tunable from 2.9 to 4.2 µm to discriminate between bacteria spores deposited on metal targets without the need of an added matrix.\textsuperscript{23} In another study, LDI of intact peptides and proteins was carried out directly from polyacrylamide gel preparations at 5.6 µm.\textsuperscript{24} Other studies include the desorption and ionization of large biomolecules using IR light sources from untreated silicon as well as aluminum, copper, stainless steel and polyethylene targets.\textsuperscript{25,26} The importance of these LDI experiments, where no matrix is added, include simplified sample preparation procedures, reduction of low mass interference that usually originates from matrix ionization and the removal of undesirable interactions that may occur between the protein and the matrix, such as matrix adducts.

1.3. Fundamental Studies of Soft Laser Desorption and Ionization

Soft LDI mass spectrometry is now one of the most widely used techniques for the study of proteomics.\textsuperscript{27,28} Because of the important role of proteins in biological organisms, there is an intense effort to understand the mechanisms of LDI with the overall goal of improving ion signal, mass measurement accuracy and ion signal resolution.\textsuperscript{29-32} By analyzing the data from experiments presented in this dissertation, questions about the desorption/ionization process for both matrix and matrix-less sample preparations can be answered. As a result of the many separate and interrelated steps in the analysis, there are many factors contributing to the success of the experiment. To gain an understanding of the fundamental processes, experimental studies have focused on parameters that can be varied in a systematic way, such as laser energy, pulse width, wavelength, and on the measurement of parameters that are indicative of the effects of these changes, such as ion signal, mass resolving power, initial ion velocity, and fragmentation.

The experiments designed to contribute information to the continuing debate over desorption/ionization process in soft LDI follow the paradigm of a systematic change of a
parameter and measuring a response to this change. In the first of these experiments, the parameter was the wavelength of a tunable IR laser and the response was the minimum laser fluence required for ionization, or threshold fluence. The second of these experiments involved changing the time between two events, in this case, two subthreshold laser pulses and measuring the number of ions generated. The information regarding the desorption/ionization process of MALDI that was obtained as well as some background information about related experiments will be presented in the next two sections.

1.3.1. Wavelength Dependence of Infrared Soft Laser Desorption/Ionization

A wavelength study using a frequency doubled Ti:sapphire laser tunable from 360 to 440 nm was the first to investigate laser energy absorption in the UV MALDI process using a continuously tunable source. The finding from this study is that the threshold fluence has an inverse dependence on the matrix absorption coefficient. If the matrix absorption coefficient is below a certain value, no ionization is observed. As the absorption coefficient of the matrix increases and the laser is tuned to shorter wavelengths, less laser energy is required for ionization up to a maximum at an absorption coefficient value of approximately $6 \times 10^4$ cm$^{-1}$. Wavelengths that generate more ions using less laser energy are considered more efficient.

Tunable IR light sources can also be used to investigate the process of laser energy absorption in IR soft LDI. A correlation between the absorption coefficient of the matrix and the production of ions has been found in past IR MALDI studies. The first of these studies used a free electron laser (FEL) to analyze the proteins bovine insulin and cytochrome C embedded in succinic, fumaric and nicotinic acid matrices. The laser was used to ionize sample material by laser absorption of $\text{O}^-\text{H}$, $\text{N}^-\text{H}$ and $\text{C}^-\text{H}$ functional groups in the 2.65 to 4.2 $\mu$m wavelength range and the $\text{C}=\text{O}$ functional group in the 5.5 to 6.5 $\mu$m range.
Sheffer, et al., studied the wavelength dependence of IR MALDI from 2.6 to 4.0 µm with a wavelength tunable optical parametric oscillator (OPO) and the protein bovine insulin in succinic acid, caffeic acid and 4-nitroaniline matrices.\textsuperscript{35} Greater ion signal was found at wavelengths near 2.9 µm and 3.4 µm corresponding to the O–H and C–H stretching vibration of the matrices, respectively. This effect occurred at lower wavelengths than the maximum absorption around 3.0 µm for succinic and caffeic acid matrices. This “blue-shift” of greater ions observed at wavelengths lower than the maximum room temperature IR absorption of the matrix was attributed to weakened or broken hydrogen bonds caused by IR laser heating.

A more recent study investigated the mid-IR wavelength dependence of a large collection of matrices for ionization of the protein cytochrome C.\textsuperscript{36} Mid-IR light from an OPO over the wavelength ranges of 1.45 to 1.75 µm and 2.7 to 4.0 µm was used and energy absorption based on matrix absorption was calculated. Greater ion signal for IR MALDI was found at wavelengths where matrix absorption in the O–H, N–H or C–H stretch region occurs. Similar to the study described above, a shift in peak ion signal to wavelengths lower than the absorption maximum of the matrix near 3.0 µm was found. As above, weakened hydrogen bonds caused by IR laser heating was given as the reason for the shift.

A wavelength study of IR soft LDI of sample preparations was also preformed.\textsuperscript{23} Since no MALDI matrix is added, IR laser light can be absorbed by the analyte, waters of hydration and the solvent used for sample preparation. Absorption by surface O–H in the case of targets made from silicon may also be possible.\textsuperscript{25,26} The study employed a custom built OPO tunable from 2.9 to 4.2 µm to analyze bacterial spores.\textsuperscript{23} Ion signal intensity of a select spore protein after IR laser ionization spanning the wavelength range of 3.05 to 3.8 µm was compared to the inverse attenuation length (proportional to the IR absorption) of mid-IR laser light of the spores.
Greater signal intensity was found for larger absorption, suggesting that the sample itself was absorbing the IR light and contributing to the ionization of spore proteins.

IR lasers have been used to ionized biomolecules directly from gels without the addition of a matrix. Direct from gel ionization of peptides and proteins was carried out using the FEL laser described above.软激光直接从凝胶中离子化的生物分子。Direct from gel ionization of peptides and proteins was carried out using the FEL laser described above.24 Soft LDI was accomplished using the C=O functional group absorption of gel components near 6 μm. In this experiment, choosing an ionization wavelength based on the absorption of sample constituents was successful. However, a more systematic study comparing the IR absorption spectrum of sample components to protein ionization over a range of wavelengths is needed to determine how the IR light is being absorbed and which wavelengths are more efficient for ionization.

In the experiment presented in Chapter 3, a wavelength tunable OPO was used to study the formation of ions using soft IR LDI. The laser wavelength was changed from 2.8 to 3.6 μm, which covers the O–H, N–H and C–H stretch regions found to coincide with ion formation in previous soft IR LDI wavelength studies. At each wavelength, the threshold fluence, or \( H_0 \), was determined from the minimum laser energy to produce protein ion signal at three times the noise level and used as a parameter to describe the efficiency of ion formation. The threshold fluence was compared to the thin film FT-IR attenuated total reflectance (ATR) absorption of the neat protein sample. Scanning electron microscopy (SEM) was used to further characterize the deposited protein thin films after irradiation by the laser at different wavelengths and fluences.

1.3.2. Two-laser IR/UV Matrix-assisted Laser Desorption/Ionization

In this experiment, processes of desorption/ionization in soft LDI was investigated by changing the delay time between two subthreshold laser pulses and measuring the ion signal. This approach is similar to experiments using lasers of different pulse widths. The similarity of
MALDI threshold fluence for UV lasers with pulse widths between 500 fs and 3 ns suggests that the energy deposited in the sample is the important factor in the MALDI process and that the rate at which the energy is deposited is not critical on the time scales probed.\textsuperscript{37,38} The influence of the rate of energy deposition in IR MALDI is less clear. A comparison of MALDI with a mid-IR free electron laser (FEL) operating at temporal pulse widths of 2 µs and 100 ns\textsuperscript{34} and a comparison of the FEL with a 200 ns Er:YAG laser at 2.94 µm\textsuperscript{39} suggests an irradiance rather than a fluence dependence of IR MALDI. The FEL results differ from those obtained in a comparison of a 6 ns mid-IR optical parametric oscillator (OPO), a switchable 100 or 185 ns Er:YAG laser, and a 75 ns Er:YSGG laser at 2.79 µm.\textsuperscript{40} In these studies, only a slight increase in threshold fluence was observed at longer pulse lengths. This result was taken to indicate a combined thermal and photomechanical desorption process with an increase in the contribution of the thermal component for longer pulses.

One of the most direct approaches to studying the time evolution of the soft LDI process is with two sub-threshold UV laser pulses.\textsuperscript{41-44} In these experiments, two nanosecond UV laser pulses are directed at the same spot on the MALDI sample target and the energy output of the lasers is adjusted so that a single pulse does not produce ion signal. The time delay between the two pulses can be adjusted up to tens of nanoseconds with single laser and an optical delay line or to longer times if two separately triggered lasers are used. The two lasers function in a kind of pump and probe configuration: the first pulse adds energy to the sample but does not create detectable ions. When the second pulse hits the sample, the threshold for ion production is exceeded and a signal is detected. By systematically varying the time delay while measuring the ion signal, the kinetics of MALDI ion formation can be recorded.
The first such study used two 337 nm nitrogen lasers with 5 ns pulse widths for two-laser MALDI;\textsuperscript{42} the protein bovine insulin in sinapinic acid (SA), 6-aza-2-thiothymine and 2,5-dihydroxybenzoic acid (DHB) matrices and the oligonucleotide decathymidylic acid in 3-hydroxypicolinic acid matrix were tested. Depending on the matrix, maximum analyte ion signal was recorded at delays between 5 to 10 ns and decayed to zero within 30 ns. Maximum analyte ion signal at non-zero delay was explained by delayed production of the precursor ions that result in the production of analyte molecules. The role of electronically excited matrix was also considered.

In another study, a 355 nm and 30 ps pulse width Nd:YAG laser with an optical delay line was used to irradiate a sample of neat DHB matrix.\textsuperscript{43} Ion formation processes with a short and long time behavior were observed. With the fast process, matrix ion signal is observed at $t = 0$ ps and this signal decays rapidly to a local minimum near 150 ps. The slower process leads to an increase in matrix ion signal to a maximum at a delay of 2 ns, after which the matrix ion signal drops to zero after a delay of 8 ns. The slow process can be explained either by physical or chemical changes in the DHB matrix after irradiation. The possibility of a delayed gas phase ion formation was also noted. Since both lasers were directed at the same location on the sample target, irradiation of desorbed sample material by the second laser pulse might have occurred several micrometers above the sample surface given an initial DHB neutral velocity of approximately 1000 m/s. Ionization of the desorbed sample material is then determined by the transfer rate of the solid into the gas phase.

Two-pulse MALDI MS of the polypeptides substance P and bovine insulin in different MALDI matrices was carried out using a single 337 nm nitrogen laser with a 4 ns pulse width and an optical delay line.\textsuperscript{44} An exponential decay of analyte ion signal from 0 to 20 ns was found.
for experiments using DHB and SA matrices. An increase in analyte ion signal to a maximum at 6.1 ns and 3.2 ns delay was observed for the matrices α-cyano-4-hydroxycinnamic acid (CCA) and 9-anthracenecarboxylic (9-ACA), respectively. Relaxation of vibronically excited matrix molecules can explain the exponential decay of analyte ion signal found for DHB and SA matrices. For CCA and 9-ACA matrices, the results are consistent with a continuous plume of analyte and matrix molecules that were formed while the temperature of the sample surface was above the sublimation point. In this case, competition for protons based on matrix proton affinities in the plume determines the delay for maximum analyte ion signal.

While the effects of electronic energy transfer and desorption plume dynamics can be effectively probed using short time scale UV laser pump-probe experiments, longer time scale thermal phenomena can be investigated using infrared lasers. For example, the kinetics of IR laser desorption of ions and neutral molecules on a microsecond time scale has been extensively studied using time-resolved laser desorption. In this experiment, the time between the laser and ion extraction pulse is varied while the ion flight time and signal is recorded to produce a time profile of the ion and desorption. Neutral molecules can be interrogated using an electron beam for gas phase ionization of desorbed species. Emission of quaternary ammonium positive ions for tens of microseconds after sample irradiation has been observed with a 10.6 μm pulsed CO₂ laser. The kinetic energy of ions emitted at later times was found to be lower than the kinetic energy of those ions formed initially, which has been taken as an indication of delayed thermal emission of preformed ions. Electron ionization of desorbed neutrals indicates that these species are emitted from the irradiated surface for more than 500 μs after the laser pulse. In Chapter 4, a study of MALDI ion formation using two-laser IR/UV MALDI is presented. Two experiments are presented: one using a 10.6 μm CO₂ laser and a 337 nm N₂ laser
and another using an optical parametric oscillator and the N\textsubscript{2} laser. In these experiments, both lasers were directed at the same target spot. This configuration differs from that of laser post-ionization, in which the initial laser pulse was directed at the surface at a 45° angle and the second laser was aligned parallel to the surface at a distance of several millimeters in order to ionize desorbed neutrals. In the IR/UV MALDI configuration, both lasers were directed at the surface at a 45° angle. The target was irradiated with the IR pulse, followed after an adjustable delay by the UV laser pulse. The laser energy was adjusted so that ion formation does not occur with either of the two laser pulses alone and the integrated ion signal was obtained as a function of the delay between the IR and UV lasers.

1.4. Infrared Laser Desorption/Ionization Directly from a Gel

The rapid expansion of the field of proteomics has created an increased demand for selective, sensitive and high-throughput methods of analysis. Since soft LDI MS has become an important analytical method in this area because of its high throughput, sensitivity and high mass accuracy, new applications to further improve analyses are desired. However, the large number and broad concentration range of the proteins present in the expressed proteome of a typical organism requires that one or more fractionation or separation steps be performed on the sample prior to mass spectrometry analysis.

One of the most commonly utilized techniques for protein separation is sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)\textsuperscript{47-51}. The molecular weight of a protein can be found from the migration distance after electrophoretic separation when compared to the migration of several marker proteins of known molecular weight that bracket the protein of interest. Currently, most proteome analyses are based on the separation of complex protein mixtures by 1D or 2D gel electrophoresis, followed by mass spectrometric analysis of proteolytic
digests of gel spots to identify individual proteins. However, this protocol requires intermediate sample processing steps such as extraction of the protein from the gel, blotting the protein onto a membrane or electroelution, which introduces a number of laborious and time-consuming manual steps that are difficult to automate.

Various efforts have been directed toward direct analysis of gel and planar chromatography separations by soft LDI MS. The mass spectrometric analysis of peptides and proteins by UV-MALDI directly from polyacrylamide gels has been reported previously. Here, ultrathin gels (less than 10 µm) were dried and affixed to the MALDI target and the matrix was added by pipette. Direct analysis of thin layer chromatography (TLC) plates has also been reported using UV and IR lasers. Crystalline matrices were used for IR and UV MALDI, and a sprayed-on glycerol liquid matrix was used for IR MALDI of the TLC plates. The advantage of the IR laser in these studies is the greater depth of penetration compared to the UV laser. As stated above, direct IR LDI mass spectrometry from polyacrylamide gels without the addition of a matrix has been reported using a 5.9 µm free electron laser. MALDI analysis of standard size gel and TLC plates requires a relatively large quantity of material due to the mismatch in size between the laser spot, which is typically a few tenths of a millimeter in diameter, in comparison to the separated material, which is between 5 and 10 mm in size.

One approach to reducing the size of the separation spot is microfluidics. There have been a number of reports of coupling microfluidic chips to MALDI-MS, both on-line and off-line. For example, open channel capillary electrophoresis (CE) has been coupled off-line with MALDI. After the separation, the solvent evaporates, matrix is added, and the sample analyzed in the mass spectrometer. In other off-line approaches, the sample is dispensed from the chip
using a piezoelectric dispenser, pumped through a spinning microfluidic disk, or sample droplets moved on a dielectric chip surface by electrowetting. In a unique on-line approach, the vacuum of the mass spectrometer source is used to pull fluid through the a microfluidic chip where it is subjected to UV MALDI as it exits the device. A second on-line approach uses a rotating stainless steel ball coupled to a on-chip capillary electrophoresis for the separation of peptides.

In Chapter 5, a novel application is presented where IR soft LDI of peptides and proteins is performed directly from a polyacrylamide gel after electrophoretic separation in a gel slab and a microfluidic chip fabricated from polymethylmethacrylate (PMMA). The additional sample processing steps of extraction of the protein from the gel, blotting the protein onto a membrane, freezing the gel or addition of an exogenous matrix prior to mass spectrometry analysis were avoided. Ionization of analytes at the wavelength of 2.94 or 2.95 \( \mu \)m was observed at room temperature using the O–H stretch vibrational absorption of the gel and the water that remains in the gel after vacuum drying. This application provides a faster, more efficient method for analyzing electrophoretically separated biomolecules using mass spectrometry.

1.5. Research Objectives

The objectives of the research presented in this dissertation were 1) to perform experiments that would help elucidate fundamental mechanisms of ion formation in IR soft LDI and 2) to develop applications that utilize useful aspects of IR soft LDI. Experiments towards achieving both of these goals were carried out using both UV and IR LDI. The studies centered around the IR LDI process for studies with and without an added matrix and are described in the following paragraphs. In addition to these experiments, the author has also helped to develop
more cost effective IR lasers for the IR LDI method, but this work will not be presented in this dissertation.

IR LDI wavelength studies of the protein, bovine insulin, were carried out to study the mechanism of laser energy absorption and ionization. As stated above, sample preparations with no added matrix help to isolate the species absorbing the laser energy because matrix absorption is not considered. At selected wavelengths, the threshold fluence, denoted $H_o$, was measured and used to generate a spectrum of ion formation efficiency that was compared to FT-IR attenuated total reflectance (ATR) spectrum of the protein sample. Scanning electron microscopy (SEM) of the irradiated spots was used to further characterize the deposited protein thin films after irradiation by the laser at different wavelengths and fluences.

Experiments designed to study MALDI ion formation were accomplished using two-laser IR/UV MALDI. In the IR/UV MALDI configuration, both lasers were directed at the surface at a 45° angle. The target was irradiated with the IR pulse, followed after an adjustable delay by the UV laser pulse. The laser energy was adjusted so that ion formation does not occur with either of the two laser pulses alone and the integrated ion signal was obtained as a function of the delay between the IR and UV lasers.

A novel application was developed where IR soft LDI of peptides and proteins was performed directly from a polyacrylamide gel after electrophoretic separation in a gel slab and a microfluidic chip fabricated from polymethylmethacrylate (PMMA). Ionization of analytes at wavelengths near 3.0 µm was observed at room temperature using the O–H stretch vibrational absorption of the gel and the water that remains in the gel after vacuum drying.
CHAPTER 2. EXPERIMENTAL

2.1. Time-of-flight Mass Spectrometry

The time-of-flight (TOF) mass analyzer is a simple means to separate ions based on their mass-to-charge ratio (m/z).\textsuperscript{71} TOF MS is typified by a large mass range and high sensitivity. Unlike scanning mass analyzers, such as sectors and ion traps, an entire mass spectrum can be obtained all at once with a duty cycle only limited by the time it takes for the ions to traverse the flight tube, typically within hundreds of microseconds. A TOF tube consists of only a chamber evacuated to a pressure of $10^{-6}$ torr or lower to prevent collisions with background gas molecules.

The length of the TOF tube is based on the desired ion separation resolution over a given mass range. Ions entering the TOF tube have roughly the same kinetic energy imparted to them after the ionization event. Therefore, the flight time of the ions depends only on their mass (m) based on the equation,

$$KE = \frac{1}{2}mv^2$$ \hspace{1cm} (1)

where KE is the kinetic energy and v is the velocity. Since KE=zeV, where z is the charge number of the ion, e is the charge of an electron and V is the acceleration voltage, equation (1) can be rearranged to solve for flight time (t) as follows given a singly charged ion (neglecting time spent in the ion source and post-acceleration at the detector):

$$t = \left(\frac{m}{2eV}\right)^{\frac{1}{2}}D$$ \hspace{1cm} (2)

where D is the field free length of the flight tube. Flight times can be calibrated for mass by using the following equation:

$$t = a + b \cdot m^{1/2}$$ \hspace{1cm} (3)
where \( a \) and \( b \) can be obtained from the \( m/z \) value of two peaks. Mass-resolving power \( (R) \) is usually reported using the full-width at half-maximum (fwhm) method and can be calculated from either flight times or masses.

\[
R = \frac{m}{\Delta m} = \frac{t}{2\Delta t}
\]  

\[ (4) \]

2.2. Linear Time-of-flight Mass Spectrometer

All experiments were performed on a custom-built linear time-of-flight (TOF) mass spectrometer. The vacuum region of the mass spectrometer contained the ion source and TOF tube, which were held at \( 1 \times 10^{-6} \) and \( 1 \times 10^{-7} \) torr, respectively, by two 1200 L/s diffusion pumps (VHS-4, Varian, Palo Alto, CA). The ion source consisted of a sample target holder followed by a grid at a distance of 19 mm and a second grid 19 mm from the first grid. Positive ions were accelerated into the TOF tube by either static or delayed extraction. In delayed extraction mode, a +17 keV acceleration voltage was applied to the sample target holder and first grid. The second grid was held at ground potential. In this configuration, ions were accelerated into the second extraction region by a +3.0 keV voltage pulse applied to the sample target holder after a time delay. A delay generator (DG-535, Stanford Research Systems, Sunnyvale, CA) and HV gate (GRX-3.OK-H, Directed Energy, Inc., Fort Collins, CO) were used to generate the voltage pulse. In static extraction mode, ions were continuously accelerated into the TOF tube by applying a +20 keV acceleration voltage to the sample target holder and grounding the two grids.

At the other end of the TOF tube, a dual microchannel plate (MCP) assembly (Galileo, Sturbridge, MA) was used for ion detection. Positive ions were accelerated into a dual 25 mm diameter MCP detector, the front of which was held at -2.2 keV. A grounded grid was located approximately one centimeter in front of the MCP. The back of the second 25 mm diameter MCP is held at -0.22 keV and the collection anode is held at ground. The field-free distance from
the first grounded lens in the ion source to the grounded grid in the detector assembly was 100 and 102 cm for delayed and static extraction modes, respectively. The detector output was coupled to the 50 Ω input of a 500 MHz digital oscilloscope (9350CM LeCroy, Chestnut Ridge, NY) for data acquisition. The digitized data were downloaded to a computer via a general purpose interface bus (GPIB), using Labview software (National Instruments, Austin, TX), for storage and further manipulation. Wavelength dependent and gel electrophoresis mass spectra were obtained under delayed extraction mode conditions whereas two-laser mass spectra were obtained in static extraction mode.

Figure 2-1 shows a schematic layout of the linear time-of-flight mass spectrometer. Two optical ports were situated on the ion source chamber to allow passage of laser light to irradiate the sample target at an angle of 45° to the sample target normal. A CCD camera with macro zoom lens was placed at a third 45° port to view placement and irradiation of the sample target. Three different lasers were used to ionize sample material based upon the experiment performed and are described below. All lasers were run at a 2 Hz repetition rate for all experiments. It should be noted that the data collection rate for the mass spectrometry is limited by the speed of the digital oscilloscope. Data collection rates on the order of 2 to 5 Hz are typical, especially when internal processing, such as averaging of multiple shots, is used in conjunction with >100,000 point data files. In addition, IR lasers can deplete solid samples within tens of laser shots unless the sample target is continuously translated.

One of the lasers used was a tunable optical parametric oscillator (OPO, Mirage 3000B, Continuum, Santa Clara, CA, USA) pumped by 1064 nm fundamental and 532 nm second harmonic of an Nd:YAG laser (Powerlite 8000, Continuum, Santa Clara, CA, USA). The OPO output a 5 ns laser pulse and was tunable from 1.45 to 4.0 µm. The IR laser was focused using a
254 mm CaF\(_2\) lens. A sapphire optical port was used. Attenuation of the laser beam was achieved by placing either a variable iris or a combination of flat optical substrates of different thicknesses into the beam path. Substrates consisted of germanium, calcium fluoride, zinc selenide, silicon or zinc sulfide. A 2500 nm long pass filter with 80 percent transmission (FXLP-0250, Janos Technologies, Keene, NH) was placed in the beam path to cut off pump laser leakage and other wavelengths generated by the optical parametric process.

![Figure 2-1. Schematic layout of the linear time-of-flight mass spectrometer (TOF-MS). A CO\(_2\) laser can be added to the setup when applicable. The delay generator (DG) triggers the high voltage (HV) gate through a fiber optic and the digital oscilloscope (DO). The sample target is viewed on a monitor by a video camera (VC).](image)

A 4 ns pulse width, 337 nm N\(_2\) laser (VSL-337ND-S, LSI, Franklin, MA, USA) was used for some experiments. Attenuation was achieved using a variable iris and a variable circular neutral density filter mounted on a rotating stage. The UV laser was focused using a 254 mm focal length fused-silica lens. A sapphire or UV fused silica entrance port window was used.

The third laser used was a pulsed, 10.6 \(\mu\)m CO\(_2\) laser (\(\mu\)-TEA CO\(_2\), LSI, Franklin, MA). The CO\(_2\) laser was used in combination with the N\(_2\) laser for two-laser experiments and was
placed in the beam path of the OPO as indicated by a dotted outline in Figure 2-1. The output beam was attenuated with a variable iris. The temporal profile of the IR laser consists of an initial 80 ns fwhm spike that contains 40% of the pulse energy followed by a 1 µs tail with a lower peak energy. The CO₂ laser was focused with a 254 mm focal length ZnSe lens. A 1 in. ZnSe flat optic was used as the material for the entrance port window in a custom-built mount.

The laser fluence was obtained by dividing the laser pulse energy by the area of laser irradiation on the sample target. Laser pulse energies were measured by placing a pyroelectric joulemeter (ED-104AX, Gentec, Palo Alto, CA, USA) in front of the laser beam. To measure the irradiation area, laser burn paper (Pacific Coast Photo, Santa Cruz, CA, USA) was attached to the sample target using double-sided tape. Three to five laser shots irradiated the paper and the burn mark was measured with a measuring microscope.

2.3. Characterization Procedures for Soft Desorption and Ionization

2.3.1. Materials

The bovine insulin (I-5500, Sigma, St. Louis, MO) protein standard was obtained and used without any further purification. The protein was dissolved in a 3:1 v/v 0.1% trifluoroacetic acid solution (TFA, 04902-100, Fisher Scientific International, Pittsburgh, PA) and methanol to a concentration of 0.8 mM. A 1.25 µm aliquot of the protein solution was deposited on a 5 x 5 piece of 0.6 mm thick untreated, polished silicon (Silicon Quest International, Santa Clara, CA) attached by conductive tape (Electron Microscopy Sciences, Ft. Washington, PA) to the target. The deposit was dried with hot air from a heat gun 5 cm from the target for 10 sec.

2.3.2. Scanning Electron Microscopy

Deposits of bovine insulin on silicon wafers were analyzed by SEM after being irradiated with the OPO. A total of three laser shots were used at different laser fluences and wavelengths.
Prior to SEM, the samples were sputter coated with a 20 nm gold/palladium (60/40) layer using a sputter coater (S-150B, Edwards, Crawley, UK). The samples were mounted on aluminum supports with conductive tape and examined at 10 or 15 kV with a scanning electron microscope (S-260, Cambridge Instruments, Cambridge, UK) located in the Louisiana State University Socolofsky Microscopy Center.

2.3.3. Fourier Transform Infrared Attenuated Total Reflectance Spectroscopy

Threshold fluences measured for mass spectra were compared to the FT-IR ATR thin film absorption of the sample. The method used for collecting IR spectra has been described previously. Briefly, all infrared spectra were recorded at 4 cm\(^{-1}\) resolution in a FT-IR spectrometer (Model 1760, Perkin-Elmer, Norwalk, CT) with a liquid nitrogen cooled mercury cadmium telluride (MCT) detector. The beam path was continuously purged with dry nitrogen. A 45° single-pass trapezoidal (SPT) ATR silicon plate (dimensions 50 x 20 x 2 mm, Harrick Scientific Corporation, Pleasantville, NY) IRE was mounted in a single-beam multiple internal reflection (MIR) attachment (Model 9, Foxboro Instruments, Foxboro, MA). Background and sample spectra were recorded by averaging 100 scans.

2.4. Two-laser Matrix-assisted Desorption and Ionization

2.4.1. Materials

The peptides gramicidin S (G-5001, Sigma) and bradykinin (B-3259, Sigma) and the proteins bovine insulin and cytochrome C (C-2506, Sigma) were used as test analytes. MALDI matrices were 4-nitroaniline (NA, 72680, Fluka, Buchs, Switzerland), DHB (G5254, Sigma), SA (D7927, Sigma), and CCA (C2020, Sigma). All chemicals were used as purchased without further purification. DHB and NA were dissolved in methanol at a concentration of 50 mg/mL. SA and CCA were dissolved in a 50/50 (v/v) methanol:acetonitrile mixture at a concentration of
50 mg/mL. A 5 to 7 µL aliquot of the matrix solution was deposited on the sample target and dried with a heat gun for 15 s at a distance of 10 cm from the sample target surface. Analyte solutions were prepared by dissolving protein samples in 95/5 (v/v) methanol:1% TFA to a concentration of 500 µM. A 3-10 µL aliquot of analyte solution was mixed with a 7-10 µL aliquot of the matrix solutions. The total volume of the mixture was deposited on the sample target and dried with hot air using the same procedure described above.

2.4.2. Laser Arrangement

A schematic diagram of the instrument configured for the two-laser IR/UV-MALDI experiments is shown in Figure 2-2. The UV laser was the 337 nm N₂ laser. The IR laser was either the 10.6 µm CO₂ laser or the OPO at a wavelength of 2.94 µm. The triggering of the lasers was varied by the DG-535 delay generator. The delay between the two lasers is represented by Δt in which negative Δt indicates that the UV laser fires before the IR laser and positive Δt indicates that the IR laser fires before the UV laser. The time jitter between the two pulses was 40 ns, which was limited by the nitrogen laser electronics.; therefore, Δt values have a plus or minus 40 ns error at approximately two standard deviations.

Spatial overlap of the beams was achieved by taping laser burn paper to the sample target, loading the target into the mass spectrometer and moving each beam until both burned the same location on the paper. The CCD camera and macro zoom lens were used to observe coarse beam overlap. Fine overlap was achieved by moving one beam around the other while watching for maximum two-laser IR/UV MALDI ion signal on the oscilloscope. Realignment of the beams to the same spatial location was carried out at the beginning of each day of experimentation. Temporal overlap was achieved by placing a photodiode (ET-2000, Electro-Optics Technology, Inc., Traverse City, MI, USA) in the beam path of the N₂ laser and the 532 nm light from the
OPO pump laser and adjusting the time delay until each photodiode signal overlapped. In the two-laser configuration using the CO\textsubscript{2} laser, the photodiode signal of the N\textsubscript{2} laser was overlapped with the rise of the CO\textsubscript{2} laser trigger TTL signal.

![Diagram of the experimental setup](image)

**Figure 2-2.** Schematic layout of the two-laser IR/UV MALDI experiments using a linear time-of-flight mass spectrometer (TOF-MS). The infrared (IR) and ultraviolet (UV) lasers are directed at the same target spot from opposite sides of the instrument. The computer (PC) controls the delay generator (DG) and digital oscilloscope (DO). Reproduced with permission © John Wiley & Sons: Little, M. W.; Kim, J.; Murray, K. K., Two-laser infrared and ultraviolet matrix-assisted laser desorption/ionization. *J. Mass Spectrom.* 2003, 38, 772-777.

### 2.5. Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

#### 2.5.1. Materials

Bradykinin and bovine insulin were obtained from Sigma Chemical Co. (St. Louis, MO). Acetic acid, methanol and \(\beta\)-mercaptoethanol were acquired from Fisher Scientific (Pittsburgh, PA). Tris was purchased from Life technologies (Gaithersburg, MD). Except when otherwise noted, all chemicals used for gel electrophoresis, including polypeptide standards, were supplied by Bio-Rad Laboratories (Hercules, CA). All chemicals were used without further purification.

#### 2.5.2. Gel Electrophoresis

Electrophoresis was carried out on a Mini-Protean 3 miniature slab gel system (Bio-Rad Laboratories, Hercules, CA). The solutions and minigels for Tris/Tricine sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were prepared according to the procedure of Schagger and von Jagow\textsuperscript{50} and run at 150 V for 70-90 min. The concentration of acrylamide in
the resolving gel was 16.5% T and the acrylamide to bisacrylamide ratio was 30:1. Here %T denotes the weight percentage of total monomer (acrylamide and bisacrylamide) whereas %C denotes the percentage of the crosslinker relative to the total monomer mass. The small-pore resolving gel was overlaid by a 7.5 %T, 3 %C stacking gel (2 cm). The thickness of the gel was approximately 750 µm. The protein samples were incubated for 30 min at 40 °C in 4% SDS, 12% glycerol, 50 mM Tris-HCl (pH 6.8) and 0.02% Coomassie Brilliant Blue G-250. A mixture of polypeptide standards (triosephosphate isomerase, 26.6 kDa; myoglobin, 16.95 kDa; α-lactalbumin, 14.4 kDa; aprotinin, 6.5 kDa; insulin B chain, 3.5 kDa; bacitracin, 1.4 kDa) was loaded onto the gel with amounts of 0.5 µg per lane. Five nmol of bradykinin and bovine insulin were loaded per gel lane. The effect of reducing agents during electrophoresis was studied by adding 5% β-mercaptoethanol. After electrophoresis, the gel was stained with 0.25% Coomassie Blue R250 in 50% methanol in water containing 10% acetic acid for 30 min and destained overnight in 10% methanol in 1% aqueous acetic acid. Gel pieces 1 to 5 mm in size containing the analyte were excised and mounted on the sample target using double-sided conductive tape (Electron Microscopy Sciences, Ft. Washington, PA).

2.5.3. Preparation of Polypeptides Spotted onto Gel Surfaces

The bradykinin and bovine insulin were dissolved in doubly distilled water containing 0.1% trifluoroacetic acid to concentrations of 5 and 3 mM, respectively. A 5 mm diameter piece of SDS-containing polyacrylamide gel was cut out and mounted on the sample target using double-sided conductive tape; 5 l of analyte solutions were applied to the gel sections. After 10 min, the gel sections were rinsed with ddH₂O.
2.6. Microfluidic Chip Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

2.6.1. Materials

Bradykinin, bovine insulin and cytochrome C were obtained from Sigma Chemical Co. (St. Louis, MO). PMMA sheets were purchased from Goodfellow Co. (Berwyn, PA). The PDMS used in this experiment was purchased from General Electric (Waterford, New York, USA). A set of RTV 615A (pre-polymer) and RTV 615B (curing agent) were mixed with a 10:1 weight ratio and degassed using a vacuum pump to remove air bubbles entrapped during mixing. The polymer was cast against the glass mold and polymerized at 100 °C for 1 h. Except when otherwise noted, all chemicals used for gel electrophoresis were obtained from Bio-Rad Laboratories (Hercules, CA). All chemicals were used without further purification.

2.6.2. Microchip Fabrication

The microfluidic chip layout is shown in Figure 2-3 and was a standard “tee” format designed using AutoCAD (Autodesk Inc., San Rafael, CA). Microfluidic chips were fabricated from PMMA wafers using a micromilling machine (Kern Micro-und Feinwerktechnik GmbH & Co. KG, Murnau, Germany). The channels were 300 µm in width and 150 µm in depth and the diameters of reservoirs were 1 mm. Two chip types were manufactured from 3 mm thick PMMA wafers: a short channel and a long channel version. The short-channel chip was 15 x 20 mm with a 5 mm short arm and 16 mm long channel and the channels crossed such that the separation channel was 14 mm long. The long-channel chip was 15 x 55 mm with 5 and 50 mm short and long arms, respectively; the separation channel was 50 mm long. Before assembly, 1 mm diameter holes were drilled through the 3 mm thick PMMA wafers to serve as reservoirs. After cleaning of the embossed microfluidic chip, a 0.5 mm PDMS cover was thermally...
annealed to the chip by clamping the assembled microfluidic chip between two glass plates and heating to 105 °C for 12 min. in a GC oven.

Figure 2-3. Schematic diagram of the gel microfluidic chip ion source. The chip is shown below inset with buffer reservoir A, analyte reservoir B, analyte waste C and buffer waste D. Ions are desorbed from the chip held behind the target L0 and ions are accelerated into the flight tube by acceleration grids L1 and L2. Reproduced with permission © Elsevier, Inc.: Xu, Y.; Little, M. W.; Murray, K. K., Interfacing capillary gel microfluidic chips with infrared laser desorption mass spectrometry. *J. Amer. Soc. Mass Spectrom.* **2006**, *17*, 469-474.

2.6.3. SDS-PAGE in Microchips

The solutions and gels for Tris/Tricine SDS-PAGE were prepared according to the procedure above. Then the sieving gel was introduced into the channel in the microfluidic chip and was polymerized for 1 hr. After polymerization, the reservoirs were flushed with Tris/Tricine running buffer. The same solution was used as a sample buffer and buffers for other
reservoirs. The device was exposed to an electrical field of 150 V/cm for 5 min. before sample loading. The samples were incubated for 30 min. at 40 °C in 4% SDS, 12% glycerol, 50 mM Tris-HCl (pH 6.8), and 0.02% Peptide and protein samples were loaded by applying voltage across the twin-T injector for 9 min. with buffer reservoirs floating. During separation, 30 V/cm was applied to both side channels to prevent leakage of excess sample into the separation channel. Electrophoresis was performed on bradykinin and bovine insulin in SDS PAGE microchips using a field of 150 V/cm that was applied for 9 min.

2.6.4. Insertion of Microchip into Mass Spectrometer

After electrophoresis, the PDMS cover was peeled off of the PMMA microfluidic chip and either the chip or the cover was mounted in a modified MALDI target holder (Figure 2-3). The target holder consisted of a 5 cm diameter, 2 mm thick circular piece of stainless steel with a 1.6 by 1 cm recess milled across the center of the holder to accommodate the microfluidic chip. A circular piece of stainless steel with the same diameter as the holder held the chip from the rear. A 20 x 1 mm slit was cut into the center of the holder to allow the laser light to irradiate the channel of the chip and allow passage of ions into the flight tube.
CHAPTER 3. WAVELENGTH DEPENDENCE OF INFRARED SOFT LASER DESORPTION/IONIZATION

3.1. Introduction

In the work described in this chapter, the wavelength tunable OPO was used to study the formation of protonated insulin ions in the H atom stretch region of the infrared. Mass spectra were obtained with wavelengths between 2.8 and 3.6 µm, which encompasses the O−H, N−H and C−H stretch absorption regions. At selected wavelengths, the threshold fluence, denoted $H_0$, was measured and used to generate a spectrum of ion formation efficiency that was compared to FT-IR ATR spectrum of the protein sample. SEM of the irradiated spots was used to further characterize the deposited protein thin films after irradiation by the laser at different wavelengths and fluences. By identifying the component of the sample that is absorbing the laser light and how the laser light effects the sample, information regarding the optimization of the IR soft LDI method as well as how the protein is ionized might be obtained.

3.2. Experimental

At each wavelength, the sample was loaded into the mass spectrometer and the laser energy was adjusted to obtain insulin [M+H]$^+$ ion signal with approximately 3:1 S/N. The laser energy was measured using the joulemeter and laser spot size was also recorded at each wavelength using photographic burn paper and a measuring microscope. The energy measurements were repeated five times with five different samples and the average laser energy at each wavelength was combined with the laser spot size to obtain the threshold fluence. The threshold fluence was determined for wavelengths between 2.8 and 3.6 µm at 50 nm increments and at 25 nm increments between 2.95 and 3.05 µm. Three measurements, one at 3.1 µm and two at 3.55 µm, were removed as outliers in accordance with a standard t-test.
3.3. Results

Infrared spectra of water, methanol, and insulin thin films are shown in Figure 3-1. The three insulin infrared spectra differ in the solvent used to dissolve the protein: water, methanol and a 3:1 (v/v) water:methanol mixture. The water spectrum has a broad absorption centered at 3 \( \mu \text{m} \) and extending from 2.7 to 3.6 \( \mu \text{m} \). The IR absorption of the methanol film extends from 3.2 to 3.6 \( \mu \text{m} \) with a weak absorption in the O–H stretch region near 3 \( \mu \text{m} \). The insulin thin film IR spectra are similar with absorption starting at 2.7 \( \mu \text{m} \) and continuing beyond the 3.8 \( \mu \text{m} \) shown in Figure 3-1 to approximately 4.5 \( \mu \text{m} \). The intense peak at 3.03 \( \mu \text{m} \) and the weaker peak at 3.26 \( \mu \text{m} \) band correspond to the N–H stretch amide bands A and B, respectively. It is important to note that the amide B peak is unique to the protein infrared spectrum. The peak at 3.4 \( \mu \text{m} \) corresponds to the C–H stretching vibration. Although the insulin mass spectra are similar, the intensity at 3 \( \mu \text{m} \) is greater in the spectrum obtained from the insulin deposited from a water solution. This is most likely due to residual water in the thin protein film.

**Figure 3-1.** FT-IR ATR spectra of water (W) (thin solid line), methanol (M) (thin dotted line) and bovine insulin (BI) dissolved in water (thick dashed line), 3:1 (v/v) water/methanol mixture (thick solid line) and methanol (thick dotted line).
Laser desorption ionization mass spectra obtained in 100 nm increments between 2.8 to 3.6 µm are shown in Figure 3-2. The laser fluence was set at 1.5 times the threshold fluence $H_0$ at each wavelength and the y-axis scale is identical for all nine mass spectra. Signal corresponding to protonated insulin ($M_r = 5733.5$ Da) is observed at wavelengths from 2.9 to 3.5 µm and is indicated as $[\text{M + H}]^+$ in Figure 3-2. Peaks observed in the mass range 300 to 4000 m/z are tentatively assigned as analyte fragments; however, the low resolution makes assignment of these peaks difficult. No peaks are observed between 50 and 300 m/z and peaks below m/z 50 are most likely the result of salt contamination. The most intense signal, both for protonated insulin as well as fragments occurs at 3.4 µm. Spectra obtained from 3.0 to 3.2 µm are a good compromise between intense analyte signal and relatively low fragment signal.

Figure 3-2. Mass spectra of bovine insulin deposited on untreated, polished silicon at infrared wavelengths between 2.8 and 3.6 µm.
A plot of the inverse of the threshold fluence, $1/H_o$, as a function of wavelength is shown in Figure 3-3. Plotting the inverse of $H_o$ facilitates the comparison of the efficiency of ionization (as indicated by low $H_o$) to the infrared absorption spectrum of the protein. The IR spectrum of a thin film of bovine insulin in a 3:1 (v/v) water:methanol mixture is shown as the solid line in Figure 3-3 (and is identical to the corresponding plot in Figure 1). Note that the IR spectrum is scaled by an arbitrary factor. The $1/H_o$ data track closely with the IR absorption although the former is somewhat narrower in extent and the maximum at 3 µm is shifted approximately 60 nm with respect to the IR absorption maximum.

![Graph](image.png)

**Figure 3-3.** Inverse threshold fluence for insulin ion formation plotted as a function of wavelength (solid circles with one standard deviation error bar) overlaid with the IR absorption spectrum an insulin thin film (thin solid line).

Figure 3-4 shows SEM images of the thin protein films after irradiation by 2.975 µm light at different fluences. Each sample was irradiated with three laser shots at the indicated fluence.
The cracks visible in the images are not caused by the laser and are apparently formed when the film dries. The threshold fluence at this wavelength is 3200 J/m$^2$ and the samples were irradiated at approximately $2/3 \, H_o$, $H_o$ and $1.5 \, H_o$. At a fluence of 2450 J/m$^2$ (Figure 3-4a), some melting at the crack edges can be seen as well as what appears to be several solidified bubbles. At 3400 J/m$^2$ fluence (Figure 3-4b) a circular feature approximately 60 µm in diameter is visible at the center of the image. Within the feature, evidence of melting and bubble formation is visible in the form of 2 to 4 µm holes and 2 to 10 µm spheres of melted material. At the center of the spot, the film appears to be completely removed. At a fluence of 5000 J/m$^2$ (Figure 3-4c), a circular feature approximately 100 µm in diameter is visible in which nearly all of the thin film has been removed. The evidence of bubbling is visible around the edges of the feature and it appears that some of this material has coalesced at the edges of the crater. Some of the material seems to have been thrown from the crater and moved several tens of micrometers away from the irradiated area.

SEM images at different wavelengths are shown in Figure 3-5. As above, the samples were irradiated with 3 laser shots. In Figure 3-5a, a wavelength of 2.85 µm and a laser fluence of 15,600 J/m$^2$ was used, which corresponds to the threshold fluence at that wavelength. Even at this relatively high laser fluence, the surface is relatively undamaged. A 50 by 130 µm area is partially ablated, but no evidence for melting is observed. Figure 3-5b shows a thin film irradiated at 3.4 µm and 5200 J/m$^2$, which corresponds to $H_o$ at this wavelength. As in the above figure, evidence of melting and bubble formation is observed in a 50 µm diameter area. The material at the center has been removed completely either by ejection or by melting and solidifying at the edge of the crater.
Figure 3-4. SEM images of bovine insulin deposited on untreated, polished silicon after irradiation by 3 laser shots of pulsed 2.975 µm IR laser light at fluences of a) 2,450 J/m² b) 3,400 J/m² and c) 4,950 J/m².
Figure 3-5. SEM images of bovine insulin deposited on untreated, polished silicon after irradiation by 3 laser shots of pulsed IR laser light at wavelengths and fluences of a) 2.85 µm and 15,550 J/m$^2$ and b) 3.4 µm and 5,200 J/m$^2$.

3.4. Discussion

The nature of the energy absorber in infrared soft laser desorption ionization has been difficult to ascertain because many compounds absorb in the IR region. With infrared MALDI, a good correlation has been found between the absorption of the matrix molecule and the relative
efficiency of ionization. Discrepancies between matrix absorption and ion generation in the 2.7 to 3.3 µm region have been explained by the absorption of free O–H groups, spectral shift due to sample heating, or by residual water or protic solvent in the sample. However, experiments carried out at different temperatures and protein hydration conditions suggest that residual solvent energy absorption is not a significant factor. These results are supported by thin film FT-IR ATR results that show no detectable solvent absorption.

In the case of laser desorption ionization with no added matrix, the energy absorber is limited to the analyte itself, residual solvent, or the target material itself. The silicon targets used in this study might be expected to present surface O–H groups that could influence the local energy absorption at the interface between target and analyte. However, it has been shown that a range of surfaces, including metals and plastics, can be used to obtain comparable mass spectra under similar conditions. The results depicted in Figure 3-1 and Figure 3-3 suggest that the analyte is the major energy absorber in the IR LDI process. The IR absorption spectra in Figure 3-1 show that water has a strong absorption between 2.8 and 3.2 µm and methanol between 3.3 and 3.5 µm. Only the insulin has an appreciable absorption peak between 3.2 and 3.3 µm and the intensity of this peak is not appreciably affected by the solvent used in the sample deposition. A comparison of the inverse threshold fluence $1/H_0$ in Figure 3-3 with the IR absorption spectra gives a clear indication that ion generation is influenced by analyte absorption rather than the solvent absorption. First, the shoulder in the $1/H_0$ plot at 3.25 µm is significant and coincides with the amide B absorption of insulin. Second, the relative intensities at 3.0 µm and 3.4 µm in the $1/H_0$ plot correlate closely with the relative intensities of the insulin IR absorption spectra. The maximum of the $1/H_0$ plot is shifted 60 nm to shorter wavelength compared to the IR absorption; this may be due to a shift in IR absorption due to transient heating that leads to
weakening hydrogen bonds in the protein.

Some indication of the mechanism of desorption can be inferred from the SEM images in Figures 3-4 and 3-5. Melting of the insulin and bubble formation occurs at the threshold fluence for ion formation and the process is strongly dependent on the wavelength. At wavelengths corresponding to high absorption, the melting occurs at low fluence; conversely, at wavelengths of low absorption, little melting is observed even at high fluences. For the desorption process at 2.975 µm where the threshold fluence \( H_o \) is 3400 J/cm\(^2\) (Figure 3-4b), the optical penetration depth of a typical protein is approximately 10 µm,\(^73,74\) leading to a volumetric energy density in the protein thin film of \( 3 \times 10^8 \) J/m\(^3\). This in turn corresponds to a molar volumetric energy density of 2000 kJ/mol. The melting enthalpy for insulin is 72 kJ/mole,\(^75\) indicating that there is sufficient energy for melting of the thin film, protein thermal dehydration and vaporization of the resulting water to form bubbles in the melted surface. This bubble formation provides enough energy to remove a large fraction of the protein thin film from the target.

The deposition of energy in the protein thin film is sufficiently rapid to place the process in the stress confinement regime of laser ablation.\(^76\) The characteristic time for acoustic energy dissipation is given by \( \tau_{ac} = 1/\alpha \ c \), where \( 1/\alpha \) is the laser ablation depth and \( c \) is the speed of sound in the irradiated material. The speed of sound in the protein film is approximately 300 m/s\(^77\) and \( \tau_{ac} \) is therefore on the order of 30 ns. As indicated above, the OPO pulse temporal width is 6 ns and acoustic dissipation of the laser energy will not occur during the pulse, resulting in stress confinement. The thermomechanical stresses associated with stress confinement would be expected to enhance the formation of bubbles resulting from cavitation and explosive boiling.\(^74\) High concentrations of course particles have been observed under stress confinement conditions of IR laser desorption.\(^76\)
The mechanism of ionization suggested by the above observations is one in which the analyte absorbs sufficient laser energy to melt and is ejected into a dense plume. Ionization may occur through the reactions of individual ions and molecules in the plume or through a cluster ionization mechanism. A fraction of the protein molecules function as a “sacrificial matrix” that absorb the laser energy and form reagent ions that react further to form protonated insulin molecules. Alternately, the protein may be ejected as clusters that form ions by charge separation of pre-formed protein ions. In either case, the protein itself appears to be acting as an intrinsic matrix.

3.5. Summary

In this chapter, mass spectra of the protein bovine insulin were presented over a 0.80 μm wavelength range between 2.80 μm and 3.60 μm. A plot of inverse threshold fluence versus wavelength closely matches the IR absorption spectrum of the protein thin film. The close correlation of the threshold fluence and the IR absorption spectrum, coupled with the presence of features in the IR spectrum that are not present in the IR spectra of the solvents used to dissolve the insulin (water and methanol) suggest that the latter are not participating in the desorption and ionization process. SEM images of the irradiated thin films show evidence of melting and bubble formation at and above the fluence corresponding to the ionization threshold. In the past, only IR absorption spectra have been used to compare the wavelength response of IR LDI. The SEM results show a better picture of what is happening to the sample at different wavelength and fluences for both on and off resonance wavelengths. The images are consistent with the melting and thermal dehydration of the insulin under stress confinement conditions. Ionization occurs either through charge separation of ejected clusters or through reagent ions formed by the decomposition of “sacrificial” insulin molecules.
CHAPTER 4. TWO-LASER IR/UV MATRIX-ASSISTED LASER DESORPTION/IONIZATION*

4.1. Introduction

In this chapter, a study of MALDI ion formation using two-laser IR/UV MALDI is presented. In these experiments, both lasers were directed at the same target spot. This configuration differs from that of laser post-ionization, in which the initial laser pulse is directed at the surface at a 45° angle and the second laser is directed parallel to the surface at a distance of several millimeters in order to ionize desorbed neutrals. In the IR/UV MALDI configuration, both lasers are directed at the surface at a 45° angle. The target was irradiated with the IR pulse, followed after an adjustable delay by the UV laser pulse. The laser energy was adjusted so that ion formation does not occur with either of the two laser pulses alone and the integrated ion signal was obtained as a function of the delay between the IR and UV lasers.

4.2. Experimental

Data were obtained using custom software to control the delay generator and digital oscilloscope via GPIB. A data acquisition sequence was initiated by setting the delay between the IR and UV laser pulses at the delay generator. Next, waveform acquisition was enabled at the digital oscilloscope and a selected number of single shot mass spectra were accumulated. For the data shown below, three to five single shot spectra were averaged. The integrated peak area was then read from the digital oscilloscope and recorded to the computer. The integration limits were set such that the entire peak was integrated from baseline before the peak to baseline after the peak. The delay time was incremented and the peak

integration process was repeated at the new delay time. To account for sample depletion and fluctuations in signal over time, the sequence of data acquisition over the full range of delay times was repeated five times for each of the delay plots shown below. Each data point results from an average of 15-25 laser shots.

4.3. Results and Discussion

4.3.1. 10.6 µm CO$_2$ / 337 nm N$_2$ Two-laser Configuration

Mass spectra obtained from a sample deposit of DHB matrix are shown in Figure 4-1. Figure 4-1a was obtained using the UV laser only, Figure 4-1b with the IR laser only, and Figure 4-1c with the IR and UV lasers at $\Delta t = 0$ µs. A weak signal from the sodium ion adduct, [M+Na]$^+$, is seen in the mass spectrum obtained from the UV laser alone (Figure 4-1a) and the IR/UV MALDI mass spectrum at $\Delta t = 0$ µs (Figure 4-1c). Strong matrix ion peaks are seen only in Figure 4-1d: the base peak at $m/z$ 137 corresponds to the loss of water from the protonated DHB, [M−H$_2$O+H]$^+$. Protonated matrix, [M+H]$^+$, and sodium ion adduct peaks are also observed. The [M−H$_2$O+H]$^+$ peak was the base peak in the IR/UV MALDI spectra at all delay times. An IR laser fluence of 6000 J/m$^2$ and a UV laser fluence of 500 J/m$^2$ were used, which represents 50% and 70% of the threshold fluence, respectively. A mass spectra similar to Figure 4-1d (and similar to those reported previously)$^{78}$ could be obtained from the DHB matrix with the UV laser alone if the pulse energy was increased above the threshold. With the IR laser alone, the sodium ion adduct, [M+Na]$^+$, was the base peak.

The area of the base peak in the matrix ion mass spectrum, [M−H$_2$O+H]$^+$, was recorded as the delay between the IR and UV lasers was systematically varied. Figure 4-2 shows the delay time dependence of the [M−H$_2$O+H]$^+$ base peak ion signal. Figure 4-2a shows the time behavior between 0 and 1 µs and Figure 4-2b that between 0 and 400 µs. The data in Figure 4-2a are also
plotted in Figure 4-2b so that the rapid rise in signal between 0 and 1 µs can be seen in the plot. The error bars correspond to one standard deviation error in the measurements. From Figure 4-2a, it is apparent that no ion signal is observed until the time delay between the two lasers is approximately 700 ns. The ion signal reaches a maximum near 1 µs and maintains that value for about 20 µs. The signal decays to zero after several hundred microseconds. The ion signal was observed at delays longer than 400 µs but was weak and not reproducible.

![Figure 4-1](image)

**Figure 4-1.** Mass spectra of DHB matrix obtained with a) UV laser only, b) IR laser only, c) IR and UV laser at Δt = 0 µs, and d) IR and UV laser at Δt = 1 µs.
Figure 4.2. Peak area as a function of delay time for DHB matrix from a) 0 to 1 µs and b) 0 to 400 µs.

Mass spectra of bovine insulin in a DHB matrix are shown in Figure 4-3. As in the matrix experiments above, Figure 4-3a was obtained using the UV laser only, Figure 4-3b with the IR laser only, Figure 4-3c with the IR and UV lasers at Δt = 0 µs and Figure 4-3d with the IR and UV lasers at Δt = 1 µs. Analyte ions are not observed with the UV or IR lasers alone (Figure 4-3a
and Figure 4-3b) or with the IR and UV lasers at a delay time of $\Delta t = 0$ µs (Figure 4-3c). The laser fluence was the same as in the DHB matrix experiment. The mass spectrum in Figure 4-3d) at $\Delta t = 1$ µs is similar to that which could be obtained with either the UV or the IR laser at an energy above the ionization threshold. The base peak in Figure 4-3d is the protonated insulin ion, $[\text{M+H}]^+$, with some contribution from the unresolved sodium ion adduct. Dimer and trimer insulin cluster ions $[2\text{M+H}]^+$ and $[3\text{M+H}]^+$ are also observed in the spectrum.

**Figure 4-3.** Mass spectra of bovine insulin in a DHB matrix obtained with a) UV laser only, b) IR laser only, c) IR and UV laser at $\Delta t = 0$ µs, d) IR and UV laser at $\Delta t = 1$ µs.
Figure 4-4 shows the delay time dependence of the protonated insulin peak area in a DHB matrix. The integration limits are sufficiently large to include alkali metal ion and matrix adducts. A similar trend is observed with the insulin analyte as for the matrix ions. Figure 4-4a shows the time behavior between 0 and 1 µs and Figure 4-4b that between 0 and 400 µs. Note that zero ion signal is offset in these figures owing to the larger integration limits, which leads to an integration of the baseline noise. The ion signal is near zero up to 700 ns, after which it increases to its maximum value near 1 µs, where it remains for up to 50 µs. The insulin ion signal decays to zero more slowly than the matrix signal. An attempt to fit the signal to an exponential decay curve was not successful because the time profile does not fit this functional form: the signal decays to zero more slowly at longer times than would be expected from a single exponential. However, it can be noted that the matrix signal drops to roughly half of its maximum value in 30 µs whereas the insulin guest ion signal decays to approximately half of its maximum value in 100 µs.

The most likely factor leading to ion formation is transient sample heating. It has been reported previously in static target cooling studies that threshold fluence for DHB laser desorption and ionization is inversely proportional to the initial sample temperature. In the case of IR/UV MALDI, the IR laser serves to heat the sample surface, after which UV MALDI proceeds from the heated surface at a higher efficiency than from the room temperature surface. As the surface cools, the efficiency drops and, because the UV laser energy is below the ionization threshold, the signal decays to zero. It is notable that the increase in signal seen in Figures 4-2a and 4-4a occurs after the energy from the 1 µs IR pulse has been deposited in the sample. The >100 µs duration of the ion signal seen in Figures 4-2b and 4-4b coincides with the
time scale of the thermal emission of neutrals from samples that were irradiated with a 10.6 µm IR laser.\textsuperscript{11}

\begin{figure}
\centering
\begin{subfigure}{0.4	extwidth}
\centering
\includegraphics[width=\textwidth]{figure_a.png}
\caption{Peak area as a function of delay time for bovine insulin from a) 0 to 1 µs and b) 0 to 400 µs.}
\end{subfigure}
\end{figure}
The rise in surface temperature, $\Delta T_{\text{surf}}$, can be calculated using the Green’s function technique for heat flow.\textsuperscript{81,82} For a thin film of DHB, the energy absorbed by the 10.6 $\mu$m laser is negligible compared with that absorbed by the stainless steel substrate and the estimated temperature rise can be calculated from the properties of the latter material.\textsuperscript{8} However, thermal heating by the IR laser might change the absorption coefficient of the sample and make this assumption less valid. The rise in surface temperature at time $t$ for a uniform light source of intensity $I_0$ is given by

$$\Delta T_{\text{surf}}(t) = \frac{2I_0}{K} \sqrt{\frac{\kappa t}{\pi}}$$

where $K$ is the thermal conductivity and $\kappa$ is the thermal diffusivity. Substituting the material parameters of stainless steel ($K = 0.14$ W cm$^{-1}$ K$^{-1}$; $\kappa = 0.03$ cm$^2$ s$^{-1}$),\textsuperscript{83,84} an irradiance of $6.5 \times 10^5$ W cm$^{-2}$ and a pulse duration of 900 ns, the estimated temperature rise is 850 K at the end of the IR laser pulse. This temperature rise is most likely an overestimate because the reflectance of the substrate has been neglected. For example, if 90% of the IR light were reflected at the metal surface, the temperature rise would be 85 K above room temperature. When cooling of the surface is considered, $\Delta T_{\text{surf}}$ value drops to 20% of its maximum value within 10 $\mu$s.\textsuperscript{81} The tailing of the ion signal to many tens of microseconds suggests a slower cooling process possibly related to melting and solidification of the matrix.

The estimated temperature increase can be compared with similar reports in the literature. Temperatures between 600 and 800 K have been estimated to be sufficient to produce alkali metal positive ions by thermionic emission after IR laser irradiation of a solid sample.\textsuperscript{8} Because sodium positive ions are not observed with the IR pulse alone under the conditions used above for IR/UV MALDI, this suggests that the maximum surface temperature in these experiments does not exceed 600 K after the IR laser pulse. The velocity distribution of neutral molecules
desorbed by 10.6 µm laser pulses at 1000 J/m² indicates a temperature of 350-450 K. The IR fluence used in the IR/UV MALDI was 6000 J/m², suggesting that the sample temperature may have exceeded 400 K. The melting point of DHB is 473 K, therefore it is likely that the matrix is at or near its melting point after being irradiated with the IR pulse. This is consistent with the calculated $\Delta T_{surf}$ if one quarter of the IR laser energy is absorbed by the substrate.

4.3.2. 2.94 µm OPO / 337 nm N₂ Two-laser Configuration

In addition to the two-laser experiments using the CO₂ laser as the IR source, experiments were also carried out with the OPO tuned to 2.94 µm. In this configuration, two-laser LDI and MALDI was tested with four MALDI matrices and the analytes gramicidin S, bradykinin, bovine insulin and cytochrome C. Two-laser analyte ion signal was obtained with DHB and SA; no two-laser matrix or analyte ion signal was found for samples prepared with CCA matrix. With the NA matrix, a large matrix ion signal was observed for a matrix fragment, but no two-laser analyte ion signal was observed. In general, analyte signal was lower and more difficult to reproduce for peptides compared to proteins.

Mass spectra for bovine insulin ($M_r = 5733.5$ Da) in DHB matrix are shown in Figure 4-5. The UV laser fluence is 55% of threshold, and the IR laser fluence is at threshold. At a delay of -100 ns (Figure 4-5a; UV fired before IR), matrix and analyte ion signal result from the IR laser only. At delay times between zero and several hundred nanoseconds, matrix and analyte ion signal are significantly larger. A typical two-laser mass spectrum obtained at a delay of 200 ns is shown in Figure 4-5b; this mass spectrum is indicative of those obtained between 0 and 400 ns. One-laser mass spectra similar to the two-laser mass spectrum in Figure 4-5b can be obtained if either the IR or the UV laser fluence alone is increased above the threshold fluence.

Figure 4-6 shows the mass spectra of bovine insulin in SA matrix. The UV laser is at
threshold, and the IR laser fluence is at the threshold to produce matrix ions. No analyte ions were produced at any IR laser fluence using SA matrix and one-laser ionization. At a delay of -100 ns, matrix ion signal in Figure 4-6a is from the UV and IR lasers, and analyte ion signal is from the UV laser only. Figure 4-6b shows a mass spectrum obtained at 200 ns that is indicative of the two-laser mass spectra obtained between 0 and 400 ns. The SA two-laser mass spectrum is similar to the DHB two-laser mass spectrum, with the exception that the analyte cluster signal is significantly lower in the former case. A UV one-laser mass spectrum similar to Figure 4-6b can be obtained if the UV laser fluence is increased, but, as indicated above, no IR one-laser mass spectrum could be obtained.

Figure 4-5. Mass spectra of bovine insulin in DHB matrix obtained with an IR and UV laser at a) $\Delta t = -100$ ns and b) $\Delta t = 200$ ns.
Figure 4-6. Mass spectra of bovine insulin in SA matrix obtained with an IR and UV laser at a) \( \Delta t = -100 \) ns and b) \( \Delta t = 200 \) ns.

In some cases, the two-laser signal was steady over time and plots of integrated signal intensity as a function of time could be obtained. In Figure 4-7, the peak area for the \([M+H]^+\) ion of bovine insulin in SA matrix is plotted as a function of delay. The non-zero baseline is due to detector noise and does not indicate analyte ion signal at negative delay times. Two-laser analyte ion signal is observed at zero delay and continues at approximately the same intensity to 400 ns and then drops to baseline after 700 ns. The higher baseline after 700 ns does not appear to be associated with analyte ion signal and may be caused by thermionic emission or gas phase UV ionization of neutrals ejected from the target by the IR laser. In contrast to SA, it was not possible to obtain reproducible delay plots for bovine insulin in DHB matrix. The large variation
in the DHB ion signal may be due to the higher IR laser fluence that was required to obtain two-laser signal.

![Graph](image.png)

**Figure 4-7.** Ion signal as a function of delay time for bovine insulin in SA matrix from -300 to 900 ns in 100 ns increments. Error bars represent one standard deviation.

Two-laser ion signal was observed for cytochrome C (M_r = 12,384 Da) in DHB matrix as shown in Figure 4-8. Results are similar to bovine insulin in DHB matrix (Figure 4-5). Figure 4-8a was obtained by adjusting the delay such that the two-laser signal was at a minimum yet the IR and UV laser fluence was sufficient to achieve two-laser MALDI. At these fluences, it was not possible to entirely eliminate the one-laser signal. The mass spectrum in Figure 4-8b was obtained at 200 ns and is indicative of the two-laser signal observed at positive delay times. Similar mass spectra to Figure 4-8b can be obtained for one-laser IR and UV MALDI at higher laser fluences.
The NA matrix was unique in that the one-laser and two-laser matrix mass spectra were different. Figure 4-9 shows mass spectra for 4-nitroaniline (\(M_r = 138.1\) Da). The UV laser fluence is 20 % of threshold. No one-laser IR LDI signal was detected for NA at any IR laser fluence; therefore, the IR laser energy was attenuated to the minimum energy necessary for two-laser LDI at 50 ns. At a delay of -10 ns, no matrix or analyte ion signal is observed (Figure 4-9a). At a delay of 50 ns, a mass spectrum with a single peak corresponding to the protonated fragment of 4-nitroaniline after the loss of NO (Figure 4-9b). When the UV fluence is increased to threshold, a different mass spectrum is obtained. The one-laser UV LDI mass spectrum for NA is shown in Figure 4-9c, which was obtained at the one-laser threshold fluence. The most
intense peak is the protonated NO loss fragment, but there are peaks corresponding to the NH$_2$ loss fragment, the protonated matrix and a fragment dimer at $m/z$ 217. The [M-NO+H]$^+$ signal was intense and reproducible and was used to obtain the delay plot shown in Figure 4-10. The signal rises rapidly beginning at zero delay to a maximum near 100 ns and then returns to baseline after approximately 500 ns.

**Figure 4-9.** Mass spectra of 4-nitroaniline matrix obtained with an IR and UV laser at a) $\Delta t = -10$ ns, and b) $\Delta t = 50$ ns and c) a UV laser alone.
Figure 4-10. Ion signal as a function of delay time for 4-nitroaniline matrix from -70 to 950 ns in 10 ns increments. Error bars represent one standard deviation.

In the previous section, it was postulated that the function of the IR laser in two-laser ionization is to heat the sample, which allows UV desorption ionization at lower laser fluences. With a larger number of matrices tested and an IR wavelength that is more strongly absorbed by the sample rather than the substrate, additional details about the two-laser IR/UV process are revealed. It is unlikely that excited electronic state processes are important in two-laser IR/UV LDI as they are in two-laser and two-pulse UV/UV LDI.\textsuperscript{42-44} It is possible that the IR laser heats the sample to its melting point: the melting points for the solid matrices used in this study range from 421 to 537 K.\textsuperscript{83} Temperatures of 600 - 800 K are normally sufficient to produce alkali metal ions by thermionic emission, as stated before.\textsuperscript{8} The presence of sodium positive ions in the IR LDI mass spectra at two-laser ionization fluences suggests that the temperature following IR
irradiation is sufficient for matrix melting. The lack of observed two-laser ionization with the CCA matrix may be due to the relatively low absorption of that matrix at 2.94 µm. A thin film of CCA has an IR absorption peak at 3.02 µm and a relatively low absorption at 2.94 µm.\textsuperscript{86} In contrast, SA, NA and DHB have a comparatively larger absorption at that wavelength.

The time behavior of the two-laser LDI is consistent with the one-dimensional heating model presented in the previous section that used a CO\textsubscript{2} laser as the IR source. That model predicts that the maximum target temperature will occur at the end of the IR laser pulse. In the CO\textsubscript{2} laser study, the full pulse is 900 ns, which coincides with the maximum in the two-laser signal for both matrix and analyte. In the current study, the IR laser pulse width is 5 ns and ion signal was observed to be coincident with the end of the IR pulse within the 40 ns jitter in the delay measurement. The drop in two-laser ion signal is interpreted as reflecting the drop in the surface temperature after the IR laser heating. In the previous section, the time for the return to baseline signal was between 100 and 300 µs; in the current study, the return to zero occurs within 1 µs. This difference could be due to the relatively low matrix absorption at 10.6 µm as compared to 2.9 µm. In the former case, the laser penetrates the thin matrix film and heats the metal substrate whereas in the latter case the energy is absorbed primarily by the thin analyte film. The bulk metal will cool more slowly than the thin matrix film.

An alternate explanation for the rapid return to baseline is that the UV laser is interacting with the expanding plume of material desorbed by the IR laser. Given a 500 m/s plume velocity, the UV laser would be expected to interact with ablated material for 100 ns or more after the IR ablation.\textsuperscript{42} As the plume moves out of the UV laser field of view, this plume irradiation will no longer be possible. However, the flight times of the matrix and analyte ions formed by two-laser MALDI are constant to better than 0.2%, suggesting that they are formed at the surface rather
than in the gas phase above the target. The anomalous two-laser mass spectrum for NA might be better explained by its high vapor pressure compared to the other matrices which could lead to a dense plume after IR laser heating. A high collision number in this dense plume could explain the high degree of fragmentation in the two-laser mass spectrum.

4.4. Summary

The two-laser IR/UV MALDI results presented in this chapter show that the efficiency of UV MALDI can be increased by first irradiating the sample with an IR laser pulse. For the CO₂/N₂ two-laser combination, the yield of matrix and analyte ions is at a maximum approximately 1 µm after the IR laser is triggered and remains at this level for several tens of microseconds before decaying to zero within several hundred microseconds. The results are consistent with IR heating of the matrix to between 400 and 600 K, after which a lower than otherwise possible UV laser pulse energy can be used to create ions. As the substrate cools, the UV MALDI efficiency declines to its room temperature value. Although it might be possible to ionize some analytes with a static heated substrate, most biomolecule analytes would not survive the heating process intact. Additionally, the temperature increase induced by the IR laser can be selected on a shot-by-shot basis simply by changing the laser attenuation. Two-laser MALDI MS studies using an OPO laser operating at 2.94 µm for the IR source and a 337 nm nitrogen laser for the UV source show two-laser matrix and analyte signal for DHB and SA matrices but not for CCA matrix. Two-laser mass LDI signal was observed for NA matrix, but the mass spectrum is not the same as the one-laser UV LDI mass spectrum. The two-laser signal occurs when the IR and UV lasers are fired simultaneously and can be obtained for delay times between the IR and UV laser of up to 500 ns. The results are interpreted as rapid heating and possibly melting of the matrix followed by efficient UV MALDI from the heated sample. There is also some indication
of UV ionization of NA molecules in the desorption plume.

The ability to manipulate the ionization of a MALDI sample on a transient and shot-by-shot basis using the two-laser technique presented in Chapter 4 has interesting implications both for the study of MALDI ionization mechanism as well as for analytical applications. There is currently an intense debate over the role of clusters in MALDI ionization and the relative contributions of thermal and photomechanical processes. With IR laser heating, it may be possible to selectively traverse the sublimation and volume ablation regimes in order to manipulate the size and internal energy of ejected clusters and the ratio of clusters to free molecules. The ability to manipulate the initial temperature of the sample on a shot-by-shot basis may also be useful for selective fragmentation of labile analytes or for reducing the effects of cluster or adduct formation. Furthermore, it may be possible to selectively enhance ionization for different classes of analytes using the proper matrix and pre-heating conditions. Additional analytical applications of IR/UV two-laser MALDI include systems in which the analyte is deeply embedded in a supporting material such as proteins separated in one and two-dimensional gels (Chapter 5), whole cells, and tissue. In these cases, it may be possible to combine the efficient material removal of IR laser desorption with the more efficient ionization of UV MALDI.
CHAPTER 5. DIRECT FROM POLYACRYLAMIDE GEL INFRARED SOFT LASER DESORPTION/IONIZATION*

5.1. Introduction

In this chapter, an application of infrared soft laser desorption and ionization is presented. Peptides and proteins are directly ionized from a polyacrylamide gel after electrophoretic separation in a gel slab and a microfluidic chip fabricated from polymethylmethacrylate (PMMA). The additional sample processing steps of extraction of the protein from the gel, blotting the protein onto a membrane, freezing the gel or addition of an exogenous matrix prior to mass spectrometry analysis were avoided. Ionization of analytes at the wavelength of 2.94 or 2.95 µm was observed at room temperature using the O–H stretch vibrational absorption of the gel and the water that remains in the gel after vacuum drying. The approach is a novel method for analyzing electrophoretically separated biomolecules using mass spectrometry.

5.2. Experimental

For direct IR LDI of gel slabs, the OPO was tuned to 2.94 µm and focused onto the sample to a spot size of approximately 200 by 300 µm. At this spot size, IR fluences ranged from 1000 to 5000 J/m². Because a large fraction of the laser shots did not provide sufficient signal, mass spectra were acquired by averaging mass spectra selected by the computer. Single shot spectra in which peaks in the mass range of the analyte were larger than 40 mV were summed, while the spectra with signals lower than this were rejected. The sample target was

continuously moved to provide a fresh area of sample for each laser shot. Here, signal loss may be due to ablation of the gel, chemical modification of the gel, desorption of the sample or desorption of the solvent. When operated in this manner, approximately 10% of the laser shots were summed. Mass spectra shown below are the summed average of five selected single laser shot mass spectra unless otherwise indicated.

For direct IR LDI of microfluidic gel chips, mass spectra were obtained in either manual or automated acquisition modes using the OPO tuned to 2.95 µm. Mass spectra were obtained manually by continuously moving the laser down the length of the channel while observing the fluorescence of the target on a video camera. Spectra were saved after 5 to 10 laser shots that generated analyte signal on the oscilloscope. Alternatively, mass spectra were obtained in automated mode by moving the chip under the laser beam using a micrometer drive and saving summed data every 5 laser shots for the entire length of the gel chip channel. The chip was moved at a rate of 1.3 mm/min and mass spectra were averaged over 5 laser shots with 50,000 data points and a flight time resolution of 1 ns per data point. The mass spectra were downloaded and stored as the experimental data collection progressed in real time. Fifty to 100 spectra were downloaded over the course of a typical experiment.

5.3. Results and Discussion

5.3.1. Direct IR LDI of Gel Slabs

The significance of sufficient optical absorption of the matrix at the laser wavelength has been recognized since the early days of MALDI.\textsuperscript{16,13} As a general rule, spectrum quality increases with absorption\textsuperscript{89}, although, once the absorption exceeds a certain value, further improvements cannot be obtained.\textsuperscript{33} Qualitatively, the best mass spectrometric performance has been obtained at wavelengths near the absorption maximum of the matrix. In this study, the
material acting as the matrix is either the gel or water held within the gel that is not completely removed by vacuum desiccation. In order to determine the relative contributions of gel and water absorption, the IR absorption spectrum of the polyacrylamide gel was investigated.

Figure 5-1 shows the FT-IR ATR spectrum of a dry 16.5% sodium dodecyl sulfate polyacrylamide gel (black solid line) taken at room temperature. As with the mass spectrometric analysis, the gel was dried under vacuum for 30 minutes before the IR spectrum was obtained. The IR spectrum of a wet gel (dashed line) is shown for comparison. The FT-IR ATR spectrum of the wet gel is similar to the FT-IR transmission spectrum of wet polyacrylamide gel previously reported. The broad peak centered around 3 μm contains the N−H and O−H stretching modes of acrylamide gel and water, respectively. The peak centered on 6 μm contains the C=O and C−N stretching modes and the N−H deformation mode of the gel and the O−H bending mode of the gel and water. The ratio of the 6 μm to 3 μm peaks in the spectra suggest that the vacuum dried gel still contains a significant fraction of water but with relatively high molar ratio of the gel to the water.

![FT-IR ATR spectra of dry (black solid line) and wet (dashed line) polyacrylamide gel.](image)

**Figure 5-1.** FT-IR ATR spectra of dry (black solid line) and wet (dashed line) polyacrylamide gel.
Initial mass spectrometry experiments were carried out with desorption and ionization of analytes deposited on polyacrylamide gels without the addition of matrix. The IR LDI mass spectra of bradykinin and bovine insulin deposited on top of the gels are shown in Figure 5-2. The base peaks in the mass spectra are protonated bradykinin (Figure 5-2a) and protonated bovine insulin (Figure 5-2b). The bradykinin mass spectrum contains intense low mass peaks associated with Na\(^+\) and K\(^+\) as well as additional peaks below 200 \(m/z\) that are most likely associated with gel components. The peak marked with an asterisk is 220 Da above the base peak in Figure 5-2a and could not be assigned to an adduct of any intact component of the gel with the bradykinin. In Figure 5-2b, there is an unresolved low mass background near \(m/z\) 2000 that may be due to the gel or to water clusters.

In order to evaluate the ability of the direct combination of gel electrophoresis and laser desorption/ionization mass spectrometry, bradykinin and bovine insulin were separated in a 16.5\%T polyacrylamide gel. An image of a gel run under the conditions used to obtain the mass spectra is shown in Figure 5-3. Tricine-SDS-PAGE allows the resolution of small proteins at lower acrylamide concentrations than glycine-SDS-PAGE.\(^5\) Using the Tris/Tricine system with 16.5\%T/3\%C narrow-pore size separation gels, bradykinin, bovine insulin, insulin A chain and insulin B chain migrate as resolved bands. The polypeptides were subsequently visualized using a Coomassie Brilliant Blue stain. The first lane contains the molecular weight size standards at 1.4, 3.5, 6.5, 14.4, 17.0 and 26.6 kDa (\textit{vide supra}). The second lane contains bradykinin and the third lane bovine insulin. The fourth lane contains bovine insulin with β-mercaptoethanol reducing agent. Note that when the reducing agent is added, both the insulin A chain and B chain are observed in the gel but the intact insulin molecule is not.
Figure 5-2. IR (2.94 µm) LDI mass spectra of a) bradykinin and b) bovine insulin deposited on a polyacrylamide gel with no added matrix.
Figure 5-3. Separation on a 16.5%T polyacrylamide gel of molecular weight standards (Lane 1), bradykinin (Lane 2), bovine insulin (Lane 3), and reduced bovine insulin (Lane 4).

Mass spectra were obtained by direct desorption and ionization of the analyte from the gels with no added matrix. Figure 5-4a shows the IR-LDI mass spectrum of bradykinin in the gel, corresponding to Lane 2 in Figure 5-3. The largest mass spectral peak associated with the analyte is the sodium cation adduct, denoted [M+Na]⁺ in Figure 5-4a. Intense Na⁺ and K⁺ peaks are observed in the low mass region. An intense peak centered on m/z 95 may be associated with glycerol. Between m/z 400 and the bradykinin [M+Na]⁺ peak, additional interference peaks, including a number of peaks separated by 12 mass units are observed. These peaks may result from fragmentation of the Coomassie dye. The largest analyte ion observed in this work was bovine insulin (Mᵣ = 5733.6); a mass spectrum of bovine insulin using IR-LDI from gels is shown in Figure 5-4b. The spectrum is characterized by large protonated molecule peak. The peaks marked with an asterisk are separated from the protonated insulin by approximately 800 m/z and are tentatively assigned as adducts of insulin with the Coomassie dye. Interfering peaks
below 1800 m/z may also be associated with the dye, possibly clustered with other gel components.

Figure 5-4. Infrared laser desorption/ionization mass spectra of a) bradykinin and b) bovine insulin following gel elution.
To denature the native protein conformation and improve the performance of the gel separation, a reducing agent is often used. A standard procedure involving the use of 5% β-mercaptoethanol during electrophoresis was used with bovine insulin. The results of this procedure are shown in Lane 4 of Figure 5-3. Here, the insulin A and B chains are clearly separated (Mr = 2239 and 3495 respectively). The 2.94 μm IR-LDI mass spectrum obtained from this separation is shown in Figure 5-5, which is the average of 10 single laser shot mass spectra. The mass spectrum of the insulin B chain shows an intense [M+H]^+ peak with a small adduct with acrylamide. (Mr = 71). The spot corresponding to the insulin A chain was not tested due to the low ionization efficiency of that peptide. The result shown in Figure 5-5 demonstrates the advantage of laser desorption mass spectrometry directly from the gel for identification of chemically modified components.

![Figure 5-5.](image)

**Figure 5-5.** Infrared laser desorption/ionization mass spectrum of bovine insulin B chain following reduction and gel elution of insulin.
Previous researchers have reported a lack of success at IR-LDI and IR-MALDI directly from a gel under conditions that are similar, but not identical to those reported above. Hillenkamp and co-workers were unable to desorb proteins directly from a gel using IR-MALDI.\textsuperscript{91} However, proteins could be ionized efficiently by IR-MALDI after electroblotting. All of the analytes tested in the IR-MALDI electroblotting work were larger than 10 kDa in mass and the quantities used for electrophoresis were between 1 and 10 pmol per lane. These previously reported results are consistent with our observation that nmol quantities of analyte per electrophoresis lane were required to obtain mass spectra. Haglund and co-workers were unsuccessful at obtaining IR-LDI mass spectra from gels at 2.9 µm, but were successful at 6 µm.\textsuperscript{24} In these experiments, the gels were cooled to 105 K rather than dried as in the data reported above. The quantity of analyte loaded on the gel was in the range of 10 nmol per lane. The fact that direct from gel ionization was successful for dried gels and not for wet ones suggests that the removal of water is essential for obtaining ionization at 2.9 µm. This is consistent with previous reports of IR-MALDI using the waters of hydration of lyophilized proteins as the matrix; it was found that water ice does not function well as a matrix due to its propensity for forming large protonated clusters.\textsuperscript{21}

5.3.2. Direct IR LDI of Microfluidic Gel Chips

In the study described in the section above, IR LDI mass spectrometry of peptides and proteins separated using conventional SDS-PAGE was performed using an 80 x 73 x 0.75 mm gel. A visualization dye was necessary to locate the position of the migrated analyte. Once located, the spot was excised and attached to the sample target using double-sided conductive tape. A wavelength of 2.94 µm from the OPO was used to desorb and ionize intact peptides and proteins without the addition of a matrix. In the method described in this section, the entire gel
chip is loaded into the mass spectrometer and irradiated by the IR laser. No visualization dyes are necessary and the size of the gel is greatly reduced. With the exception of the visualization dye and its solvents, the materials are identical to the previous section.

All of the capillary gel microfluidic mass spectra were obtained after the analytes had been electrophoretically transported through a closed chip channel. Operation with a closed channel requires that the PDMS cover be removed from the PMMA microfluidic chip prior to insertion of the chip into the mass spectrometer. When removing the PDMS cover from the PMMA chip, sections of the gel tended to adhere to the surface of the PDMS. With some amount of care the entire gel lane could be extracted intact from the chip and initial mass spectra were obtained by mounting the PDMS cover in the mass spectrometer. Figure 5-6a shows the IR-LDI mass spectrum of the peptide bradykinin (M_r = 1060.2) desorbed and ionized from the PDMS cover. The bradykinin was loaded into the chip at a concentration of 2.5 mM and injected into the chip channel using a field of 150 V/cm for 9 min. The spectrum is the result of 10 laser shots at 2.95 µm wavelength. The base peak of the spectrum is assigned to the singly-protonated bradykinin molecule, [M+H]^+. The sodium adduct, [M+Na]^+, is present in the spectrum, but at low intensity; less than 5% as intense as the protonated peptide. An adduct of acrylamide with bradykinin was observed 71 m/z to the high m/z side of the base peak. Several peaks were observed below m/z 400 that were tentatively assigned to ionized gel components. No visualization dyes were used, which may account for the lower number of peaks in the low m/z region as compared to the results in the section above.

Although IR-LDI from the PDMS resulted in excellent mass spectra, it was difficult to obtain an intact gel along the full length of the channel. Furthermore, the flexible cover could not
Figure 5-6. Mass spectra of bradykinin in a gel microfluidic chip obtained by 2.95 µm infrared laser desorption/ionization of a) the PDMS cover and b) the PMMA microfluidic chip.
be easily translated with respect to the laser desorption spot, which prevented interrogation of the full length of the gel. In some cases, it was possible to remove the PDMS cover and leave the gel intact in the channel. Laser desorption/ionization from the PMMA gel microfluidic chip is shown in Figure 5-6b. The sample loading and electrophoresis conditions were identical for Figure 5-6a and 5-6b. The spectra in Figure 5-6 are similar in that the protonated bradykinin is the base peak and the low m/z region of the mass spectrum is nearly free of interfering peaks. However, the analyte signal is approximately ten times lower in Figure 5-6b and the intensity of the acrylamide adduct peak is nearly identical to the [M + H]⁺ peak intensity. By observing the ablation of the gel using the video camera and later inspection of the irradiated gel, the depth ablated by each laser shot can be estimated at approximately 50 µm. Because the laser is continuously moved across the surface of the gel, the entire volume of gel in the 150 µm deep microchannel is not removed by the IR laser. The OPO penetration depth together with the better quality of the mass spectra obtained from the PDMS cover slip suggests that the analyte is migrating to the bottom of the gel channel and is more accessible to the desorption laser when the gel is removed from the channel. Another possibility is the difference in the ion extraction field between the recessed gel channel and the protruding gel on PDMS cover slip, which may affect the efficiency of ion extraction.

An estimate of the detection limit for the microfluidic gel chip was obtained using a 100 µM bradykinin solution loaded onto the chip using 150 V/cm for approximately 9 min. and electrophoretically driven through the microfluidic chip channel. The mass spectrum corresponding to this injection is shown in Figure 5-7 and represents the minimum quantity of peptide required to obtain a mass spectrum from the gel chip channel. When the quantity of analyte is reduced, the ratio of [M+H]⁺ to the acrylamide adduct drops. The relative intensity of
the $b_8$ fragment ion at m/z 906 is also proportionally larger, possibly due to the higher laser energy used with the lower concentration sample. The volume of the channel intersection is approximately 10 nL. If it is assumed that the volume is filled with analyte solution, then a 1 pmol quantity of analyte was injected. This represents greater than 1000 fold improvement over the detection limit for the gel slab work presented in the previous section. This estimate does not include the volume of the channel displaced by the gel medium (which would decrease the estimate) or the possible effects of stacking in loading the channel (which would increase the estimate).

![Mass Spectrum](image.png)

**Figure 5-7.** Single shot 2.95 µm infrared laser desorption/ionization mass spectrum of 1 pmol bradykinin injected into the gel channel.

The IR-LDI mass spectra of bovine insulin electrophoretically run through the gel microfluidic chip is shown in Figure 5-8. The concentration of the solution that was loaded onto the chip was 2.5 mM and the material was injected into the channel using 150 V/cm for 9 min. The base peak in the mass spectrum is assigned to the singly, protonated insulin molecule
An adduct of acrylamide with bovine insulin molecule is observed as well as several other adducts in a small cluster of peaks centered around 6100 Da. The peaks at \(m/z\) 6037, 6110, and 6178 are most likely adducts of SDS (\(M_r = 288.5\)), SDS+acrylamide (\(M_r = 359.6\)) and SDS+bisacrylamide (\(M_r = 442.7\)), respectively. As is typical of spectra obtained with no added matrix, there are few peaks in the \(m/z\) region below 500 Da. Insulin was the largest mass protein that could be reliably detected on the gel chip. Mass spectra of the protein cytochrome C (\(M_r = 12,362\)) could be obtained with difficulty, but the mass-resolving power and signal were too poor for absolute assignment of peaks (Figure 5-9).

![Mass Spectrum of Bovine Insulin](image)

**Figure 5-8.** Infrared laser desorption/ionization mass spectrum of bovine insulin injected into a capillary gel chip.

A separation of the bradykinin and bovine insulin was accomplished in a gel microfluidic chip and the chip was analyzed in an automated fashion in the mass spectrometer. The chip was driven by the motor at a rate of 150 \(\mu\)m/min and mass spectra were recorded continuously by the
oscilloscope and downloaded to the computer in real time. A total of 78 mass spectra were recorded and these are represented by the two dimensional contour plot in Figure 5-10.

![Mass Spectrum Image](image)

**Figure 5-9.** Infrared laser desorption/ionization mass spectrum of cytochrome C injected into a capillary gel chip.

The x-axis in Figure 5-10 corresponds to ion flight time whereas the y-axis corresponds to distance scanned on the chip channel. The m/z scale is indicated at the bottom of the plot.

Intensities in the mass spectra are represented by grayscale shade: dark regions correspond to high intensities and light areas correspond to low intensities. Peaks from the gel in the m/z region below 500 appear as vertical stripes between 2 and 5 µs flight time. Because these peaks appear in all mass spectra, regardless of the presence of analyte, their intensity gives an indication of the baseline ion signal at a given point on the gel channel. It can be seen that the signal between 0 and 6 mm is good, but then drops off. This may be due to the gel being partially removed from the channel in this region. The bradykinin is observed at a flight time of 17 µs and the insulin at a flight time of 40 µs. Both bands are spread out over several mm, probably due to the analytes
sticking on the channel wall as they migrate through. The bradykinin is observed from 1 to 3 mm and the insulin is highest in intensity between 0 and 2 mm. The substantial EOF as well as potential interactions between the polypeptides and the channel walls can induce band spreading over the distance of the microchannel. The effect of solute-wall interactions and a biopolymer’s electoosmotic flow has been reported for DNA separations in polymer microchips.\textsuperscript{92-95}

Dispersion of the analytes over a large distance in the microchannel translates into less material under the laser at any given position, which adversely affects the achievable detection limit.

**Figure 5-10.** A two-dimensional representation of a 2.95 µm laser desorption/ionization mass spectrum scan of a gel microfluidic chip loaded with a mixture of bradykinin and bovine insulin. The chip was translated past the laser and the mass spectra were recorded automatically.

### 5.4. Summary

The direct coupling of 2.94 µm infrared laser desorption/ionization mass spectrometry with polyacrylamide gel electrophoresis with no added matrix has been demonstrated in this
chapter. Because this method did not require the addition of matrix, many of the intermediate processing steps were avoided, and the method provided potential advantages in speed and reduced complexity. Although mass spectra of peptides and proteins have been obtained directly from gels, the practical application of this technique was restricted by the limit of detection. It was necessary to load nanomole quantities of peptide and protein analytes into each band for a successful analysis. In a typical gel electrophoresis analysis, the amount of proteins loaded per gel band is a factor of 10 or more lower. On the other hand, the amount of gel required for IR LDI analysis was relatively small since the laser irradiates a small fraction of gel band.

The key to minimizing sample consumption and optimizing the limit of detection was to move the gel separation to a microfluidic chip format. IR LDI from a capillary gel microfluidic chip has been demonstrated using a 2.95 µm pulsed infrared laser and no added matrix. The gel chips were machined in a simple tee structure from PMMA and covered with an annealed PDMS cover. After electrophoresis, the PDMS cover was removed and the gel inserted into vacuum and ablated with the pulsed laser to form ions. The gel could be analyzed when attached either to the PDMS cover or in the PMMA chip. Ionization from the PDMS resulted in approximately 10 times larger ion signal, possibly due to the greater exposed area resulting from the removal of the gel from the microfluidic channel. Excellent mass spectra were obtained from pure mass standards bradykinin and insulin using pmol quantities of material injected in the gel channel. The mass range for this approach was limited to less than 10 kDa. An automated analysis of an off-line separation shows broad electrophoretic bands, suggesting that the analyte was sticking to the polymer as it migrated through the chip.
CHAPTER 6. CONCLUSIONS AND FUTURE DIRECTIONS

6.1. IR Soft LDI MS

In this dissertation, two objectives were presented with the goal of answering questions about the desorption/ionization process in IR soft LDI and demonstration of a unique application of the IR soft LDI analytical method. Successful completion of these objectives will contribute more information to studies already completed in the mass spectrometry community to improve upon the IR soft LDI method for the analysis of proteins. Knowing more about the fundamental aspects of the desorption/ionization process could lead to improvements in analytical performance criteria such as mass accuracy, mass-resolving power and sensitivity. Development of novel applications using IR soft LDI opens up new ways of analyzing proteins which could further the field of proteomics.

The most promising result of these studies is the use of intrinsic matrix materials for ionization. As shown in the wavelength studies in Chapter 3, soft ionization of proteins is possible without an added matrix using a mid-IR wavelength that is absorbed by the analyte. The implications of this study include simplified sample preparation procedures and the removal of interfering matrix ion signal from the mass spectrum. With the greater penetration depth of the IR laser compared to UV lasers and the direct ionization of the sample material without a matrix, a better way to analyze samples is possible. The direct-from-gel application presented in Chapter 5 is one example. Molecules present in the gel material are sufficient to absorb the mid-IR laser light and allow soft ionization of peptides and proteins without the addition of a matrix. The laser can also penetrate deep into the sample to desorb and ionize the analytes embedded in the gel. The nanomole sensitivity of the gel slab experiments was greatly improved by moving to a microfluidic chip format, however, band broadening in the microfluidic chip limit further
sensitivity improvements. Optimization of electrophoretic separation parameters and replacing the plastic chip with a glass chip would reduce the band broadening and concentrate more analyte into a smaller band.

The analytical applications of the two-laser studies are less obvious; however, a few implications of the technique can be interfered. The ability to manipulate the ionization of a MALDI sample on a transient and shot-by-shot basis could change the size and internal energy of ejected clusters and the ratio of clusters to free molecules. Systematically changing cluster formation from desorption can provide information about the laser desorption/ionization process. Experiments have been designed using a particle sizer to obtain this information.\textsuperscript{96,97} It also might be possible to selectively fragment labile analytes or reduce the effects of cluster or adduct formation. Furthermore, it may be possible to selectively enhance ionization for different classes of analytes using the proper matrix and pre-heating conditions.

6.2. Future Directions

In addition to direct-from-gel IR LDI applications, soft ionization of peptides and proteins in cell and tissue samples is possible without adding a matrix. Information regarding the identity, location and functionality of proteins in these samples can be obtained by combining IR soft LDI with a technique called MS tissue imaging.\textsuperscript{98} This technique involves rastering the laser used for analysis over an entire cross section of tissue section looking for potential biomarkers. Biomarkers are proteins, drugs and/or metabolites that appear throughout the tissue whose location might indicate a functionality of the protein in response to a biological process. Color-coding the ion signal of the biomarkers provides an image map of the biomarkers location. In order to produce a highly resolved image map, the laser spot size must be focused to a size small enough to achieve the desired spatial resolution. This fact coupled with the size of the tissue
cross section would require many tens of thousands of laser shots to complete the experiment, a very time consuming analyses with the low repetition rate lasers found in most MALDI mass spectrometers.

Most MS tissue imaging studies using the MALDI technique, currently, employed high repetition rate UV lasers to speed up the analysis time required for tissue imaging. Two problems arise with the use of high repetition rate UV lasers for MALDI imaging analyses: the shallow penetration depth of the UV lasers and spatial relocation of sample constituents after matrix deposition. Many of the proteins embedded in the sample being analyzed are located hundreds of nanometers below the surface. UV lasers can only penetrate a few nanometers into the sample due to the high absorptivity of the MALDI matrix and constituents in the sample. UV MALDI requires the addition of a matrix in order to ionize protein molecules without fragmentation. Adding droplets of matrix in solution to the top of the sample can move sample constituents to other parts of the sample, therefore losing spatial information.

Ongoing research has centered on developing a cheap, tunable mid-IR laser that is capable running at a repetition rate in the kHz range. Also, a custom-built mass spectrometer is being designed to irradiate the target from behind. This arrangement called, transmission geometry, allows the use of a microscope objective lens to focus the laser spot size to a few micrometers in diameter. The high repetition rate laser and the transmission geometry mass spectrometer solve throughput problems and increase spatial resolution. IR lasers can alleviate both of the problems associated with UV MALDI tissue imaging because IR MALDI analyses can be performed without the addition of a matrix and the penetration depth of IR lasers can reach embedded proteins. Other benefits include faster analyses due to reduced sample preparation steps and small molecule analyses due to the absence of low mass matrix ionization.
In addition to application improvements using IR soft LDI without the addition of a matrix, IR/UV two-laser MALDI can also provide new ways of analyzing samples. In future experiments, analytes deeply embedded in a supporting material such as proteins separated in one and two-dimensional gels, whole cells, and tissue will be performed. The idea is to combine the efficient material removal of IR laser desorption with the more efficient ionization of UV MALDI.
REFERENCES


89. Horneffer, V.; Dreisewerd, K.; Lüdemann, H. C.; Hillenkamp, F.; Läge, M.; Strupat, K., Is the incorporation of analytes into matrix crystals a prerequisite for MALDI-MS? A study of


APPENDIX 1. TWO LASER TRIGGER SETUP

Figure A1-1. Schematic of OPO/N_2 laser combination trigger setup. All connections are made with 50 Ω BNC cables. To change the delay between the two laser pulses, increase or decrease the delay of Channel A on the DG-535 #1. The OPO Fire/Charge cable is made up of two 50 Ω BNC cables with BNC female connectors at one end and a 9-pin RS-232 connection at the other. The center of the charge BNC is connected to pin 1 and the shield wire is connected to pin 2. The center of the fire BNC is connected to pin 3 and the shield wire is connected to pin 4.
Figure A1-2. Schematic of CO$_2$/N$_2$ laser combination trigger setup. All connections are made with 50 Ω BNC cables. To change the delay between the two laser pulses, increase or decrease the delay of Channel A on the DG-535.
APPENDIX 2. LETTERS OF PERMISSION

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Mark Warren Little was born in Atlanta, Georgia, on March 16, 1976. He graduated in 1998 with a Bachelor of Science degree in chemistry at Wake Forest University. He also received a minor in mathematics at the same institution. In 1999, he enrolled in the doctoral program for chemistry at Emory University. While at Emory University, he received the Teaching Assistant of Year Award in 2000. In 2001, he transferred to the doctoral program for chemistry at Louisiana State University. His degree professor at Louisiana State University was Dr. Kermit K. Murray. Mr. Little has published three first author publications and four coauthor publications and has presented at nine science conventions under his degree professor. He is a member of the American Society for Mass Spectrometry and the American Chemical Society. Mr. Little is currently a candidate for the degree of Doctor of Philosophy in chemistry, which will be awarded at the December 2006 Commencement.