Photonic and Ablation-based Ionization for the Analysis of Single Cells and Organelles

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Photonic and Ablation-based Ionization for the Analysis of Single Cells and Organelles

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Dedication

In loving memory of my Abuelito,

Gustavo A. Pasquel.

As a chemical engineer, my Abuelito was thrilled when he heard I was going to pursue a doctorate degree in Chemistry. Although he didn’t get to see my dissertation to completion, I know he would be proud.

This dissertation is dedicated to my parents, Marc and Mariana Stolee. I would not be where I am today without their life-long support, love, and encouragement. They instilled in me the values of hard work and integrity, and the confidence that I can accomplish anything that I put my mind to. I am forever grateful for the endless personal sacrifices my parents have made to educate and guide me throughout my life, and couldn’t have done this without them!

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Abstract

Photonic and Ablation-based Ionization for the Analysis of Single Cells and Organelles

A systemic understanding of the dynamic cellular processes in living organisms requires highly sensitive techniques that can rapidly detect and identify diverse biomolecules. Owing to the specificity and sensitivity of mass spectrometric analysis, it has become an indispensable tool for chemical analysis. The metabolic analysis of single cells and subcellular organelles is an emerging field that aims at studying cellular heterogeneity and its effect on the response of an organism to disease and environmental influences. Technical advances in mass spectrometry are required to make significant strides in this field. This dissertation describes my achievements based on photonic nanostructures and mid-infrared laser ablation for the mass spectral analysis of single cells and organelles.

Chapter 1 stresses the importance of single and subcellular analysis and describes how mass spectrometry is being used in metabolomics. An introduction to vacuum and ambient ionization techniques is given, with an emphasis on novel photonic and ablation-based methods.

Chapter 2 presents the use of laser-induced silicon microcolumn arrays, which exhibit nanophotonic ion production in laser desorption experiments, for the adjustable structure specific fragmentation of small molecules. The nanophotonic interactions enable a novel way for peptide activation without the use of an additional step.

Chapter 3 introduces the development of another nanophotonic ion source, nanofabricated nanopost arrays, that are tailored to exhibit resonant ion production.
The effects of changing the dimensions of the posts, particularly the aspect ratio, on the ion yields are discussed in detail. Insight into the nanophotonic processes that play a role in desorption and ionization is also given.

Chapter 4 explores the fundamental desorption and ionization mechanism of nanopost arrays in detail. The effects of surface chemistry and the plane of polarization of the desorption laser on ion yields and fragmentation are studied.

Chapter 5 focuses on the development of an ablation-based ambient ionization technique for the metabolic analysis of small cell populations and single cells. This method is based on the mid-infrared ablation of cells held in a capillary and ionization by an electrospray.

Chapter 6 describes the combination of cell microdissection with laser ablation electrospray ionization for subcellular analysis. Differences in the metabolite distributions between two regions of a cell are discovered.

Chapter 7 evaluates the current state of the art in single and subcellular metabolomics by mass spectrometry. As these scientific innovations will likely face major challenges in the future, suggestions on improvements and future directions to overcome some of these barriers are given.
Table of Contents

Dedication .................................................................................................................. iii
Acknowledgements .................................................................................................... iv
Abstract ...................................................................................................................... vi
List of Figures ............................................................................................................. xiv
List of Tables ............................................................................................................ xvi

Chapter 1. Introduction ................................................................................................. 1

1.1 Overview ................................................................................................................. 1
1.2 Mass spectrometry for metabolomics ................................................................. 2
1.3 Single cell and subcellular analysis ...................................................................... 3
1.4 Advanced ionization techniques ......................................................................... 5
   1.4.1 Laser desorption ionization in vacuum ......................................................... 5
   1.4.2 Ambient ion sources ..................................................................................... 9
1.5 Purpose of this investigation .............................................................................. 12
1.6 References ........................................................................................................... 13

Chapter 2. High Energy Fragmentation in Nanophotonic Ion Production by Laser-Induced Silicon Microcolumn Arrays .................................................................................. 16

2.1 Abstract ................................................................................................................ 16
2.2 Introduction .......................................................................................................... 17
Chapter 3. Tailored Silicon Nanopost Arrays for Resonant Nanophotonic Ion Production

3.1 Abstract ................................................................. 49
3.2 Introduction ............................................................ 50
3.3 Experimental ............................................................ 51
  3.3.1 NAPA fabrication ................................................ 51
  3.3.2 Mass spectrometry ............................................. 52
3.4 Results and discussion .............................................. 52
Chapter 6. Direct Observation of Subcellular Metabolite Gradients in Single Cells by LAESI Mass Spectrometry .................................................131

6.1 Abstract ................................................................. 131

6.2 Introduction ........................................................... 131

6.3 Experimental ......................................................... 133

   6.3.1 Laser ablation electrospray ionization mass spectrometry .......... 133
Biography ........................................................................................................ 170
List of Publications .......................................................................................... 171
Awards .............................................................................................................. 173
Conference Proceedings .................................................................................. 174
Intellectual Property ......................................................................................... 177
List of Figures

Chapter 1

Figure 1.1 Illustration of laser desorption ionization on NAPA ......................... 7
Figure 1.2 Depiction of ambient analysis by LAESI ........................................ 10

Chapter 2

Figure 2.1 Side view image of microcolumns captured by SEM ....................... 24
Figure 2.2 Peptide ion fragmentation in laser desorption from LISMA structures .. 27
Figure 2.3 Fluence dependent survival yields of a preformed ion .................... 37
Figure 2.4 Internal energy distributions for LISMA ...................................... 40

Chapter 3

Figure 3.1 SEM images of NAPA .................................................................... 54
Figure 3.2 Positive and negative mode mass spectra ..................................... 56
Figure 3.3 Ion yield resonances for NAPA ..................................................... 59
Figure 3.4 Mass spectrum and desorption threshold of a preformed ion .......... 63
Figure 3.5 Mass spectrum and survival yield of a preformed ion .................. 66

Chapter 4

Figure 4.1 SEM image of high aspect ratio NAPA ........................................ 81
Figure 4.2 Mass spectra of P14R showing the emergence of fragmentation .... 84
Figure 4.3 Survival yield of a preformed ion as the polarization angle is rotated .. 87
Figure 4.4 Molecular structure of vitamin B_{12} .......................................... 89
Figure 4.5 Ion yields for vitamin B_{12} fragments as a function of polarization .... 92
Figure 4.6 Survival yields of a preformed ion from native and derivatized NAPA .. 96

Chapter 5

Figure 5.1 Schematics of plume collimation and LAESI setups ..................... 110
Figure 5.2 Limit of detection in plume collimation experiments ..................... 113
Figure 5.3 Spectra obtained from small cell populations ............................... 117
Figure 5.4 Spectra from six cells and a single animal cell ............................. 120
Figure 5.5 Images of mid-IR ablation in a capillary .................................... 124

Chapter 6

Figure 6.1 Schematic of the subcellular LAESI setup with microdissection ....... 137
Figure 6.2 Microscope images taken during cell microdissection .................... 139
Figure 6.3 Spectra obtained from a single cell, cytoplasm, and a cell nucleus .... 141
Figure 6.4 Intensities of metabolites detected in the cytoplasm and nucleus ..... 146
Figure 6.5 S-plot obtained from cytoplasm and nuclei .................................. 149
Figure 6.6 Data from organelle extraction and electrospray ionization .......... 154
List of Tables

Chapter 2

Table 2.1 Fragmentation patterns of protonated bradykinin ......................... 30
Table 2.2 Protonated leucine enkephalin fragmentation................................. 32

Chapter 3

Table 3.1 Fragmentation of peptides in laser desorption ionization from NAPA ... 68

Chapter 6

Table 6.1 Tentative assignments for metabolites detected in Allium cepa ........ 144
Table 6.2 Metabolites identified by OPLS-DA data analysis............................. 151
CHAPTER 1

INTRODUCTION

1.1 OVERVIEW

A systemic understanding of the dynamic cellular processes in living organisms requires highly sensitive techniques that can rapidly detect and identify diverse biomolecules. Owing to the specificity and sensitivity of mass spectrometric analysis, it has become an indispensable tool for chemical analysis. The ionization step, in which ions from the sample are produced, is critical in mass spectrometric analysis. Matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) are the two most widely used soft ionization techniques, i.e., they generate intact molecular ions with minimal fragmentation.[1-3] However, because matrix interferences and ion suppression effects limit the use of MALDI for low molecular weight biomolecules, there has been a push for developing matrix-free platforms based on nanostructured materials that can efficiently ionize small molecules, including metabolites, in vacuum. At atmospheric pressure, electrospray ionization is effective for the analysis of liquid samples, but it typically requires some sample preparation and is not amenable to studying the composition of live tissues and cells. Consequently, over the last decade a large number of ambient mass spectrometry methods have been developed. Technical advances in both vacuum and ambient ion sources are required to make significant strides in metabolomics, particularly for applications in single and subcellular analysis.
1.2 MASS SPECTROMETRY FOR METABOLOMICS

Metabolomics experiments require the detection and analysis of a large number of diverse small molecules, or metabolites, that are involved in the biochemical processes of a cell. The identification and quantification of the metabolites in a biological system can give insight into the metabolic pathways affected by external stimuli and stresses, such as diseases, while taking into account the history of the organism and environmental changes. The comprehensive detection of the metabolome, which contains molecules present in a wide range of concentrations and with varying chemical complexity and physical properties, requires an unbiased, highly sensitive, and high-throughput technique. Currently, nuclear magnetic resonance (NMR) and mass spectrometry techniques coupled with separations-based methods are most widely used in metabolomics studies.[4-6] Although NMR enables high-throughput, non-destructive and unbiased chemical analysis, mass spectrometry offers qualitative and quantitative analysis with higher sensitivity, a necessity for volume-limited samples.

Conventionally, various enrichment and separation techniques are used prior to mass spectral measurements. These steps can be time-consuming, require more sample than is available, and may provide biased results. Extensive sample preparation can also lead to alterations in the metabolite profiles and may not provide an accurate snapshot of the metabolome.

There is a large variety of traditional and emerging ionization techniques that are currently being used for metabolomics studies. For example, matrix-assisted laser desorption ionization (MALDI) has been used to analyze lipid profiles and image exogenous and endogenous metabolites in tissue sections.[7, 8] While conventional MALDI is performed in vacuum, atmospheric-pressure infrared MALDI enables chemical imaging of metabolites in the ambient environment.[9] However, interferences from the use of a matrix make it difficult
to analyze and quantify the low molecular weight metabolites. Matrix-free vacuum techniques, based on nanostructured surfaces, overcome this challenge and have been used to analyze metabolites in biological samples with high sensitivity.[10-13] Samples in solution, such as cell extracts and biofluids, can be analyzed without separation or the application of a matrix by electrospray ionization mass spectrometry.[14] The emergence of ambient ion sources, triggered by desorption electrospray ionization (DESI) [15] in 2004, has enabled the direct analysis of biological samples, including tissues and cells, with no sample preparation steps, in their native environment. Applications include the identification of potential biomarkers in lung cancer by urine analysis, the direct analysis of Torpedo californica electric organ to study metabolites involved in electric synapses, and the detection of ovarian cancer by the metabolic profiling of blood.[16-18]

1.3 SINGLE CELL AND SUBCELLULAR ANALYSIS

Single cell studies give insight into biological variability and metabolic phenomena in heterogeneous cell populations. Chemical transport in a cell is affected by molecular crowding and active transport, resulting in the heterogeneous distribution of metabolites within subcellular domains. The metabolic analysis of both single cells and their organelles is required to fully understand the growth, function, and response of a biological system.

Separation-based methods, particularly capillary electrophoresis, are commonly used to separate compounds from single cells or subcellular fractions prior to detection by fluorescence, electrochemistry, or mass spectrometry, among others.[19] Fluorescence microscopy and spectroscopy, based on fluorescent labeling, is highly sensitive and has been used to follow certain metabolite distributions in single cells and organelles.[20, 21] Electrochemical approaches are
also being applied for single cell analysis, for example, to understand exocytotic behavior of individual cells.[22, 23] Although sensitive, these methods can only provide information on a select number of targeted metabolites in any given experiment.

Mass spectrometry can be used to detect a large number of chemically diverse metabolites in complex samples. For example, capillary electrophoresis with electrospray ionization has been used to profile individual neurons [24]; secondary ion mass spectrometry has been applied to follow phospholipid domains during biological fusion [25]; and matrix-free laser desorption ionization has been utilized to analyze a single cancer cell in vacuum [26]. However, most of these techniques require extensive sample preparation or the use of vacuum environment. Laser ablation electrospray ionization (LAESI) has recently enabled the in situ analysis of large single cells (e.g., plant cells) in their native environment.[27] While other techniques have demonstrated single cell sensitivity, there are a limited number of single cell metabolomics studies by mass spectrometry, particularly in the ambient environment. Chapter 5 describes my efforts to increase the sensitivity in LAESI with the goal of nearing single mammalian cell analysis.

Although certain metabolite distributions can be followed by fluorescence microscopy or SIMS [28], most subcellular work relies on fractionation based on cell disruption and differential centrifugation to separate the organelles by their density. While this process preserves the distribution of large molecules, e.g., proteins, metabolite concentration gradients can be easily disrupted. The analysis of a single cytosolic lipid droplet has been demonstrated by using a nanospray emitter for droplet retrieval and subsequent analysis by nanospray mass spectrometry.[29] Similarly, granules from single cells have been extracted with a nanospray tip while being monitored and directly electrosprayed to analyze their content by a mass spectrometer.[30] In these studies sample dilution with organic solvents was
necessary, and this likely disturbs the metabolite concentrations. Subcellular analysis in the ambient environment has recently been demonstrated by combining cell microdissection with LAESI (Chapter 6).[31]

1.4 ADVANCED IONIZATION TECHNIQUES

The advent of matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) in the late 1980s enabled the routine analysis of intact biomolecules. While these methods laid the foundation for modern mass spectrometry of biomedical samples, a growing number of new techniques have been developed in the past two decades to overcome some of their inadequacies. The development of new technologies continues as we search for ways to improve the methods of single cell and subcellular metabolomics. Some of the recent and most widely used vacuum and ambient methods are briefly discussed below.

1.4.1 Laser Desorption Ionization in Vacuum. The MALDI technique that utilizes a UV absorbing matrix to facilitate the volatilization and ionization of embedded analyte molecules during laser ablation, has become an indispensable soft ionization technique.[1, 2] MALDI efficiently ionizes large biomolecules resulting in high sensitivity; however, matrix interferences in the low mass range hinder the analysis of metabolites and small molecules. To enable small molecule analysis with high sensitivity and minimal interferences, a variety of nanostructured matrix-free platforms have emerged.

Desorption ionization on silicon (DIOS) was one of the first widely accepted matrix-free approaches.[32] Other laser desorption ionization sources include nanostructure initiator mass spectrometry (NIMS) [26], graphite-assisted laser desorption ionization [33], laser-induced silicon microcolumn arrays (LISMA) [34], and silicon nanopost arrays (NAPA) [35], among others. Detailed studies of the
desorption and ionization mechanisms and the unique characteristics of ion production from LISMA and NAPA are described in Chapters 2, 3, and 4.

Interactions between pulsed laser radiation and nanostructured materials, with dimensions ranging from 1 nm to 500 nm, result in enhanced desorption and ionization of organic and biomolecular adsorbates due to processes that are absent on the macroscale, e.g., confinement effects and nanophotonic interactions, such as surface plasmon generation (as illustrated in Figure 1.1).[13, 36] In structures with dimensions commensurate with the wavelength of the laser radiation, polarization dependent ion yields and adjustable fragmentation of the molecular ion are observed.[37-39] Depending on the surface chemistry, structural dimensions, and laser pulse properties, the laser-nanostructure interactions can be varied to enable efficient ionization of metabolites.

Laser desorption ionization from silicon NAPA is depicted in Figure 1.1. The driving force behind desorption and ionization on the nanoscale is the energy deposition from the laser pulse into the nanostructure. Simply put, energy redistribution within the nanostructures and between the substrate and adsorbates facilitates desorption and the formation of ions which are detected by a mass spectrometer. In the case of the analysis of cells by NAPA, laser energy deposition also facilitates cell lysis.[40]

In addition to minimizing interferences in the low mass range, nanostructured surfaces offer simplified sample preparation compared to MALDI, a wide dynamic range for quantitation, and ultrahigh sensitivity, with detection limits as low as 800 yoctomoles.[41] Metabolic applications include the detection of phosphorylated molecules from a cell extract with single cell sensitivity [10], the identification of catecholamines in lymphocytes [11], and the detection of morphine from untreated urine [12]. Imaging of lipids and metabolites in tissue, as well as single cell analysis, has been made possible by nanostructure-based mass spectrometry.[26, 33, 42, 43]
Figure 1.1 Illustration of single cell analysis by laser desorption ionization on nanopost arrays (NAPA) in vacuum. (Image adopted from the cover art associated with Reference [31] by permission of Professor Akos Vertes and the PCCP Owner Societies from Phys. Chem. Chem. Phys., 2012, 14, 8453-8471, DOI: 10.1039/C2CP00038E.)
Similar to MALDI, a major limitation of the nanostructure-based approach to laser desorption ionization is the requirement of a vacuum environment and, although it is minimal, the necessity for some sample preparation. The introduction into vacuum severely limits the size and type of sample that can be studied. Furthermore, the low pressure environment can be detrimental to some biological samples, indeed it is more appropriate to perform experiments in native-like conditions, i.e., in the ambient environment.

1.4.2 Ambient Ion Sources. Although electrospray ionization is performed in the ambient environment, it requires a liquid solution of the sample and/or extensive sample preparation so it is not classified as an ambient technique. The methods of direct analysis, including ambient ion sources, enable rapid, high-throughput, label-free analysis of unprepared samples at atmospheric pressure. More importantly, in vivo or in situ chemical characterization of a broad class of molecules from biological samples, such as live tissues and cells, becomes possible.

Ambient techniques span from those based on liquid extraction, e.g., DESI, to plasma sampling and ionization, e.g., direct analysis in real time (DART) [44], to laser based techniques, e.g., laser ablation electrospray ionization (LAESI) [45], among a host of others.[46] When operating in the ambient environment, an important tradeoff is a loss of sensitivity compared to the vacuum-based techniques. Typical detection limits in the ambient environment are in the low femtomole range. Metabolomic applications vary from the direct detection of cocaine from a human finger [47], to in vivo analysis of hydrocarbons from a male fly [48], to two- and three-dimensional imaging of metabolites in mammalian and plant tissue.[49]

A schematic of brain tissue analysis by LAESI is shown in Figure 1.2. Mid-IR laser radiation at 2940 nm is strongly absorbed by biological samples due to
Figure 1.2 Depiction of ambient analysis by laser ablation electrospray ionization. Inset in the bottom left corner shows the absorption coefficient of water, which has a maximum absorption at 2940 nm, based on data published by Irvine and Pollack and reproduced with permission from ref. [50]. Copyright [1968], Elsevier.
their high water content. As shown in the inset of Figure 1.2, water has a strong absorption maximum at 2940 nm. Thus, LAESI utilizes a mid-IR laser operating at this wavelength to generate a plume of sample-related particulates, droplets, and vapor. A portion of the ejected plume is intercepted by an electrospray plume, ionized, and detected by a mass spectrometer. Chapters 5 and 6 describe variants to the LAESI technique to improve the sensitivity and to enable subcellular analysis.

1.5 PURPOSE OF THIS INVESTIGATION

The present work discusses my efforts to develop and characterize novel ionization sources for single cell and subcellular metabolomics. The laser desorption mechanism and fragmentation of peptides and preformed ions generated from LISMA was studied in Chapter 2. This provided some insight into the fundamental driving forces in laser desorption ionization from nanophotonic sources, but in order to get a better grasp on the laser-nanostructure interactions involved, tailored structures were made. The development of NAPA yielded some unexpected ionization characteristics and enabled ultrasensitive analysis in the vacuum environment (described in Chapters 3 and 4). Subsequently, NAPA was used for single cell analysis. For in situ analysis of cells, I developed a method to collimate the ablation plume in LAESI for higher sensitivity in the ambient environment (Chapter 5). Finally, subcellular analysis was achieved by combining cell microdissection with LAESI (Chapter 6).
1.6 REFERENCES


2.1 ABSTRACT

Laser-induced silicon microcolumn arrays (LISMA) exhibit nanophotonic ion production in laser desorption ionization experiments (Walker et al., Angew. Chem.-Int. Ed. 2009, 48, 1669) for small to medium-size molecules. Although these surfaces are known to promote fragmentation of adsorbates at high laser fluences, the nature, extent and origin of peptide ion decomposition remains unknown. Here we demonstrate that peptide ions, e.g., bradykinin, leucine enkephalin, angiotensin I, substance P and various tripeptides, desorbed from these substrates show an increasing degree of fragmentation as the fluence is raised. Compared to other ion fragmentation methods, LISMA shows similarity to high-energy collision activated dissociation (CAD), ion decomposition produced by metastable atom beams and surface induced dissociation (SID). While some of the produced fragments are associated with high-energy decomposition channels, for example, the abundant \( a_5 \) fragment produced from singly protonated bradykinin ion, other ions in the same spectra (e.g., the ammonia loss from the protonated bradykinin ion) are predominantly produced by low energy processes. To explore the role of internal energy in the fragmentation of ions produced from LISMA, the survival yields of eight
benzyl-substituted benzylpyridinium thermometer ions were also studied as a function of laser fluence and surface derivatization. The corresponding internal energies were determined using the Rice-Ramsperger-Kassel-Marcus formalism. On both native and silane-derivatized surfaces, the thermometer ions showed stable internal energy values over a wide range of laser fluences. This presented a strong contrast to the behavior of the peptides that yielded high-energy fragments at increased fluence. As the thermometer ions did not record an increase in internal energy, the enhanced fragmentation of the peptides was indicative of alternative high-energy mechanisms.

2.2 INTRODUCTION

Micro- and nanostructured silicon surfaces can be produced by femtosecond and nanosecond laser irradiation.[1-2] Repeated exposure of a silicon wafer to picosecond laser pulses creates two-dimensional arrays of protrusions called laser-induced silicon microcolumn arrays (LISMA) that serve as efficient platforms for soft laser desorption ionization.[3] These mesostructured surfaces, also termed black silicon, possess uniformly high absorptivity in a broad wavelength range (from 200 nm to the mid-IR).

In laser desorption ionization experiments, LISMA exhibit highly polarization dependent ion production.[4] This behavior is explained in terms of nanophotonic interactions that show similarities to the behavior of optical antenna arrays. The ionization mechanism on these structures and their ability to induce peptide fragmentation at elevated fluences remains unexplained.

Near-field fluorescence measurements in the vicinity of nanoscopic protrusions on silicon indicate a strong enhancement of the electric field, \( \mathbf{E} \), at a distance \( r \) from the origin:[5]
\[ \mathbf{E} = -\kappa |\mathbf{E}_L| \left( \frac{a}{r} \right)^3 \mathbf{r} \]  

(1)

where \( \mathbf{E}_L \) is the electric field vector of the laser radiation, \( a \) is the radius of curvature of the protrusion, and \( \mathbf{r} \) is a unit vector pointing away from the protrusion. The enhancement factor, \( \kappa \), for a 3:1 aspect ratio protrusion is approximately 6, which translates into a laser irradiance enhancement of \( \sim 36 \).[5] Thus the adsorbed molecules and the desorbed ions in the vicinity of the microcolumns experience very strong fields and radiation intensities. These conditions can contribute to the ionization and, through ion activation, to the fragmentation processes.

Other soft ionization sources, such as matrix-assisted laser desorption ionization (MALDI) produce low internal energy peptide ions with minimal fragmentation.[6-8] The need for complementary soft ionization methods, capable of small molecule analysis, has led to the introduction of nanomaterials and nanostructures that facilitate desorption and ionization without using a matrix. Recently, desorption ionization on silicon (DIOS) based on a nanoporous silicon substrate[9] and nanostructure-initiator mass spectrometry (NIMS)[10] were introduced as versatile laser desorption ionization substrates. Fragmentation of preformed ions on DIOS showed reduced sensitivity to laser fluence changes.[11]

Peptide ions produced by these ionization platforms remain intact in the source region of the mass spectrometer. To induce their fragmentation, an additional activation step is required. These include collision activated dissociation (CAD),[12-13] blackbody infrared radiative dissociation (BIRD),[14] surface induced dissociation (SID),[15-17] electron capture dissociation (ECD),[18-19] electron transfer dissociation (ETD)[20] and ion decomposition produced by metastable atom beams.[21-23] Fragmentation of model peptides, e.g., bradykinin, leucine enkephalin, angiotensin I and substance P, have been extensively studied by all of these methods.
Thermometer ions (TI), such as benzyl-substituted benzylpyridinium cations, are preformed ions with a single decomposition channel that, in a laser desorption ionization experiment, can report on internal energy transfer in the desorption step separately from the energy gain due to ionization.[24-25] The survival yield method based on TI has been utilized to gauge the internal energy of ions generated by electrospray ionization,[26-32] MALDI,[6-7, 33-36] DIOS,[11, 37] desorption electrospray ionization,[38] and silicon nanowires (SiNW).[39]

Other methods developed for internal energy measurements include the deconvolution method that correlates the internal energy distribution, \( P(E) \), to the decomposition spectrum and the breakdown curve.[40] According to the thermometer ion method, \( P(E) \) can be estimated from the ion abundances measured in unimolecular decomposition with known thermochemical parameters.[41-42] A modified version of the thermometer ion method, the above mentioned survival yield method, is based on the correlation between the survival yield, \( SY \),

\[
SY = \frac{I(M^+)}{I(F^+) + I(M^+)}
\]  

(2)

where \( I(M^+) \) and \( I(F^+) \) are the abundances of the molecular ion and fragment ion, respectively, and the critical energy of the molecular ion.[43]

Nanophotonic ion production from LISMA structures shows structure specific peptide fragmentation at high laser fluences.[3] This sets LISMA apart from MALDI and many other soft ionization techniques. The polarization and incidence angle dependence of energy deposition into LISMA structures was explained by axial currents in the microcolumns induced by the electromagnetic radiation of the desorption laser.[4] The related energy dissipation is similar to Ohmic losses in antenna arrays. Biomolecular adsorbates on the microcolumns are rapidly desorbed from the heated surface. Desorption of the TI results in the direct production of ions, whereas most peptides require an additional ionization step. This difference enables
us to discern if the origin of peptide fragmentation is linked to the internal energy gained during desorption.

The desorbed components in the plume can be ionized and activated through homogeneous and surface reactions. These processes include photoionization due to the local electric field, interactions with the hydroxy-terminated sites on the silicon microcolumns, proton transfer from solvent residues, and reactions with the electrons emitted from the silicon surface.\[9, 44] At high laser intensities hydrogen-free radicals may form, due to the interaction of the elevated electron density with protons from the solvent residues, potentially causing in-source decay and peptide fragmentation.\[3, 45] As it is shown in eq 1, the microcolumns can also induce local enhancements in the electromagnetic field that might also promote fragmentation.

In this chapter, we survey the type and abundance of fragments produced from peptide ions generated on LISMA substrates by laser radiation at increased fluence levels. By comparing the fragmentation of model peptides in LISMA ion production to results with low and high-energy ion activation methods, we can discern the nature of the processes involved. We also probe the energy content of TI generated from LISMA and compare it to fragmentation patterns for peptides. The fluence dependence of fragmentation from these two types of adsorbates reveals the relationship between peptide fragmentation and internal energy effects on these nanophotonic structures. Studying the energy transfer into the preformed TI from LISMA gives insight into the desorption process and enables comparisons with other soft laser desorption ionization methods. Chemical surface modifications are used to alter the interaction energy between the ions and the LISMA surface, and the resulting changes in the efficiency of energy transfer are evaluated.

2.3 MATERIALS AND METHODS
2.3.1 Materials and Sample Preparation. Low resistivity (~0.001-0.005 Ω•cm) p-type silicon wafers were purchased from University Wafer (South Boston, MA). Deionized water (18.2 MΩ•cm) was produced with an E-pure system (Barnstead, Dubuque, IA), whereas reagent grade methanol was obtained from Sigma. Bradykinin (Arg-Pro-Pro-Gly-Ser-Phe-Pro-Phe-Arg), leucine enkephalin (Tyr-Gly-Gly-Phe-Leu), angiotensin I (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu), substance P (Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH₂), and tripeptides Gly-Phe-Ser and Tyr-Gly-Gly were purchased from Sigma and used without further purification. Gly-Phe-Leu was purchased from Synbiosci (Livermore, CA). Chloride salts of eight benzyl substituted benzylpyridinium ions (with 4-nitro- (4N), 4-chloro- (4C), 4-fluoro- (4F), 4-methoxy- (4MO), 3-methoxy- (3MO), 4-methyl- (4M), 3-methyl- (3M), and 2-methyl- (2M) substituents) were custom synthesized by Celestial Specialty Chemicals (Nepean, Ontario, Canada) and 1.0×10⁻⁵ M solutions were prepared daily in 50% methanol. For laser desorption ionization experiments 1.0 µL of these solutions was deposited on LISMA substrates and dried under ambient conditions.

2.3.2 Synthesis of LISMA and Surface Derivatization. A detailed description of LISMA production can be found elsewhere.[3] Briefly, the silicon wafers were cleaved into chips of 9 mm² surface area, cleaned with methanol and deionized water, air-dried, attached to the bottom of a Petri dish with double-sided tape, and submerged in 5 mL of deionized water at a depth of ~2.6 mm. Microcolumns were generated by irradiating the silicon chips with 600 laser shots of 22 ps length from a frequency tripled Nd:YAG laser (PL2143, EKSPLA, Vilnius, Lithuania) at 0.13 J/cm² fluence. For surface characterization of the laser processed spot, a JEOL JSM-840A (Peabody, MA) scanning electron microscope (SEM) and a Hitachi FE-SEM S-4700 (Hitachi, Pleasanton, CA) were used.
For derivatization, the LISMA surfaces were oxidized with ozone, placed in a Petri dish, and covered with 15 µL of (pentafluorophenyl) propyldimethylchlorosilane (PFPPDCS) (Gelest, Inc., Morrisville, PA). Subsequently they were baked in an oven for 30 minutes to create a perfluorophenyl (PFP)-derivatized LISMA surface. The derivatized LISMAs were rinsed thoroughly with methanol and deionized water and air-dried.

2.3.3 Instrumentation and Data Acquisition. For the peptide fragmentation experiments, a high resolution reflectron time-of-flight mass spectrometer (Axima CFR, Shimadzu-Kratos, Manchester, UK) was used. A home-built linear time-of-flight mass spectrometer (TOF-MS) with delayed extraction and a 4 ns pulse length nitrogen laser was used for the thermometer ion and survival yield experiments. The detailed description of the instrument can be found elsewhere.\[46\] The laser fluence was adjusted by a variable attenuator (935-5-OPT, Newport, Fountain Valley, CA) and calculated from the laser focal area and pulse energy, measured by a burn mark on photographic paper, and with a pyroelectric joule meter (model J4-05, Molelectron, Portland, OR), respectively.

For each sample, twenty spectra were taken at ten different locations on the LISMA for a total of 200 spectra. Peak areas of molecular and fragment ions were then integrated and used to calculate experimental survival yields. The survival yields were converted into experimental rate coefficients, $k_{exp}$,

\[
k_{exp} = \left( \frac{1}{\tau} \right) \ln(SY)
\]  

where $\tau$, the reaction time in the acceleration region, is estimated as 100 ns for all TI.[6]
2.3.4 Rate Coefficient Calculations. The vibrational frequencies for the TI were previously calculated using AM1 semiempirical molecular orbital calculations with PC Spartan version 1.3 (Wavefunction, Irvine, CA)[6] and the critical energy values of the TI were obtained from the literature.[24, 26] The unimolecular decomposition rate coefficient, \( k(E) \), for a particular energy, \( E \), can be calculated with the Rice-Ramsperger-Kassel-Marcus (RRKM) theory,

\[
k(E) = \frac{G^*(E)-E_0}{\hbar N(E)}
\]

where \( E_0 \) is the critical energy, \( G^*(E-E_0) \) is the number of states between \( E \) and \( E_0 \) in the transition state, and \( N(E) \) is the density of quantum states at energy \( E \).[47] The computer program MassKinetics Scientific, Version 1.9, was used for the RRKM calculations.[48]

2.4 RESULTS AND DISCUSSION

2.4.1 LISMA Morphology. SEM imaging before and after laser irradiation confirmed that during the ion production experiments the microcolumns remained intact. Figure 2.1 shows a few columns from a typical LISMA structure used in this study. Measurements on the SEM images indicated an average diameter, periodicity and height of 400, 600 and 800 nm, respectively. Thus the aspect ratio of the microcolumns was 2:1, which at the column surface resulted in a field enhancement factor of \( \kappa \approx 4 \) and an enhancement of the laser irradiance of \( \sim 16 \). According to eq 1, moving away from the surface the enhanced field decays as \( (a/r)^3 \), where the microcolumn tip radius of curvature, \( a = \sim 200 \text{ nm} \).

The dimensions of the LISMA are commensurate with the 337 nm wavelength of the desorption laser. Based on the polarization and incidence angle dependence of the ion yield demonstrated earlier,[4] these columns are heated by the axial current induced in them by the parallel component of the electric field in the laser radiation.
**Figure 2.1** Side view of silicon microcolumns in a LISMA structure captured by SEM confirms that the column dimensions are commensurate with the laser wavelength. Reprinted with permission from ref. [49]. Copyright [2010], American Chemical Society.
From the SEM images, it is also evident that the individual microcolumns exhibit coarse textural features that increase the surface area.

2.4.2 LISMA Spectra of TI and Peptides. Mass spectra of all the TI desorbed from native and PFP-derivatized LISMA were obtained for a range of laser fluences. The fluence threshold for detectable ion production was ~26 mJ/cm\(^2\) that exhibited slight variations. The molecular ion peak, M\(^+\), and a single fragment peak due to pyridine loss, F\(^+\), were observed, indicating that the energy deposited into the TI at the threshold fluence was at or above the critical energy of the fragmentation channel. The spectral identifications were straightforward as no external interferences, such as silicon clusters or alkaline adducts, were detected even at high fluences. Relative peak intensities in the TI spectra remained largely unchanged throughout the studied fluence range between 26 mJ/cm\(^2\) and 44 mJ/cm\(^2\).

Bradykinin spectra at low fluences showed predominantly the quasi-molecular ion peaks (protonated and sodiated) (see the black trace in Figure 2.2A). As the fluence was raised, an increasing number and abundance of structure specific fragments were observed (see the wine, olive and blue traces in Figure 2.2A). At the highest fluence level, we detected the \([M-NH_3+H]^+\), \(y_8\), \(y_7\), \(a_5\), \(a_6\), \(d_6\) and \(c_5\) ions as major fragments, as well as \(c_8\), \(Y_8\), \(a_8\), \(Y_7\), \(w_{a7}\), \(a_{7}\), \(y_{6}\), \(y_5\) and \(v_5\) as minor breakdown products (see Figure 2.2B).

Extensive fragmentation was observed for other peptides, such as leucine enkephalin (see Table 2.1 for the list of fragments and their intensities), angiotensin I, substance P, and tripeptides Gly-Phe-Ser, Gly-Phe-Leu and Tyr-Gly-Gly. In regular MALDI experiments without collisional activation, none of these peptides exhibited fragmentation. The fluence dependent fragmentation of bradykinin and the other small peptides was in sharp contrast to the TI fragmentation that remained unchanged in a wide fluence range.
Figure 2.2 Peptide ion fragmentation in laser desorption from LISMA structures. (A) Bradykinin shows increasing variety and abundance of fragments as the laser fluence is increased. The molecular ion survival yield falls rapidly (see inset). (B) Enlarged segment of the high laser fluence (145 arbitrary units) spectrum in the 500 < m/z < 1000 range shows the identification of all of the major and minor fragments. Reprinted with permission from ref. [49]. Copyright [2010], American Chemical Society.
(A)

(B)
The bradykinin fragments produced by LISMA at high laser fluences show major deviations from the species generated by low-energy CAD[12] or by BIRD[14] activation of the singly protonated molecule. Table 2.1 compares the fragments produced by some of the known activation methods. The fragmentation spectra on LISMA is clearly different from low-energy CAD and BIRD, and the electron capture dissociation (ECD) of the doubly protonated bradykinin.[18-19] There are, however, remarkable similarities to the spectra produced by high-energy CAD,[13] metastable atom beams[21-22] and surface induced dissociation (SID) at 50 eV primary ion energy.[15] Of these three latter techniques, metastable helium atoms, He(2^1S) and He(2^3S), generate abundant a_5 and c_5 species that are also prevalent in the LISMA spectra. The excitation energies of the He(2^1S) and He(2^3S) metastables are 20.6 and 19.8 eV, respectively. Inspecting the high-energy CAD and the LISMA spectra reveals that both contain major peaks for the a_5, a_6 and y_7 ions with kinetic energy losses of 22.9 eV, 19.7 eV and 11.1 eV, respectively, in the CAD experiment.[13] The correlation between the LISMA and the SID ions is the strongest. In the 520 < m/z < 910 range all of the significant ions reported by the two methods are the same. The similarity in fragmentation between the three high-energy techniques and LISMA means that the production of most fragments in LISMA is likely a high-energy process.

In contrast to these three high energy techniques, however, the LISMA spectra contain significant amounts of [M-NH_3+H]^+ ions characteristic of low-energy CAD and BIRD spectra. The presence of this fragment suggests that, in addition to the high-energy processes responsible for the a-series and internal fragment ions, a low-energy mechanism is also operational. To our knowledge, there is no ion activation method available that can access low- and high-energy fragments in the same experiment. The LISMA spectra of leucine enkephalin also revealed the presence of fragments associated with high-energy processes. Table 2.2 lists the
TABLE 2.1 Comparison of protonated bradykinin fragmentation patterns for LISMA at high fluence with various methods of ion activation. The primary ion is singly charged unless noted otherwise. Reprinted with permission from ref. [49]. Copyright [2010], American Chemical Society.
<table>
<thead>
<tr>
<th>Activation method</th>
<th>Conditions</th>
<th>Major fragments</th>
<th>Kinetic energy loss in CAD (eV)</th>
<th>Energy (eV)</th>
<th>Minor fragments</th>
</tr>
</thead>
<tbody>
<tr>
<td>LISMA at high fluence</td>
<td></td>
<td>([M-NH_3+H]^+), (y_{8r}), (y_{7r}), (a_{5r}), (a_{6r}), (d_{a6r}), (c_5)</td>
<td></td>
<td></td>
<td>(c_{8r}), (Y_{8r}), (a_{8r}), (Y_{7r}), (w_{a7r}), (a_{7r}), (y_{6r}), (y_{5r}), (v_5)</td>
</tr>
<tr>
<td>CAD at low energy(^a)</td>
<td>213(^\circ) C, 20 s</td>
<td>([M-NH_3+H]^+)</td>
<td>1.3</td>
<td></td>
<td>(y_{7r}), (Y_{6r}), (y_{5r}), (b_{8r}), (b_2)</td>
</tr>
<tr>
<td>BIRD(^b)</td>
<td>191(^\circ) C, 45 s</td>
<td>([M-NH_3+H]^+)</td>
<td>1.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECD(^c) for ([M+2H]^{2+})</td>
<td></td>
<td>(c_5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CAD at high energy(^d)</td>
<td>Energy loss measured</td>
<td>(a_5)</td>
<td>22.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(a_6)</td>
<td>19.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(a_8)</td>
<td>15.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b_{6r})</td>
<td>12.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b_8)</td>
<td>17.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(y_7)</td>
<td>11.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(b_8+OH)</td>
<td>5.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Metastable atoms(^e)</td>
<td>He(2(^1)S)</td>
<td>(a_{3r}), (a_{5r}), (a_{4r}), (c_{5r}), (x_6)</td>
<td>(\sim20)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>He(2(^3)S)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SID(^f)</td>
<td>50 eV</td>
<td>(y_{8r}), (a_5)</td>
<td></td>
<td></td>
<td>(a_{6r}), (y_{5r}), (a_{7r}), (w_{a7r}), (Y_{7r}), (a_8)</td>
</tr>
</tbody>
</table>

\(^a\)Low energy CAD data from McLuckey and coworkers.\[^{12}\]

\(^b\)BIRD data from Williams and coworkers.\[^{14}\]

\(^c\)ECD data from Barran and coworkers,\[^{19}\] and Smith and coworkers.\[^{18}\]

\(^d\)Kinetic energy loss data from Glish and coworkers.\[^{13}\]

\(^e\)Metastable atom beam data from Berkout\[^{21}\] and Doroshenko.\[^{22}\]

\(^f\)SID data from Wysocki and coworkers.\[^{15}\]
**TABLE 2.2** Protonated leucine enkephalin fragmentation for LISMA, high-energy CAD and SID. Reprinted with permission from ref. [49]. Copyright (2010), American Chemical Society.

<table>
<thead>
<tr>
<th>Fragmentation</th>
<th>LISMA, high-energy CAD</th>
<th>LISMA, high-energy SID</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leucine enkephalin</td>
<td>[Data]</td>
<td>[Data]</td>
</tr>
<tr>
<td>Fragment</td>
<td>LISMA abundance</td>
<td>Kinetic energy loss in CAD (eV)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>----------</td>
<td>-----------------</td>
<td>------------------------------------------</td>
</tr>
<tr>
<td>$L$</td>
<td>high</td>
<td></td>
</tr>
<tr>
<td>$F$</td>
<td>high</td>
<td></td>
</tr>
<tr>
<td>$Y_i$</td>
<td>medium</td>
<td></td>
</tr>
<tr>
<td>$Y$</td>
<td>high</td>
<td></td>
</tr>
<tr>
<td>$GF-28$</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>$a_2$</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>$GF$</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>$b_2$</td>
<td>medium</td>
<td></td>
</tr>
<tr>
<td>$GGF-28$</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>$GGF$</td>
<td>low</td>
<td></td>
</tr>
<tr>
<td>$b_3$</td>
<td>high</td>
<td>7.4</td>
</tr>
<tr>
<td>$y_2$</td>
<td>low</td>
<td>4.4</td>
</tr>
<tr>
<td>$y_3$</td>
<td>absent</td>
<td>3.6</td>
</tr>
<tr>
<td>$a_4$</td>
<td>low/medium</td>
<td>5.7</td>
</tr>
<tr>
<td>$b_4$</td>
<td>low</td>
<td>3.5</td>
</tr>
<tr>
<td>$MH^+$</td>
<td>medium</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Kinetic energy loss data from Glish and coworkers.[13]

<sup>b</sup>SID threshold energy data from Laskin.[16]
observed fragments and their abundances. The formation of the highly abundant $b_3$ and the $Y$ and $F$ immonium ions require relatively higher energy than the $b_4$ and $a_4$ fragments that are present with low to medium abundance. The energetics of the various fragmentation channels is revealed by threshold energies[16] measured in SID and kinetic energy loss data[13] for high-energy CAD in Table 2.2.

Similarities between the leucine enkephalin fragmentation patterns in LISMA and SID spectra recorded at 50 eV primary ion energy were especially pronounced. Comparable correlation was observed for the fragments of protonated angiotensin I. At elevated laser fluence, LISMA produced over 20 structure specific fragments from this ion, half of which were also found in the 100 eV SID spectra of the angiotensin I.[17] The similarities between LISMA and SID spectra raise the possibility of a mechanistic relationship between the two techniques. The energetic ions produced in the troughs of the LISMA structure can collide with the microcolumn surfaces before being extracted into the mass spectrometer.

2.4.3 Survival Yields in LISMA Ion Production. To explore the significance of internal energy transfer in LISMA, the survival yield method was applied to a set of TI. Survival yields were calculated using eq 2 for laser fluences ranging from 26-44 mJ/cm$^2$. The survival yield values correlated with the critical energy of the TI, where ions with larger critical energies exhibited higher survival yields. Survival yields for all native and all but the 4M derivatized LISMA substrates remained unchanged as the fluence was increased. The survival yield of the 4M ion on PFP-derivatized surface was constant up to 36 mJ/cm$^2$ and slightly declined above that.

To compare the energy transfer in ion production from LISMA with other ionization methods, the survival yields for 4M ions desorbed from PFP-derivatized DIOS and from α-cyano-4-hydroxycinnamic acid (CHCA), a MALDI matrix,[39] were plotted along with our findings in Figure 2.3A. The threshold laser fluence required to
desorb the TI from LISMA at 26 mJ/cm² is slightly higher than that of DIOS and MALDI (∼24 mJ/cm²). Above the threshold, the preformed TI released by MALDI showed a sharp decline in survival yield that was in contrast with the steady behavior of TI desorbed from LISMA (see Figure 2.3A). This could be interpreted as an increase in internal energy for the MALDI process, compared to an unchanged internal energy for LISMA desorbed ions as the laser fluence increased.

These results indicate that LISMA exhibits internal energy transfer similar to DIOS, but with somewhat higher survival yields. Xiao et al. recently reported fluence dependent survival yields for TI desorbed in DIOS experiments.[37] They explained this discrepancy by the more than two orders of magnitude higher background pressure in their vacuum system compared to ours. Background pressure and surface adsorbates are known to have a strong influence on ion yields and fragmentation in laser desorption from silicon surfaces.[50]

Our findings on the fluence dependence of peptide mass spectra produced by MALDI and LISMA seem to contradict our observations with TI. While MALDI time-of-flight mass spectra of peptides show little or no fragmentation, the mass spectra of bradykinin and other small peptides in LISMA experiments indicate declining survival yields as the fluence is raised. In other words, although increasing the laser fluence does not seem to affect the internal energy of the TI desorbed from LISMA, it does promote the fragmentation of peptides. The inset in Figure 2.2 demonstrates rapid decline for the survival yield of bradykinin with increasing fluence. The observations for these two molecular classes cannot be reconciled if the peptide fragmentation is solely attributed to the unimolecular decomposition of excited ions.

A possible explanation for this apparent contradiction is the presence of an alternative fragmentation pathway. In an earlier study based on the fragmentation pattern of the P₁₄R synthetic peptide, we hypothesized that the in-source fragmentation on LISMA substrates was induced by hydrogen radicals produced via
the recombination of photoelectrons emitted from the silicon and protons generated from the residual solvent.\textsuperscript{[3]} According to the hypothesis, similar to a mechanism advanced for ECD, the hydrogen radicals attacked the peptide backbone and induced several pathways of fragmentation. These reactive fragmentation channels did not require the elevated internal energies necessary for the commonly utilized CAD. Comparing the ECD spectra for bradykinin and the other peptides in this study to the fragments produced by LISMA, however, brings this mechanism into question. As it is seen in Table 2.1, ECD spectra are rich in \( c \)- and \( z \)-series ions, whereas LISMA spectra are dominated by \( a \)- and \( y \)-series ions. Alternative explanations can be based on the combination of free radical and CAD or SID-type channels as well as the field enhancement effects around the microcolumns and the differences between the transient dipole moments of the peptides and the TI.

\textbf{2.4.4 Surface Derivatization.} By altering the surface chemistry of LISMA through silylation, less polar and more hydrophobic surfaces were produced, which resulted in higher ion yields compared to native LISMA. As it can be seen for the 4F benzylpyridinium ion in Figure 2.3B, in addition to enhanced ion production, derivatized LISMA surfaces also increased the survival yield. During the interaction of the LISMA with the laser radiation, the microcolumns heat up and impart some of their energy to the adsorbates.\textsuperscript{[4]} This energy transfer is modulated by the adsorbate-surface interaction. Native LISMA have terminal hydride and silanol groups that delay the release of polar adsorbates resulting in an increased energy transfer. The suppression of energy transfer to the TI on silylated LISMA is the consequence of reduced interaction energy between the adsorbate and the microcolumns.

The 4F benzylpyridinium ion exhibits the most pronounced survival yield difference between desorption from the native and the PFP- derivatized surfaces. The
Figure 2.3 (A) Fluence dependence of 4M benzylpyridinium TI survival yields drop precipitously in the case of MALDI from CHCA matrix[6] (■), but remain mostly unchanged for desorption from PFP-derivatized LISMA (●) and PFP-derivatized DIOS[11] (▲). (B) The flat survival yield curve for 4F benzylpyridinium ions from native LISMA surfaces (●) is shifted to higher values for PFP-derivatized LISMA (▼). Reprinted with permission from ref. [49]. Copyright [2010], American Chemical Society.
sharp reduction in energy transfer to the 4F ion is likely related to the weak fluorine-fluorine interactions with the PFP-derivatized layer compared to the strong affinity to the terminal hydride or silanol groups on native LISMA. Of the studied TI, the 4F ion is also the least polarizable, leading to weaker ion surface interactions. This translates into the faster release of these ions and reduced energy transfer.

2.4.5 Internal Energies. Internal energy values for the TI were derived from the survival yield data using the RRKM theory. The internal energies of the TI desorbed from native LISMA were obtained by comparing the experimental rate coefficients of decomposition determined through eq 3 with the RRKM curves expressed by eq 4 determined through eq 3 with the RRKM curves expressed by eq 4. Except for the 4Cl case, the derived internal energy values, $E_{\text{int}}(4\text{MO}) = 3.97 \text{ eV}$, $E_{\text{int}}(4\text{M}) = 4.85 \text{ eV}$, $E_{\text{int}}(2\text{M}) = 4.91 \text{ eV}$, $E_{\text{int}}(3\text{M}) = 4.91 \text{ eV}$, $E_{\text{int}}(3\text{MO}) = 8.18 \text{ eV}$, $E_{\text{int}}(4\text{Cl}) = 4.48 \text{ eV}$ and $E_{\text{int}}(4\text{F}) = 8.33 \text{ eV}$, correlated well with the critical energies for their fragmentation. The internal energies determined as a function of laser fluence in the 26 to 44 mJ/cm$^2$ range remained essentially unchanged.

When compared with MALDI from CHCA, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid) and 2,5-dihydroxybenzoic acid matrixes, TI desorbed from LISMA generally exhibited higher internal energies. In the MALDI experiments, the internal energies were dependent on the laser fluence. As the eight TI exhibited a range of critical energies (from 1.3 eV to 2.2 eV), with the survival yield method we were able to extract the internal energy distributions at a particular laser fluence. The results are shown in Figure 2.4 for native (solid line) and derivatized (dashed line) LISMA with no corrections for the kinetic shift. At a laser fluence of 36 mJ/cm$^2$, the mean of the internal energy distribution in Figure 2.4 was slightly higher for native LISMA than for silane derivatized LISMA, with values of 1.73 eV and 1.59 eV respectively.
**Figure 2.4** Internal energy distributions of TI desorbed from native LISMA (solid line in black) and PFP-derivatized LISMA (dashed line in red) at a laser fluence of 36 mJ/cm² and MALDI from CHCA matrix at a laser fluence of 26 mJ/cm² (dotted line in olive color, data adopted from ref 39). Reprinted with permission from ref. [49]. Copyright [2010], American Chemical Society.
The 0.14 eV lower internal energy on the derivatized surface corresponded to a significant increase in the survival yield. For comparison, the internal energy distribution of MALDI from CHCA matrix at a laser fluence of 26 mJ/cm$^2$ is also shown.$^{[39]}$ The mean internal energy distribution, 1.18 eV, was lower than derivatized and native LISMA, as expected from the increased survival yield values shown in Figure 2.3A. In addition the internal energy distribution from CHCA is broader than from LISMA, which may indicate a more dense plume.

The internal energies and their distributions for TI desorbed from native and derivatized LISMA structures indicated that the ion activation mechanism operational for peptides did not work for these benzylpyridinium ions. As the internal energy of TI did not change with increasing fluence, the declining survival yield of peptide ions at elevated laser fluences probably did not stem from an increase in their internal energy induced by the desorption process.

### 2.5 CONCLUSIONS

Due to their role in the structural studies of biomolecules, new ionization methods are vigorously investigated. Mesostructured surfaces, such as LISMA, are the basis of emerging techniques for the laser desorption ionization of a broad class of molecules. LISMA is the first ionization platform to exhibit nanophotonic ion production for mass spectrometry. In contrast to most conventional methods, laser desorption ionization of peptides from LISMA induces adjustable structure specific fragmentation without additional ion activation techniques. From the high fluence mass spectra it is clear that the related processes take place in the ion source of the mass spectrometer (in-source decay). Thus, the half-life time of the activated species must be comparable to the residence time of the ions in the ion source region (<100 ns).

Model peptides desorbed from LISMA showed fluence dependent fragmentation. At low laser fluences mostly quasi-molecular ions were observed. As
the fluence was raised, the abundance and variety of structure specific peptide fragments increased. The resulting fragment ions were compared to the dissociation products, and related energetics, formed in other methods of ionization, such as low- and high-energy CAD, BIRD, ECD, SID and interactions with metastable helium atoms. Surveying the fragment ions produced by LISMA, we discovered that both low energy and high-energy channels were active. Similarities in the high fluence LISMA mass spectra of small peptides (bradykinin, leucine enkephalin, etc.) to conventional SID spectra indicated that a similar activation mechanism, i.e., ion collisions with the microcolumn surfaces, might play a role in ion activation.

To discern if the internal energy of the ion gained in the desorption step plays a role in the fragmentation, the survival yields of TI were analyzed. We found that the internal energy transfer from LISMA and thus the degree of TI fragmentation were independent of the applied laser fluence. Furthermore, silane-derivatization of LISMA decreased the surface-adsorbate interaction and resulted in higher survival yields. These results for TI are in conflict with the strong fluence dependence of the survival yield for peptides.

Earlier studies indicated that nanophotonic structures, such as LISMA, enable new ways to couple laser energy into the desorption and ionization process. In this chapter, we showed that the interaction of laser radiation with LISMA also provides a novel way for peptide ion activation. This method accessed both low energy and high-energy fragmentation channels.

The mechanism of ion activation remains unresolved. Likely factors in this process include the enhancement of the electromagnetic field near the microcolumns, surface activation due to ion-surface collisions, and reactive channels, including hydrogen radical attack on the peptide backbone. At this point none of these mechanisms provides an explanation for all of the experimental findings in this study. For example, if surface activation plays a role in the peptide fragmentation
why does the survival yield of the TI stay unchanged in a broad fluence range? To explain the presence of low energy and high-energy fragments in the peptide spectra, one might have to consider the combined effect of several mechanisms.

To gain further insight into the role of these effects in ionization, nanofabricated structures with tailored column diameter, height and periodicity can be produced. For example, by changing the aspect ratio of the columns to 10:1, the local field enhancement can be increased to \( \kappa \approx 13 \) and correspondingly the near-field laser irradiance is raised by a factor of \(~169\). Thus, following the peptide fragmentation with changing column aspect ratio can indicate the significance of near-field effects. Increasing the periodicity enlarges the trough width and reduces the contribution of surface collisions. Lowering the delay time in delayed extraction experiments results in shorter residence time in the ion source that, in turn, limits the impact of reactive fragmentation channels. In addition to the residual solvents that are always present on these surfaces, other reactants can be introduced into the structures to promote ionization and/or fragmentation through reactions.

Due to the features of LISMA discussed in this paper, their potential applications include the preparation of diverse ionic species. The ability of this nanostructure to induce peptide fragmentation without the use of CAD, ECD or other activation method, presents a simple approach to structural elucidation in chemical analysis.

### 2.6 ACKNOWLEDGEMENTS

The authors are thankful for the financial support from the Chemical Sciences, Geosciences and Biosciences Division, Office of Basic Energy Sciences, Office of Science, U.S. Department of Energy (DE-FG02-01ER15129) and from Protea Biosciences Inc. Support from the Department of Energy does not constitute an endorsement of the views expressed in the article. The MassKinetics Scientific
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2.7 REFERENCES


CHAPTER 3

TAILORED SILICON NANOPOST ARRAYS FOR RESONANT
NANOPHOTONIC ION PRODUCTION


3.1 ABSTRACT

Nanostructures that have dimensions commensurate with the wavelength of the electromagnetic radiation exhibit near-field effects and, as optical antennas, can couple laser radiation to the local environment. Interactions between laser radiation and photonic structures at elevated laser intensities give rise to the production of positive and negative ions from adsorbates. In this work we demonstrate that tailored silicon nanopost arrays (NAPA) exhibit resonant ion production. Ion yields from posts with subwavelength diameter show sharp resonances at high aspect ratios and these posts show reduced laser fluence threshold for ionization. The resonant enhancement in ion intensities can be modulated by adjusting the post aspect ratio. Posts with diameters at or below the thermal diffusion length demonstrate high surface temperatures due to the radial confinement of the deposited energy. As a consequence enhanced fragmentation, i.e., a lower survival yield of the molecular ions is observed. These nanophotonic ion sources can control the degree of ion fragmentation and could eventually be integrated with micromachined mass spectrometers and microfluidic devices.
3.2 INTRODUCTION

Quasiperiodic columnar silicon nanostructures offer low reflectivity in a wavelength range spanning from 200 nm through mid-IR\cite{1} to the terahertz region.\cite{2} Examples include laser-induced silicon microcolumn arrays (LISMA)\cite{3} produced by femtosecond laser surface structuring\cite{4} and silicon nanotip (SiNT) arrays fabricated by plasma etching.\cite{2} These high aspect ratio structures offer sub-band gap light absorption with a corresponding photocurrent, broadband anti-reflection properties, efficient electron emission\cite{5} and superhydrophobic behavior.\cite{6-7}

Nanoscopic protrusions on silicon surfaces are known to result in the local enhancement of electromagnetic radiation that, for a 10:1 aspect ratio column, can reach an intensity gain close to 200 in the near field.\cite{8} Metal nanostructures can exhibit additional enhancements through surface plasmon resonances and operate as optical antennas.\cite{9-11} These structures demonstrate resonant energy absorption\cite{12} that is sensitive to polarization\cite{13} and antenna length\cite{10}, and their near-field response can be tuned through altering their geometry.\cite{14} Near-field radiation induced fluorescence has been demonstrated in biological membranes\cite{15} and single molecules\cite{16} opening the way for the microscopy and spectroscopy of subwavelength domains. At higher laser fluences materials brought to the proximity of these enhanced fields can undergo ablation\cite{17-18} that can include the nanoscopic structure producing the enhancement itself.\cite{19} In particular, gold nanoparticle ablation induced by the near field sets in between 9 and 12 mJ/cm\(^2\), whereas melting only commences at 15 mJ/cm\(^2\).\cite{7, 19}

When the dimensions of a nanostructure are on the order of the wavelength of light, the evanescent components of the electromagnetic field and near-field effects need to be considered.\cite{20-21} Localization and confinement of the radiation around the nanostructure, as a result of external excitation (i.e., laser light), result
in very strong near-fields. Molecules near or on the surface can respond to the enhanced field by desorbing and ionizing.[17, 22-23]

In recent studies we have demonstrated that photonic nanostructures with quasiperiodic features, such as LISMA, exhibit nanophotonic ion production.[24-25] This includes polarization-dependent ion yields and adjustable fragmentation (through both high- and low-energy channels) by simply increasing the laser fluence. The mechanism of nanophotonic ion production likely involves multiple steps and is not well understood.

In order to systematically study the interactions between the nanostructure and laser radiation that contribute to desorption and ionization, it is important to control the nanostructure dimensions. Nanofabricated silicon nanopost arrays (NAPA) are similar to LISMA in their chemical composition and overall morphology. However, due to the nanofabrication used in their production, we have a greater control over the relevant dimensions such as nanopost height and diameter.

Here we study the effects of varying the NAPA post diameters, heights, and periodicities on the ion yields of small molecules. We demonstrate ion yield resonances, producing gains of up to 55, for certain post aspect ratios and we show that as the diameter of the posts decrease, the resonant aspect ratio increases. Using preformed ions, we find that the desorption threshold decreases for higher aspect ratio posts, indicating the importance of near fields in the vicinity of the posts. Lastly, fragmentation of small peptides is induced by increasing the laser fluence.

### 3.3 EXPERIMENTAL

#### 3.3.1 NAPA Fabrication. Silicon NAPA with post diameters between 50 nm and 600 nm and periodicities from 200 nm to 1200 nm were nanofabricated in square patterns with sides of 500 µm. The computer generated patterns were transferred to ZEP520A spin-coated low resistivity (0.001-0.005 Ω·cm) p-type silicon wafers by
electron beam lithography (JEOL JBX-9300). The exposed resist was removed and the wafer was descummed in oxygen plasma (Technics RIE system). An electron beam evaporator was used to deposit 10 nm of chromium on the wafer and an acetone bath was used to remove the remaining resist and chromium layer on the unexposed resist. High aspect ratio posts, with heights ranging from 200 nm to 1500 nm were produced by reactive ion etching (Oxford PlasmaLab 100 RIE system). Scanning electron microscope (SEM; FEI Nova Nanolab 600 DualBeam) images were taken to inspect the NAPA after nanofabrication, see Figure 3.1. Optionally, these NAPA were derivatized with (perfluorophenyl)-propyldimethylchlorosilane (PFP) by first exposing them to ozone and then baking them with the PFP.

**3.3.2 Mass Spectrometry.** Following the nanofabrication process, the laser desorption ionization properties of the NAPA were confirmed using a Bruker Daltonics Autoflex II reflectron time-of-flight mass spectrometer (TOF-MS). Adsorbate solutions of substance P, bradykinin, leucine enkephalin, angiotensin I, GFL, verapamil, and the 4-methyl-benzylpyridinium (4M) thermometer ion (TI) were prepared at a concentration of ~1 mg/mL in 50% methanol. A 0.5 µL aliquot of a solution was deposited onto the NAPA surface and air dried. Averaged mass spectra were acquired from 100 laser shots in reflectron mode. More detailed experiments, aimed at the fluence dependence of the ion yield and the fragmentation behavior, were conducted on a Kratos Axima III TOF-MS with a curved field reflectron. The Kratos instrument used a nitrogen laser focused to ~100 µm diameter spot with adjustable laser fluence on the NAPA target. Averaged spectra were acquired from 200 laser shots in reflectron mode using a 2.5 kV extraction voltage with a 100 ns delay and a 20 kV accelerating voltage.

**3.4 RESULTS AND DISCUSSION**
3.4.1 Nanopost Arrays (NAPA). Ion yields were systematically studied as a function of NAPA post diameters, $D = 50$-600 nm, heights, $H = 200$-1500 nm, and periodicities, $P = 200$-1200 nm. Even for high aspect ratio posts, such as $H/D = 10$, reactive ion etching yielded uniform post dimensions with minimum tapering and vertical walls, as shown in the SEM image in Figure 3.1A. For comparison, posts with low aspect ratio, $H/D = 1.8$, are shown in Figure 3.1B. Posts are fabricated on a square grid of 500 µm X 500 µm, yielding approximately 2,200,000 posts for $P = 337$ nm (Figure 3.1A) and approximately 346,000 posts for $P = 850$ nm (Figure 3.1B). The 10 nm layer of chromium on the top of the posts, visible in the SEM images, was necessary to aid in the nanofabrication of high aspect ratio posts. Since these NAPA share similar nanophotonic ion production characteristics as LISMA, which are made solely of silicon, the chromium caps on NAPA do not seem to contribute to their nanophotonic nature.

3.4.2 Laser Desorption Ionization from NAPA. Small organics and biomolecules, deposited on the NAPA structures, are efficiently desorbed and ionized by 337 nm nitrogen laser radiation of $\sim 20$ mJ/cm$^2$ and above. Figure 3.2A depicts the high mass region of the low fluence positive mass spectrum of the neuropeptide substance P (RPKPQFFGLM). The peptide sequences are described by a one letter code and the fragmentation nomenclature follows the conventions introduced by Biemann[26]. Protonated molecules are produced with high abundance accompanied by low amounts of alkalinated products and a fragment corresponding to $m/z$ 14 loss. In the low mass region ($m/z < 560$), backbone cleavage ($a_2$, $b_2$), internal fragments (PK-28/KP-28, QF, KPQ-28) and immonium ions (R-87 or P-17, K/Q, K/Q-28, M-28, R-45, F-28, K/Q-45) are prevalent. Other small peptides (bradykinin (RPPGFSPFR), leucine enkephalin (YGGFL), angiotensin I (DRVYIHPFHL), and GFL, etc.) give similar results.
**Figure 3.1** SEM images of nanopost arrays with dimensions of (A) D = 100 nm, H = 1000 nm, and P = 337 nm and (B) D = 500 nm, H = 900 nm, and P = 850 nm which corresponds to an aspect ratio of 10 in (A) and 1.8 in (B). Images show uniform post dimensions, vertical walls, and chromium caps on the top of the posts.
Figure 3.2 (A) Positive ion mass spectrum of substance P from NAPA exhibits peaks corresponding to the molecular and quasimolecular ions with high resolution, as shown by the isotope distribution of the molecular ion in the inset. Reprinted with permission from ref. [27]. Copyright [2010], American Chemical Society. (B) Negative ion mass spectrum of citric acid from NAPA with D = 175, P = 575, and H = 1000 nm. The structure of citric acid is shown in the inset. Reprinted with permission from ref. [28]. Copyright [2010], American Institute of Physics.
Small organics composed of preformed ions (organic salts), like verapamil hydrochloride, produce very clean spectra (not shown) dominated by the protonated molecule, with negligible fragmentation corresponding to the loss of the 3,4-dimethoxyphenylmethyl moiety. Preformed ions exist as charged entities already in the solid phase or as an adsorbate. Thus they do not require an ionization step and can be used to probe the desorption process separately. The desorption of verapamil from the NAPA structure is found to be very efficient resulting in an ultralow detection limit of 6 attomoles.

Negative ions can also be generated from NAPA. Figure 3.2B shows a mass spectrum of citric acid obtained in negative ion mode from NAPA with $D = 175$ nm, $P = 575$ nm, and $H = 1000$ nm. The deprotonated molecular ion is the most abundant peak and a few other small fragments are also detected.

The reusability of NAPA was investigated by taking mass spectra of various peptides repeatedly from the same substrate and sonicating it in methanol and water baths between the experiments. Our results showed minimal cross contamination between runs and no nanopost damage was observed after sonication. The minor cross contamination is likely due to the ultra low limit of detection, pointing to the importance of thorough cleaning between experiments. Furthermore, storage of the NAPA structures for over 1.5 years did not impact the performance of these structures. These results indicate the potential for reusability for these structures and stability in the ambient environment.

### 3.4.3 Ion Yield Resonances and Fragmentation

To explore the impact of NAPA geometries on peptide ion production, the yields of quasimolecular ions are followed for a variety of nanofabricated structures. Of the three main parameters, $D$, $H$, and $P$, the post heights have the strongest influence on the ion yields. Figure 3.3A shows the yields of substance P ions as a function of the post aspect ratio, $H/D$, for
Figure 3.3 (A) High aspect ratio posts exhibit ion yield resonances for substance P. (B) Modest gains are observed as the periodicity shifts from, $P = 450$ nm (■) to $P = 600$ nm (▼) for posts with $D = 200$ nm. Reprinted with permission from ref. [27]. Copyright [2010], American Chemical Society.
different post diameters. Compared to the H/D = 1 case, the large diameter posts, D = 500 nm, have modest ion yield maxima at a low aspect ratio of 2.4 that shows a factor of 7 increase. As the posts become thinner, D = 200 and 100 nm, the maxima shift to higher aspect ratios, H/D = 6 and 10, respectively, and the ion yields show more dramatic gains. The largest gain, a factor of 55 compared to the H/D = 1 case, is observed for D = 200 nm at an aspect ratio of six. In terms of post height, the D = 500 and 200 nm posts show maximum ion yields at H = 1,200 nm and the D = 100 nm post is most efficient at H = 1,000 nm. This corresponds to H/λ ≈ 3.0 to 3.6 and an optimum gain of 55.[7]

This resonance-like behavior is analogous to the aspect ratio-dependent gains in the near-field intensity observed for spheroidal silicon protrusions through fluorescence.[8, 29] Studying the effect of probe length on field enhancement around an apertureless near-field probe, Bohn and coworkers have found that for a tip radius of curvature R_c = 10 nm the intensity enhancement of κ^2 ≈ 225 is the highest at a/R_c ≈ 12, where a is the semimajor axis length of the spheroid. They attributed the drop-off of the enhancement at higher aspect ratios to the emergence of internal resonances in the probe. For the protrusion with R_c = 5 nm the enhancement factor continues to grow up to κ^2 ≈ 250 at a/R_c ≈ 20, the highest studied aspect ratio. The general trend in this data is similar to our ion yield observations. Slender posts in both cases produce strong enhancement in the signal at aspect ratios that shift to higher values for smaller diameters. Despite the similarities, the interactions of the posts with the laser radiation are significantly different in the two studies. This is directly manifested in the corresponding skin depth, δ, values. While the silicon in the fluorescence study has a skin depth of ~1100 nm, i.e., their near-field probe is practically transparent, in our work, δ ≈ 84 nm resulting in strong absorption of the incident light in the posts. Further differences arise from the interactions between the posts in our large arrays.[7]
The effect of the NAPA periodicity on the ion yields of substance P was studied as a function of aspect ratio for posts with D = 200 nm, P = 450 to 600 nm, and H/D = 1 to 7.5. Figure 3.3B shows data from periodicities of 450 nm and 600 nm and maximum ion yields are observed for H/D = 6. The lowest ion yields at H/D = 6 are observed for P = 450 nm and the highest are observed for P = 600 nm, with the amplitude of the gains of 18 and 30, respectively. This indicates a very modest dependence on periodicity, but a very strong dependence on post aspect ratio. The observed resonances are likely due to the near-fields around a post rather than interactions between other posts in the array.[7]

To observe the role of the aspect ratio on desorption rates, salts of thermometer ions were deposited on NAPA structures with different post aspect ratios. The thermometer ions, such as 4-methyl-benzylpyridinium (4M), in these salts exist in the ionic form already as an adsorbate. Therefore, by observing their ion yields in their mass spectra (shown in Figure 3.4A) we can directly monitor the desorption process, separate from the ionization step. Figure 3.4B depicts the threshold fluence necessary to produce 4M ions from NAPA structures of increasing aspect ratio. As the fragmentation of the thermometer ion reflects elevated surface temperatures of the substrate and at the fragmentation threshold fluence their decomposition is negligible, it is thought that the related surface temperatures are below the value required for fragmentation. Thus, the decrease in the desorption threshold for higher aspect ratios is not the consequence of elevated temperatures and this effect can be viewed as the direct manifestation of enhanced near fields for higher aspect ratios.

A near-field equivalent of the “shadowing effect,” that is observed in the far-field for macroscopic objects, may also occur in the case of nanoscopic structures.[30] In NAPA this shadowing effect may be present if the periodicity of the structures is too small or if the structures are too tall. Similar to near-field
**Figure 3.4** (A) Mass spectrum showing the 4M thermometer ion and negligible amount of fragments at the threshold laser fluence. (B) Threshold laser fluence required to desorb ions from NAPA of increasing aspect ratios, H/D, shows a declining trend. Reprinted with permission from ref. [28]. Copyright [2010], American Institute of Physics.
fluorescence experiments where shadowing results in a decrease in fluorescence, in nanophotonic ion sources shadowing may reduce the desorption yields.

In thin posts, e.g., D = 100 nm, additional enhancement of the desorption rate at a given fluence level can be attributed to higher surface temperatures resulting from energy confinement. When the diameter of the posts falls below the thermal diffusion length, the dissipation of the deposited laser energy through heat conduction is hindered. This results in elevated surface temperatures.[31] In the mass spectra of the 4M thermometer ion (Figure 3.5A), this effect is manifested in reduced survival yields, i.e., the fraction of ions that remains intact, at elevated fluences (Figure 3.5B). Although this phenomenon is not the result of a nanophotonic interaction, due to the post dimensions that exhibit such effects, the energy confinement is often observed in the corresponding structures.

Adsorbates close to the surface experience strong electric fields and radiation intensities that, depending on the aspect ratio of the posts, can be up to 200 times higher than the incident laser intensity. These conditions can promote ionization and induce fragmentation through a yet unknown mechanism. Table 3.1 summarizes the fragment ions produced by a nitrogen laser pulse on a typical NAPA from model peptides GFL and substance P. In addition to some of the a, b and y-series ions commonly observed in high energy collision activated dissociation (CAD)[32] and in surface induced dissociation (SID)[33], the decomposition products observed from NAPA include internal fragments and immonium ions. In place of the commonly observed ammonia loss from the protonated molecule at low energies, the loss of 14 Da is detected. Based on the size distribution and the nature of the fragments it seems that low energy and high energy decomposition channels are operational simultaneously. Currently only nanophotonic ionization techniques, LISMA and NAPA, produce peptide molecular ions and their structure specific fragments without additional ion activation.
**Figure 3.5** (A) Mass spectrum showing the fragmentation channels of the 4M thermometer ion at a laser fluence above the threshold. (B) As the laser fluence increases the survival yield of the 4M ions desorbed from NAPA declines. Reprinted with permission from ref. [28]. Copyright [2010], American Institute of Physics.
Table 3.1 Fragmentation of small peptides in laser desorption ionization from NAPA substrates (D = 200 nm, H = 1000 nm and P = 500 nm for substance P and D = 200 nm, H = 800 nm and P = 500 nm for GFL). Reprinted with permission from ref. [27]. Copyright [2010], American Chemical Society.
<table>
<thead>
<tr>
<th>Peptides</th>
<th>Quasi-molecular ions</th>
<th>14 loss</th>
<th>a ions</th>
<th>b ions</th>
<th>c ions</th>
<th>y ions</th>
<th>Y ions</th>
<th>Internal fragments</th>
<th>Immonium ionsᵦ</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFL</td>
<td>M+H, M+Na, M+K</td>
<td>M+H-14</td>
<td>a₂</td>
<td>b₂</td>
<td>c₂</td>
<td>y₁, y₂</td>
<td>Y₁</td>
<td></td>
<td>F-28</td>
</tr>
<tr>
<td>Substance P</td>
<td>M+H, M+Na, M+K</td>
<td>M+H-14</td>
<td>a₂, a₂⁻</td>
<td>b₂</td>
<td>c₂</td>
<td>PKPQQ-28, PQQ, KPQ-28</td>
<td>R-87 or P-17, K/Q, K/Q-28, M-28, R-45, F-28, K/Q-45</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ᵦThe peptide fragmentation nomenclature follows the conventions introduced by Biemann[26].

ᵦImmonium ions undergo consecutive losses of 17, 12, 29, etc. This notation starts from the intact immonium ion of a residue and marks the losses in nominal mass units.
3.5 CONCLUSIONS

Nanophotonic ion sources, such as LISMA, offer a new means of coupling laser radiation with nanostructures.[24] The ability of such nanostructures to exhibit localized electromagnetic resonances results in a unique ionization mechanism. In order to systematically study the desorption and ionization process, we nanofabricated NAPA so that we could produce high aspect ratio structures with a range of periodicities. The strong near-fields in the vicinity of slender posts facilitates the desorption and ionization of small molecules. Resonant ion production for high aspect ratio posts resulted in 55-fold signal enhancement.

Furthermore, the threshold fluence for ion generation is also lower for slender structures. This is explained in terms of energy confinement in structures thinner than the thermal diffusion length. As a consequence, the surface temperatures of these posts sharply increase with decreasing diameter. This results in enhanced energy transfer to the adsorbate, and higher ion yields and internal energies. As a result, NAPA-based ion sources offer adjustable fragmentation not available for ion sources currently used in the structure elucidation of peptides and proteins. The traditional techniques rely on CAD or other ion activation techniques to produce structure specific fragments.

Of the three varied parameters, the periodicity has the least significant impact on the ion yields. Only a marginal dependence is observed, where larger periodicities produce slightly higher ion yields. This indicates that the localized field enhancements around the posts contribute to desorption and ionization and the shadowing effect is detrimental to the production of ions.

The power to shape the laser light-nanostructure interaction through structural properties and to efficiently optimize the resulting enhanced ion yields is unique to nanophotonic ion sources. Further understanding of such nanophotonic ion sources can lead to the development of very efficient sources tailored for specific
needs. Beyond their obvious use for ion production in mass spectrometry, they are also candidates for energy harvesting and as solar cell materials. In addition, due to their small dimensions, these sources are amenable for integration with microfluidic separation devices and miniaturized mass spectrometers.

### 3.6 ACKNOWLEDGEMENTS

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3.7 REFERENCES


CHAPTER 4

POLARIZATION DEPENDENT FRAGMENTATION OF IONS
PRODUCED BY LASER DESORPTION FROM NANOPOST ARRAYS

Based on material published by J.A. Stolee and A. Vertes in


4.1 ABSTRACT

Tailored silicon nanopost arrays (NAPA) enable controlled and resonant ion production in laser desorption ionization experiments and have been termed nanophotonic ion sources (Walker et al., J. Phys. Chem. C, 2010, 114, 4835–4840). As the post dimensions are comparable to or smaller than the laser wavelength, near-field effects and localized electromagnetic fields are present in their vicinity. In this chapter, we explore the desorption and ionization mechanism by studying how surface derivatization affects ion yields and fragmentation. We demonstrate that by increasing the laser fluence on derivatized NAPA with less polar surfaces that have decreased interaction energy between the structured silicon substrate and the adsorbate, the spectrum changes from exhibiting primarily molecular ions to showing a growing variety and abundance of fragments. The polarization angle of the laser beam had been shown to dramatically affect the ion yields of adsorbates. For the first time, we report that by rotating the plane of polarization of the desorption laser, the internal energy of the adsorbate can also be modulated resulting in polarization dependent fragmentation. This polarization effect also resulted in selective fragmentation of vitamin B12. To explore the internal energy of NAPA generated ions, the effect of the post aspect ratios on the laser desorption thresholds and on
the internal energy of a preformed ion was studied. Elevated surface temperatures and enhanced near fields in the vicinity of high aspect ratio posts are thought to contribute to desorption and ionization from NAPA. Comparison of the fluence dependence of the internal energies of ions produced from nanoporous silicon and NAPA substrates indicates that surface restructuring or transient melting by the desorption laser is a prerequisite for the former but not for the latter.

4.2 INTRODUCTION

Nanophotonics is a vigorously developing discipline because the nanostructures commensurate with the wavelength of the radiation confer unique capabilities for a wide range of applications such as telecommunications and biosensing.[1-2] A new approach is to make use of nanophotonic interactions to produce ions for mass spectrometric analysis.[3-4] When an electromagnetic field is coupled with nanostructures, strong light confinement and near-fields enhance the light-matter interactions[5] and can cause material desorption, ionization and ablation.[6] When biomolecules are adsorbed on nanostructures, such as laser-induced silicon microcolumn arrays (LISMA)[7] or nanopost arrays (NAPA)[3], similar effects are thought to contribute to the desorption and ionization of these molecules. At this time, LISMA and NAPA are the only ion sources shown to exhibit nanophotonic ion production.

Matrix-assisted laser desorption ionization (MALDI) is an established ionization technique for the mass analysis of intact large biomolecules.[8] Due to the spectral interferences introduced by matrix-related ions in the low mass range, a multitude of matrix-free ionization platforms have emerged.[9-13] Desorption ionization on silicon (DIOS), which utilizes a nanoporous silicon
substrate, has received the most attention due to its excellent sensitivity, simplicity, and high throughput.[14] The surface morphology of DIOS substrates, mainly the pore size and porosity, have been shown to play a large role in the efficiency of desorption and ionization of adsorbates.[15-16]

Nanophotonic ion production provides new opportunities compared to traditional ionization sources. For example, NAPA dimensions can be tailored to optimize ion production. Ion yield resonances were observed for nanoposts with specific high aspect ratios that exhibit strong local fields and elevated surface temperatures.[3]

Molecular ion yields from LISMA and NAPA also exhibit maxima as a function of polarization, analogous to the optical properties of nanoantennas.[3, 17-20] This can be attributed to strong axial absorption of the electromagnetic field in the case of p-polarized beams, resulting in high ion yields, and insufficient excitation of the axial modes in the case of s-polarized radiation, producing little to no molecular ions. In contrast, polarization had no effect on the ion yields from MALDI.[3]

A third feature of nanophotonic ion sources is that the degree of fragmentation can be modulated by the laser fluence. At low laser intensities exclusively the molecular ion is observed, whereas at increased laser intensities structure specific fragmentation takes place.[3, 21] Enhanced electromagnetic fields around the nanostructures, surface activation due to ion-surface collisions, and new reactive fragmentation channels are all thought to influence ionization and fragmentation from LISMA and NAPA. These mechanisms are fundamentally different from MALDI that produces low internal energy ions[22-23] and commonly relies on an additional activation step to induce fragmentation.[24] Similarly, DIOS produces molecular ions with some fragmentation and the ion internal energy is not affected by the desorption laser fluence.[12, 25]
Control over molecular fragmentation is advantageous for elucidating the structure of biomolecules and for peptide sequencing. A crucial parameter governing the extent of fragmentation is the internal energy of the primary ions.[26] The ion internal energy can be gauged by applying the survival yield (SY) method to thermometer ions (TI).[26-27] These preformed ions allow the desorption step to be probed separately from the ionization step. The SY of the molecular ion is derived from the mass spectrum and corresponds to the ratio the molecular ion intensity to the total ion intensity (the sum of the molecular and fragment ion intensities). This method has been used to investigate ion internal energies from a variety of ionization sources including electrospray ionization,[28] MALDI,[22-23, 29-32] DIOS,[16, 25] and LISMA.[21]

Fragmentation studies are not limited to thermometer ions, and the ion activation mechanisms and fragmentation of vitamin B\textsubscript{12} have also been studied by MALDI[33-34], DIOS[35], plasma desorption[36], and laser desorption ionization.[37] In these experiments, vitamin B\textsubscript{12} acquires sufficient internal energy to readily fragment. Studying the propensity of vitamin B\textsubscript{12} fragmentation and comparing this with other methods can also give insight into the internal energy transfer from NAPA. A better understanding of the desorption and ionization mechanism from nanophotonic ion sources might enable us to adjust the transfer of laser energy for controlled ion yields and fragmentation.

In this chapter we use a threefold approach to study the desorption and fragmentation of adsorbates from NAPA. First, we investigate the use of derivatized NAPA for reduced fragmentation at low to moderate laser powers to gain an understanding of the influence of surface chemistry on ion disintegration. Second, we explore the effect of polarization on the ion yields and survival yields of thermometer ions. This gives insight into the polarization dependence of energy deposition and fragmentation on NAPA surfaces. The applicability of
polarization dependent fragmentation to heterocyclic macrocycles is shown by studying the fragmentation pathways of vitamin B$_{12}$. Thirdly, we systematically vary the NAPA post aspect ratios to study their effect on the laser threshold and fragmentation during the desorption process. Our results show that fragmentation can not only be adjusted by varying the laser fluence and changing the NAPA post aspect ratios, but also by rotating the plane of polarization of the desorption laser.

4.3 EXPERIMENTAL

4.3.1 Materials. Aqueous stock solutions of Pro$_{14}$-Arg (P14R) from Protea Biosciences, Inc., (Morgantown, WV) and vitamin B$_{12}$ (cyanocobalamine) purchased from Sigma Chemical Co. (St. Louis, MO) were prepared in 50% methanol at 1 mg/mL concentration and were diluted as necessary. The chloride salt of the 4-methyl-benzylpyridinium (4M) thermometer ion (TI) was custom synthesized by Celestial Specialty Chemicals (Nepean, Ontario, Canada). Stock solutions were prepared in 50% methanol and diluted to 0.7 µM. All solutions were made using deionized water (18.2 M Ω·cm) produced with an E-pure system (Barnstead, Dubuque, IA) and reagent grade methanol purchased from Sigma Chemical Co. (St. Louis, MO). For the derivatization of the NAPA, (perfluorophenyl)-propyldimethylchlorosilane was purchased from Gelest, Inc. (Morrisville, PA).

4.3.2 Nanofabrication of NAPA. A detailed description of NAPA fabrication can be found elsewhere.[3] Briefly, silicon wafers with a range of resistivities (0.005-100 Ω·cm) were spin-coated with Zep520A resist. Patterns designating the top view of NAPA, with diameters, D, between 100 nm and 250 nm and periodicities, P, of 337 nm and 674 nm, were rendered on the wafers by e-beam lithography (JBX-9300,
JEOL, Peabody, MA). The exposed resist was then removed and the wafers were coated with 10 nm of chromium using vacuum deposition. Following the removal of the unexposed resist that is coated with chromium, posts of ~1000 nm heights were produced with an Oxford PlasmaLab100 RIE reactive ion etching system. For surface characterization of the structures a dual beam scanning electron microscope (SEM) (Nova Nanolab 600, FEI, Hillsboro, OR) was used.

Optionally, perfluorophenyl (PFP)-derivated NAPA were produced by oxidizing the surfaces by exposure to ozone, covering the oxidized NAPA with ~15 µL of (perfluorophenyl)-propyldimethylchlorosilane, and baking the surfaces in an oven for 30 min. The derivatized NAPA were thoroughly rinsed with methanol and deionized water and air-dried.

4.3.3 Mass Spectrometry. A 0.5 µL drop of the sample solution was deposited on the NAPA surface and air-dried prior to mass spectrometry experiments. Laser desorption and ionization was performed using a high-resolution curved field reflectron time-of-flight mass spectrometer (Axima CFR, Shimadzu-Kratos, Manchester, UK). Averaged spectra were acquired from 100 laser shots in reflectron mode using delayed ion extraction with 2.5 kV extraction voltage, 20 kV accelerating voltage, and a 100 ns delay.

Experiments using plane polarized laser beams were conducted using a home-built linear time-of-flight mass spectrometer. Radiation from a 4 ns pulse length nitrogen laser ($\lambda = 337$ nm) (VSL-337ND, Laser Science Inc., Newton, MA) was polarized by a Glan-Taylor calcite polarizer. A variable neutral density filter was used to maintain a constant pulse energy while the plane of polarization was rotated. The areas of sample specific peaks were integrated from averaged spectra to obtain the ion yields.
4.4 RESULTS AND DISCUSSION

Figure 4.1 shows a representative SEM image of a NAPA segment with post diameter, D, periodicity, P, and height, H, of 100 nm, 337 nm, and 1000 nm respectively. The dimensions of NAPA are precisely controlled in the nanofabrication process and the resulting high aspect ratio structures have vertical walls with no noticeable taper. SEM images also verified that the NAPA remained intact after laser desorption experiments, although for posts with diameters smaller than the thermal diffusion length transient melting is still possible.[38]

4.4.1 Fragmentation from Surface Derivatized NAPA. It has been shown that both low- and high-energy fragmentation channels are active in NAPA.[3] To further explore this finding, we introduce chemical derivatization to change the interaction energy between the adsorbate and the surface. Silylation is a reliable method for the chemical modification of silicon surfaces and the diversity of available reagents provides a means to control the polarity of the substrate. Perfluorophenyl (PFP)-derivation creates less polar and more hydrophobic surfaces and results in decreased interaction energy between the structured silicon substrate and the adsorbate. As a consequence, for other silicon-based platforms lower laser desorption thresholds have been observed.[14, 21]

Similarly, NAPA derivatized with PFP results in up to ten times higher peptide molecular ion yields than native NAPA (data not shown). Although the mechanism is unclear, native NAPA is also capable of producing structure specific peptide fragmentation. To characterize fragmentation from derivatized NAPA, the labile synthetic peptide Pro14-Arg (P14R \textit{m/z} of 1533.86) was used. P14R has a basic arginine at the C-terminus and in MALDI experiments it is known to undergo post-source fragmentation at the proline residues.[39] The nomenclature of
Figure 4.1 After reactive ion etching, SEM imaging confirms the uniform distribution and negligible tapering of high aspect ratio posts in a NAPA with \( D=100 \) nm, \( P=337 \) nm, and \( H=1000 \) nm. Reprinted with permission from ref. [40]. Copyright [2011], Royal Society of Chemistry.
peptide fragmentation is described in ref. [41].

Figure 4.2A shows the mass spectrum of P14R from PFP-NAPA at relatively low laser power (75 in arbitrary units). The most abundant peak is the sodiated P14R molecule. Additionally, C_{14} ions are present due to the loss of the arginine residue. This indicates that the low-energy fragmentation channels may already be activated at this power. As the laser power is increased to 80, high mass P14R fragments begin to appear (Figure 4.2B). A significantly increased overall abundance of fragment ions can be observed if the laser power is further increased to a high value of 130 (Figure 4.2C), whereas the molecular and quasimolecular ions decrease in intensity. These fragments correspond to sodiated a-type ions, a result of proline residue cleavage from the N-terminus, and b-type ions that appear with lower intensity (not labeled).

This latter fragmentation pattern dramatically differs from that of MALDI, in which the intensity of the y-type ions precipitously diminishes as subsequent prolines are lost. In the case of PFP-NAPA, the fragment ion intensities increase with sequential proline losses. This suggests that at this laser power, high-energy fragmentation channels are activated.

**4.4.2 Polarization Dependent Fragmentation.** The polarization of the desorption laser has a profound effect on the ionization processes in nanophotonic ion sources. For peptides and small organic molecules, p-polarized radiation generates high molecular ion yields, whereas s-polarized laser pulses result in little or no ion production.[3] To investigate the effect of polarization on the internal energy transfer on NAPA during the desorption step, the survival yield method was applied using the 4M thermometer ion. As 4M is a preformed ion, it enables us to separate the desorption and the ionization steps and focus only on the former. The mass spectra of 4M exhibit a single fragmentation channel and
Figure 4.2 (A) Mass spectrum of P14R from PFP-NAPA at a laser power of 75 (arbitrary units) shows minimal fragmentation. (B) As the power is increased to 80 fragments begin to emerge. (C) At a laser power of 130, structure specific fragmentation, corresponding to the sodiated N-terminal fragment ions (a- and c-type), is observed. Reprinted with permission from ref. [40]. Copyright [2011], Royal Society of Chemistry.
the only peaks that are present are the molecular ion and a fragment ion, which is the result of a pyridine loss.

First, the total ion yield of the 4M thermometer ion was measured as the polarization angle, $\phi$, was rotated from s- to p-polarized. The inset in Figure 4.3 shows that the total ion yield of 4M exhibits a maximum for the p-polarized ray and the ion yield decreases significantly as the beam is rotated to s-polarized. At 180° there is a sharp maximum with an order of magnitude increase compared to the s-polarized signal. This implies that the angle of polarization affects the ability of NAPA to absorb the laser energy resulting in efficient desorption of the adsorbate.

In the laser desorption process, the energy transfer from NAPA to the adsorbate can be assessed by monitoring the SY as a function of the angle of polarization. Figure 4.3 shows a pronounced minimum in the SY when the desorption laser is p-polarized. As the plane of polarization is rotated from s- to p-polarized, the SY starts from 1, indicating no fragmentation, and decreases to 0.4, indicating significant fragmentation. This drastic fall in the SY indicates a significant rise in the internal energy that is a strong function of the polarization. As a consequence, the polarization can be adjusted to vary the degree of fragmentation while still maintaining sufficient ion yields.

The polarization dependence of ion yields from NAPA reveal that the axial currents induced in the posts play a large role in the desorption process. As the 337 nm radiation corresponds to 889 THz, the DC conductivity of the silicon wafers should be irrelevant in the energy deposition. Indeed, desorption experiments on NAPA with a range of DC resistivities between 0.005 $\Omega \cdot$cm and 100 $\Omega \cdot$cm revealed minimal change in the SYs (results not shown).

To test the application of adjusting fragmentation via polarization, a neutral molecule, vitamin B$_{12}$ (see Figure 4.4), was chosen as a model compound.
Figure 4.3 As the polarization angle, $\phi$, of the desorption laser is rotated from s-polarized (at 90°) to p-polarized (180°) the degree of fragmentation of the 4M TI, represented by the survival yield, reaches a minimum. The inset shows that the ion yield of the 4M TI rises sharply for p-polarized radiation. Reprinted with permission from ref. [40]. Copyright [2011], Royal Society of Chemistry.
**Figure 4.4** Vitamin B$_{12}$ molecular structure showing the losses associated with four of the fragment ions detected in NAPA experiments. Fragment ion (1) corresponds to [(M + K)-CN-DMBI-pentose-PO$_4$-CH$_2$CONHCH$_2$CHCH$_3$]$^+$ at m/z 913, (2) is [M-CN-DMBI-pentose-PO$_4$-C$_3$H$_6$]$^+$ at m/z 931, (3) is [M-CN-DMBI-pentose-PO$_4$]$^+$ at m/z 972 and (4) is [M-CN-DMBI-pentose-PO$_3$]$^+$ at m/z 989, where DMBI is dimethylbenzimidazole. Reprinted with permission from ref. [40]. Copyright [2011], Royal Society of Chemistry.
Vitamin B$_{12}$ was selected because it fragments readily in laser desorption experiments and comparisons can be made with other methods that have studied its fragmentation. Direct laser desorption ionization of vitamin B$_{12}$ from polished silicon produced no molecular ions from the adsorbate. With PFP-NAPA, positive mode mass spectrometric experiments at high laser power resulted in 9 fragments along with the molecular ion. These previously observed species include two of the three fragment ions detected with DIOS, four of the five ions observed with traditional MALDI, and eight of the thirteen ions produced in a postionization experiment.[35, 42] At lower laser fluences the four most abundant peaks observed are at m/z 913, m/z 931, m/z 972, and m/z 989 that correspond to fragments (1), (2), (3) and (4), respectively, in Figure 4.4. The molecular structure of vitamin B$_{12}$ along with these four losses is shown in Figure 4.4 and a mass spectrum of these peaks is shown in the inset of Figure 4.5.

The ion yields of two fragment peaks from PFP-NAPA were studied as a function of the polarization angle (see Figure 4.5). While most of the fragment intensities only exhibit a weak maximum for the p-polarized beam, the peak at m/z 972 shows a strong resonance. This fragment is formed by the loss of the 5,6-dimethylbenzimidazolyl nucleotide, which is the main fragmentation channel in these experiments indicating that the corresponding C-O bond is the weakest in the structure. Therefore it exhibits the strongest fragmentation response to increasing the internal energy of the molecule. This species is also a characteristic vitamin B$_{12}$ fragment in DIOS experiments, and its formation has been explained by the absorption maximum of the molecule at 361 nm and the dissociation of the photoactivated molecule.[35] In the case of NAPA, however, the fragmentation likely follows different pathways. As we have shown in the case of 4M TI, the enhanced energy deposition into the posts for p-polarized beams results in higher internal energy. This mechanism is also expected to be active for
Figure 4.5 Ion yields for two of the most prominent vitamin $B_{12}$ fragments from PFP-NAPA as a function of polarization angle, $\varphi$, at a constant fluence. The mass spectrum in the inset shows the peaks of four vitamin $B_{12}$ fragments and the ion yields of the two peaks with symbols are shown. The numbers above the peaks correspond to the losses shown in Figure 4.4. Reprinted with permission from ref. [40]. Copyright [2011], Royal Society of Chemistry.
the vitamin B<sub>12</sub> molecule. In addition, the field enhancement around the posts is also accentuated for the p-polarized case, potentially resulting in stronger field ionization.[43] Thus, in addition to changing the laser power, the polarization of the laser can also be utilized for the adjustable fragmentation of a molecule.

Other mechanisms of vitamin B<sub>12</sub> ion excitation have been proposed. In UV-MALDI, the internal energy transfer was found to primarily depend on the matrix used, and photodissociation was found not to play a role in the fragmentation.[33] Likewise, photodissociation does not explain the polarization dependent fragmentation from NAPA. Instead, the interaction of the electromagnetic field with the posts clearly plays a large role in the ionization and fragmentation of the molecules desorbed from NAPA.

Optical antennas show strong resonances when the electromagnetic field is polarized along the axis of the antenna structures whereas no such resonances are observed when the light is perpendicular to the antenna’s long axis.[19, 44] In NAPA, the polarization dependence has been explained in terms of strong axial absorption of p-polarized laser radiation. Results from both the thermometer ion and vitamin B<sub>12</sub> studies show that the internal energy of the adsorbate is preferentially increased for p-polarized radiation, as shown by the increased fragmentation. The fragmentation and ion yield resonances from NAPA can both be explained by the energy deposition into the posts and the electric field intensity enhancements around them. The polarization dependence of fragmentation shows the importance of the electric field enhancements on fragmentation, insight that cannot be derived from the fluence dependence of fragmentation alone.

4.4.3 Post Aspect Ratio and Surface Polarity Effects. To further our understanding of the desorption mechanism, SYs from native and derivatized
NAPA with post aspect ratios (H/D) ranging from 10 and 4 with periodicities of 337 nm and 674 nm were investigated. The points with the lowest fluence for the SY curves in Figure 4.6 also represent the desorption threshold for the corresponding post geometry. For both native and PFP-derivatized NAPA, the minimum laser fluence required to desorb the 4M TI shifts to higher values as the post diameter increases, i.e., as H/D decreases (see Figure 4.6). For example, in the case of the thinnest posts with diameters of 100 nm (H/D = 10), 4M is desorbed with a fluence of less than 5 mJ/cm$^2$, whereas almost 60 mJ/cm$^2$ is required to desorb 4M from 250 nm posts (H/D = 4). This 12-fold change in the desorption threshold fluence can be attributed to enhanced near fields in the vicinity of the high aspect ratio posts and to the higher surface temperatures observed for posts with diameters below the thermal diffusion length.[45-46]

For the range of post diameters studied, SYs from native NAPA decrease as the laser fluence is increased, Figure 4.6A. This decline correlates to higher internal energy transfer due to elevated post surface temperatures at higher laser fluences. In addition, when the post diameters are smaller than the thermal diffusion length, dissipation of the deposited laser energy in the posts is obstructed by radial confinement. From Figure 4.6A it can be observed that the SY drops at a faster rate as the post diameters are decreased (see the slopes of the fitted lines in Figure 4.6A). The thinner posts have lower conductive thermal loss, reach higher maximum surface temperatures and heat more uniformly than the thicker ones. These high surface temperatures provide a stronger driving force for the energy transfer to the adsorbate.

The effect of surface derivatization on the internal energy of the 4M TI is shown in Figure 4.6B. It is evident that PFP-derivatization of NAPA reduces the internal energy transfer to the adsorbate, as indicated by the generally higher SYs. The SY values either remain close to one for thinner posts or decrease only
Figure 4.6 Survival yields of 0.35 pmol of 4M TI from (A) native NAPA and (B) PFP-derivatized NAPA with $P = 337$ nm for $H/D = 10$ (●), 8 (▲), and 6.7 (▼), and $P = 674$ nm for $H/D = 5.7$ (○), 5 (▼), 4.4 (Δ) and 4 (■), and a resistivity of $10^{-100}$ Ω•cm. In panel (A) the SYs from native NAPA show a steep decline, from values close to 1 to between 0.5 and 0.6, as the laser fluence is increased. In (B) the SYs from PFP-NAPA with $P = 337$ nm remain close to 1 whereas SYs from NAPA with $P = 674$ nm decrease from 1 to 0.8. In contrast, the SY of 35 pmol of 4M TI desorbed from PFP-derivatized DIOS substrates is significantly lower, $SY = \sim 0.4$, and it shows no change with increasing fluence (data from ref. 26). Reprinted with permission from ref. [40]. Copyright [2011], Royal Society of Chemistry.
moderately to around 0.8 for the thicker ones. In addition, the threshold laser fluence shifts to slightly lower values for derivatized NAPA. These effects are due to the reduced interaction energy between the polar TI and the derivatized NAPA surface that is apolar.

The SY values and their fluence dependence for 4M desorbed from PFP-NAPA are in stark contrast to observations on PFP-DIOS, for which a constant SY of around 0.4 has been observed independent of the laser fluence. In DIOS experiments the lack of fluence dependence on the internal energy of the ions was attributed to the limited interaction time between the DIOS surface and the adsorbates due to rapid heating. Energy deposition in NAPA will also cause the post temperatures to increase rapidly resulting in the fast desorption of the TI. Therefore, this does not explain the differences in the magnitude and fluence dependence of the SYs between the two methods. This drastic disparity might be attributed to differences in the morphology of the DIOS and NAPA structures. While DIOS exhibits 20 to 100 nm pores separated by pore walls of similar thickness, the studied NAPA structures have 100 to 250 nm posts separated by approximately twice as wide troughs. The thin pore walls of the DIOS structure reach temperatures above the melting point in the fluence range of interest. These high temperatures result in the reduced SYs observed for DIOS. For the significantly thicker posts of NAPA, however, the surface temperatures remain lower at similar fluences explaining the higher SYs.

These observations and Figure 4.6B point to the fundamental difference between the ion production from DIOS and NAPA substrates. Whereas for DIOS surface restructuring or transient melting by the desorption laser seems to be a prerequisite for ion production, for NAPA there is no such requirement. These differences are reflected in the absolute values and the fluence dependence of the SYs.
4.5 CONCLUSIONS

Nanophotonic ion sources such as LISMA and NAPA offer new means of coupling laser radiation to nanostructures for ion production. The interaction between the electromagnetic radiation and the nanoposts can be manipulated to induce desorption, ionization, and fragmentation. We demonstrate that NAPA enables adjustable structure specific fragmentation as a function of the laser fluence. This allows enhanced structure identification in the mass spectrometry of organic molecules and for peptide sequencing without using an additional ion activation step to induce fragmentation.

We have found that the desorption yield of preformed ions and neutral molecules strongly depend on the polarization of the laser beam and for the first time we have also demonstrated that the internal energy transfer, and consequently the degree of fragmentation, are also affected by polarization. It was shown that certain fragment ions are more sensitive to polarization, and this may lead to a method that can preferentially fragment a molecule. This enhanced control over fragmentation opens new possibilities in structure elucidation of organic compounds using mass spectrometry. In particular the ability to adjust fragmentation via the polarization angle might obviate the need for collisional activation in tandem mass spectrometry. Conversely, simple single stage mass spectrometers can be used to explore the structure of simple organic compounds.

Investigating the impact of the NAPA dimensions on the internal energy of adsorbates gave insight into the mechanism of desorption from NAPA. Thermal confinement in thin posts leads to more efficient desorption and fragmentation. Findings show that the internal energy of the adsorbate increases as the fluence is raised. This is consistent with biomolecule fragmentation at high laser fluences. High surface temperatures and enhanced near fields around the posts can contribute to the desorption and ionization. By modifying the surface polarity, the
interaction energy between the adsorbate and NAPA is affected, and less internal energy is transferred to the thermometer ions.

Comparison of the ion yields and SYs from NAPA and DIOS substrates reveals fundamental differences. Whereas restructuring and transient melting are a prerequisite for ion production from DIOS, they do not seem to play a role in the case of NAPA.

We have demonstrated that nanophotonic interactions offer new means for the production of ions from organic molecules and biopolymers with enhanced control over their internal energy. The NAPA substrates in this study are an example of such nanophotonic ion sources. Better understanding of desorption and ionization from these ion sources will enable even greater control of the ion yields.

4.6 ACKNOWLEDGEMENTS

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4.7 REFERENCES


CHAPTER 5

Toward Single Cell Analysis by Plume Collimation in Laser Ablation Electrospray Ionization Mass Spectrometry

Based on submitted material by J.A. Stolee and A. Vertes

5.1 ABSTRACT

Ambient ionization methods for mass spectrometry have enabled the in situ and in vivo analysis of biological tissues and cells. When an etched optical fiber is used to deliver laser energy to a sample in laser ablation electrospray ionization (LAESI) mass spectrometry, the analysis of large single cells becomes possible. However, because in this arrangement the ablation plume expands in three dimensions, only a small portion of it is ionized by the electrospray. Here we show that sample ablation within a capillary helps to confine the radial expansion of the plume. Plume collimation, due to the altered expansion dynamics, leads to greater interaction with the electrospray resulting in increased ionization efficiency, reduced limit of detection (by a factor of ~13, reaching 600 amol for verapamil), and extended dynamic range (6 orders of magnitude) compared to conventional LAESI. This enhanced sensitivity enables the analysis of a range of metabolites from small cell populations and single cells in the ambient environment. This technique has the potential to be integrated with flow cytometry for high throughput metabolite analysis of sorted cells.

5.2 INTRODUCTION

The analysis of metabolite distributions in biological systems can give insight into the physiological and disease states of an organism and is critical to understanding its
response to environmental stimuli and drugs. However, the detection of the over ~2,700 currently known metabolites present in the human metabolome at widely varying concentrations requires the use of a high-throughput technique that can analyze physically and chemically diverse small molecules.[1] Due to the selectivity and quantitative capabilities of mass spectrometry, it has become an indispensable tool in the emerging field of metabolomics.[2, 3]

Compositional heterogeneity within cell populations can be used to characterize different phenotypes on fewer and fewer cells, ultimately at the single cell level.[4] New cell isolation methods, such as laser capture microdissection, are being developed to extract cells prior to chemical analysis by mass spectrometry.[5] Mass spectrometric techniques for single cell analysis include matrix-assisted laser desorption ionization, secondary ion mass spectrometry has been used to image the subcellular distributions of lipids, and capillary electrophoresis coupled with electrospray ionization (ESI) has enabled the analysis of metabolites from single neurons.[6-8] While these methods facilitate the use of mass spectrometry for single cell analysis, they require elaborate sample preparation and cannot follow the dynamic processes involved in cellular metabolism. Thus, techniques with in situ capabilities, high sensitivity, and a wide range of quantitation are becoming increasingly important.

To obtain an accurate snapshot of the metabolite profiles in a sample, extensive efforts are devoted to developing ionization techniques for the rapid analysis of biological specimens in their native environment.[9] Since the introduction of desorption electrospray ionization (DESI)[10] in 2004, numerous ambient ion sources have emerged ranging from those based on spray extraction, e.g. DESI, to plasma sampling and ionization, e.g., direct analysis in real time (DART)[11], to laser based techniques, e.g., laser ablation electrospray ionization (LAESI)[12], among a host of others.[13]
Some ambient methods, e.g., LAESI currently show promise for in situ single cell analysis. Optical fibers provide a convenient and robust method for microsampling by laser pulses. When an etched optical fiber was used to deliver laser energy to a sample in LAESI, the analysis of large single cells with an average volume of ~1 nL became possible.[14] This technique provided information on coexisting cell phenotypes and their metabolic differences in heterogeneous populations.[15, 16] However, due to their minute sample volumes (as low as a few femtoliters) and the limited number of some molecules, the analysis of smaller cells becomes more challenging.

LAESI utilizes 2.94 µm wavelength laser radiation that is strongly absorbed by the native water content present in biological samples. Rapid energy deposition by a pulsed laser in the ambient environment initiates surface evaporation, shock-wave emission, and, at elevated fluences, phase explosion.[17, 18] These processes result in a radially expanding plume of vapor and fine droplets originating from the sample that is ionized by an electrospray plume of highly charged droplets. Because the ablation plume expands in three dimensions, only a small portion of it is ionized by the electrospray plume, resulting in low ionization efficiencies.

Here we present sample ablation experiments performed in a capillary to minimize the radial expansion of the ablation plume. We demonstrate that by altering the ablation plume dynamics we are able to achieve higher ion yields and increased sensitivity probably due to an improved overlap with the electrospray plume. Plume collimation in LAESI enables the detection of metabolites from small populations of human and animal cells including single cells from the latter. We use a diverse group of cells to demonstrate the feasibility of this method, including β-cells derived from the rat pancreas that are responsible for insulin secretion and are important in diabetes, epithelial cells from the buccal mucosa of a human, megakaryoblast cells derived from the human bone marrow, and individual sea
urchin eggs. Biologically relevant metabolites are detected from these diverse set of cells.

5.3 MATERIALS AND METHODS

5.3.1 Plume Collimation in Laser Ablation Electrospray Ionization. Mass spectrometry experiments were conducted in positive ion mode with orthogonal acceleration time-of-flight mass spectrometers. Small cell populations were studied on a Q-TOF Premier instrument, whereas the single cell experiments were conducted on a Synapt G2-S system (Waters CO., Milford, MA). Both of them were equipped with a home-built electrospray system was described elsewhere.[12] Briefly, a syringe pump (Physio 22 or 11 Plus, Harvard Apparatus, Holliston, MA) was operated to supply 50% methanol solution with 0.1% acetic acid (v/v) to a tapered tip emitter at a rate of 300 nL/min. A power supply (PS350, Stanford Research System, Inc., Sunnyvale, CA) was used to apply high voltage to the emitter to generate the electrospray. The emitter was placed on axis with the mass spectrometer orifice, with its tip located 10 to 12 mm away from it. In conventional LAESI, a plano-convex focusing lens was used to focus laser radiation at 2.94 µm wavelength provided by a Nd:YAG laser driven optical parametric oscillator (Opolette 100, Opotek Inc., Carlsbad, CA).

In the plume collimation experiments, laser radiation was delivered through a germanium oxide (GeO₂) optical fiber (450 µm core diameter, HP Fiber, Infrared Fiber Systems, Inc., Silver Spring, MD) with a tip etched in a 2% nitric acid solution as described earlier.[14] Optical fibers with a 200 µm core diameter were also tested. Borosilicate and quartz capillaries with inner diameters, ID, ranging from 0.6 mm to 1 mm (Sutter Instrument, Novato, CA) were cleaved to lengths, L, ranging from 2 mm to 6 mm with a sapphire scribe and used to hold the liquid sample. The
capillary was held in place with a fiber clamp (HFF001, Thorlabs, Newton, NJ) and its position was adjusted by a three-axis translation stage. Ablation was performed by inserting the optical fiber tip into the liquid sample that is held inside the capillary, as shown in Figure 5.1A. The fiber was held by a fiber chuck and positioned with a five-axis translator (BFT-5, Siskiyou Corporation, Grants Pass, OR). Similar to conventional LAESI, the ablation products were ionized by an electrospray.

### 5.3.2 Chemicals
HPLC grade methanol and water were from Alfa Aesar (Ward Hill, MA) and acetic acid was from Fluka (St. Louis, MO). Verapamil, bradykinin, and reserpine were from Sigma Aldrich (St. Louis, MO) and stock solutions were prepared in 50% methanol.

### 5.3.3 Cell Culturing and Sampling
Epithelial β-cells (RIN5mF) from the rat pancreas were kindly provided by Aleksander Jeremic of the George Washington University and megakaryoblast cells (MEG-01) were purchased from ATCC (CRL-2021, Manassas, VA). Cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (ATCC, Manassas, VA) in a 5% humidity controlled incubator at 37 °C. Prior to experiments, β-cells were detached from the culture dish with trypsin-EDTA (Invitrogen, Grand Island, NY) and suspended in medium. The medium was removed, and the β-cells were suspended in a 10× diluted phosphate buffered saline (PBS) solution. Prior to experiments the number of cells in the capillary was determined under a microscope. Human buccal mucosa epithelial cells were collected from a healthy subject. For the experiments with the megakaryoblast cells, the medium was removed and the cells were suspended in 10× diluted PBS solution.
5.3.4 **Single Cell Experiments.** Unfertilized sea urchin eggs (*Lytechinus pictus*) kept frozen at -80 °C in artificial seawater were revived and diluted in HPLC water. The sea urchin eggs did not visibly lyse. Single sea urchin eggs were extracted from the solution using a manually pulled glass capillary with an inner diameter ~90 µm which was held and controlled by a microdissection system (TransferMan NK2 and CellTram Air, Eppendorf, Hauppauge, NY). The selected cell was deposited into a 0.5 µL droplet of HPLC water and the droplet containing the cell was subsequently pipetted into a capillary for the plume collimation LAESI experiments.

5.4 **RESULTS AND DISCUSSION**

5.4.1 **Plume Collimation: Controlling Factors.** In the plume collimation experiments the capillary length and diameter, the position of the capillary relative to the electrospray and mass spectrometer orifice, and the fiber parameters were systematically varied to obtain optimal ion yields. Using verapamil solution samples, it was found that the capillary length, which ranged from 2 to 8 mm, and the inner diameter, which ranged from 0.6 mm to 1 mm, had minimal impact on the ion intensities. As the outer diameter of the fiber was 450 µm, it was easiest to work with capillaries having inner diameters larger than 750 µm or larger. The fiber tip was inserted approximately 200 µm into the sample solution prior to the onset of ablation.

The schematics in Figure 5.1 illustrate the major differences between the plume collimation experiments and conventional LAESI experiments. Collimation within the capillary results in a quasi-one-dimensional plume expansion (see Figure 5.1A), whereas conventional LAESI (depicted in Figure 5.1B) yields a freely expanding hemispherical ablation plume. Because of limited volume of the collimated plume, the position of the capillary relative to the electrospray had a strong effect on the signal intensity. The capillary and the electrospray emitter were kept in the same
Figure 5.1 (A) Schematic top-view of LAESI with plume collimation. An etched optical fiber is inserted into a glass capillary of inner diameter, ID, and length, L, that contains the sample. Upon ablation in the capillary, a collimated plume emerges (shown in blue) and is ionized by an electrospray. Ablation in the capillary leads to a forward directed plume. (B) Schematic side-view of conventional LAESI. The laser beam is focused by a lens onto the sample. Due to the hemispherical expansion of the ablation plume, only a small portion of it is intercepted by the electrospray.
plane by maintaining their elevation equal in the z direction and the intercept of their axes located approximately 4-5 mm from the mass spectrometer orifice (see x in Figure 5.1A). The end of the capillary was approximately 10 mm away from the intercept (see y in Figure 5.1A). This position provided the highest verapamil signal intensities probably due to the maximum overlap between the two plumes. Moving away from the optimized geometry, the signal intensities decreased by up to two orders of magnitude or became undetectable.

In the plume collimation experiments, an etched optical fiber tip is used to deliver the laser energy into the sample. We also explored how the sharpness of the fiber tip affected the ablation in the capillary. It was found that a flat, cleaved fiber end did not deliver sufficient energy for the ablation of the sample. Sharp fibers, with approximately 20 µm tip diameters, provided the best energy coupling for ablation.

5.4.2 Analytical Figures of Merit with Plume Collimation versus Conventional LAESI. To discover the effects of plume collimation on the figures of merit, we compared the measured ion intensities with that of conventional LAESI for a range of analyte concentrations. Solutions of verapamil were prepared in 50% methanol with 0.1% acetic acid from a stock solution of 1.2 mM. A droplet of ~0.5 µL was deposited inside a capillary with an inner diameter of 750 µm. An optical fiber with a tip diameter of ~20 µm was used to deliver laser pulses with 20 Hz repetition rate to the sample until the entire sample was ablated. The mass of the capillary was measured with a semi-microbalance (MS105DU, Mettler Toledo, Columbus, OH) before and after experiments to account for any sample residue remaining in the capillary. A new capillary was used for every solution, and each measurement was repeated three times. Mass spectra obtained during the ablation were averaged and the intensity of the molecular ion was recorded.
**Figure 5.2.** Verapamil ion intensities as a function of solution concentration measured by LAESI with plume collimation (in black) and conventional LAESI (in gray; from Ref. [12]). Plume collimation enables the detection of verapamil with a dynamic range of 6 orders of magnitude versus 4 decades for conventional LAESI. As shown in the inset, at the limit of detection the molecular ion of verapamil exhibits a signal-to-noise ratio greater than 3.
As shown in Figure 5.2, the limit of detection for verapamil using LAESI with plume collimation was at $1.2 \times 10^{-9}$ M concentration, which corresponds to 600 attomoles of analyte. The inset in Figure 5.2 shows that at the limit of detection, the signal to noise ratio is greater than 3. Using linear regression, a correlation coefficient of 0.986 was obtained. This limit of detection is 13 times lower than the value found for conventional LAESI that exhibits a limit of detection of 8 fmol measured at $2.4 \times 10^{-7}$ M concentration. Furthermore, LAESI with plume collimation exhibits a dynamic range extended to 6 orders of magnitude versus the 4 decades found for conventional LAESI.

These greatly improved figures of merit are attributed to delivering more of the ablated sample into the electrospray plume for enhanced ion production. Similar results were obtained with other analytes, such as reserpine and bradykinin. To test the utility of the improved sensitivity, cell populations of diminishing size were analyzed.

5.4.3 Analysis of Mammalian Cells. In situ mass spectrometric analysis of mammalian cells was performed using LAESI with plume collimation. Apart from the obvious advantage of better sensitivity, using a capillary to hold the sample has a few additional benefits. The presence of water is a prerequisite for sample ablation in LAESI. Since in these experiments the cells are suspended in a droplet and held in a capillary, the evaporation rate is significantly decreased compared to conventional LAESI. This allows for the analysis of smaller sample volumes resulting in reduced dilution of the cell contents by the suspending medium. Furthermore, because of the improved sensitivity, a cell suspension, rather than a cell pellet, can be used for analysis.

Potential metabolites from mammalian cells were identified using the accurate mass of the monoisotopic peak, isotope distributions, and literature
searches. As the purpose of this investigation was to demonstrate the detection of metabolites from small cell populations and single cells, the identification of the metabolites is not discussed.

Rat insulinoma β-cells (RIN5mF) with an average cell diameter of 10 µm, corresponding to a single cell volume of ~500 fL/cell, were analyzed with LAESI mass spectrometry utilizing plume collimation. Figure 5.3A shows a spectrum obtained from fewer than 100 β-cells, corresponding to a total cell volume of ~50 pL, suspended in a 0.5 µL droplet of diluted PBS buffer solution. Similar to conventional LAESI, singly charged protonated and potassiated ions were primarily observed. Approximately 35 ions were detected from this sample and more than half of the small molecules have been tentatively assigned to biologically relevant metabolites such as primary amino acids (e.g., alanine at m/z 90.04 and proline at m/z 116.05), nucleobases (e.g., uracil at m/z 135.01 and adenine at m/z 174.02), and phospholipids (e.g., phosphatidylcholine PC(34:1) at m/z 760.54). As expected, a greater number of metabolites were detected with higher signal intensities from more concentrated cell suspensions. Since these cells are adherent, they have an affinity to the inner wall of the capillary and many of them remained there even after ablation. This made the analysis of fewer than 100 cells difficult.

Megakaryoblast cells (MEG-01) that primarily grow in suspension have a lower affinity to the inner wall of the capillary than β-cells. While some of the adherent megakaryoblast cells are large, with diameters up to 50 µm, the average cell diameter of a suspended cell is 18 µm (corresponding to a single cell volume of ~3 pL/cell). Using plume collimation in LAESI, signal was obtained from as few as 20 megakaryoblast cells or a total cell volume of ~60 pL (see spectrum in Figure 5.3B). In this small cell population, spermidine, at m/z 146.15, was one of the significant peaks. Its selected ion chromatogram is shown in the inset of
Figure 5.3. (A) A representative mass spectrum obtained from <100 epithelial β-cells from rat insulinoma by LAESI with plume collimation. The spectrum exhibits a large number and variety of peaks. Microscope image in the inset shows β-cells which are ~10 µm in diameter. (B) A mass spectrum from <20 megakaryoblast cells. The inset shows the selected ion chromatogram of the spermidine ion at m/z 146.15 detected in samples containing 50 to 10 cells.
Figure 5.3b. It can be observed that as the number of cells is decreased from 50 to 10 the intensity of this ion also decreases. Other peaks that were detected in larger cell populations include spermine (m/z 203.22) and phospholipids (e.g., phosphatidylcholine PC(36:4) at m/z 782.48).

5.4.4 Toward Single Cell Analysis. Buccal epithelial cells are somewhat larger than typical mammalian cells, with sizes ranging from 30 to 50 µm that, assuming spherical shape, corresponds to volumes of 14 to 65 pL/cell. In order to produce a sample with a small cell population, a dense cell suspension was diluted in water until the desired concentration was reached. There was no observable cell lysis when the cells were diluted in water. Figure 5.4A shows the signal obtained from the detection of 6 cheek cells (a total cell volume of 84 to 390 pL) enabled by plume collimation. Many of the detected metabolites have been tentatively assigned to metabolites present in a typical diet or those involved in the gut metabolism. For example, peaks at m/z 219.01, 365.10, and 527.17 have been tentatively assigned to a hexose, a disaccharide, and a trisaccharide, respectively. However, the obtained spectra exhibited day-to-day variations, probably due to differences in diet, thus for this study it is more appropriate to have cells that are cultured in a controlled environment.

Frozen sea urchin eggs, with diameters of 90 to 100 µm or volumes of 400 to 500 pL, were thawed and diluted in HPLC water. Individual sea urchin eggs were selected using the micromanipulator system and deposited into a capillary for LAESI mass spectrometry with plume collimation. Peaks corresponding to the electrospray background and HPLC water were subtracted from the sea urchin egg spectrum. Numerous ions were detected from a single sea urchin egg (see Figure 5.4B). Certain regions of the spectrum, i.e., m/z < 137 and m/z > 160, were enlarged to indicate the number of peaks present. Some of the peaks have
Figure 5.4. (A) Mass spectrum obtained by LAESI with plume collimation from six cheek cells contained in water. The inset shows a microscope image of the cheek cells. (B) Mass spectrum of a single sea urchin egg by LAESI with plume collimation. The inset shows the image of a single egg in the capillary before ablation.
been tentatively assigned to metabolites such as glycine \((m/z\ 76.04)\), trimethylamine dimer \((m/z\ 141.14)\) and lipids (e.g., doubly charged phosphatidylcholine PC(35:4) or PC(36:3) at \(m/z\ 403.76\) and doubly charged phosphatidylserine PS(38:7) or PS(37:0) at \(m/z\ 422.73\)) that are known to be abundant in sea urchin eggs.\[19\] These results demonstrate the feasibility of this technique for the analysis large individual animal cells.

**5.4.5 Plume Dynamics.** During the ablation process in LAESI a plume of particulates, droplets, and vapor is formed. It appears that the angular distribution of the expanding ablation plume has a strong effect on the ion intensity. In order to understand the mechanistic differences contributing to the increased ionization efficiencies due to plume collimation, we compare the ablation plume dynamics with that of conventional LAESI.

When a nanosecond mid-IR laser pulse of sufficient fluence impinges on an aqueous sample, the nonlinear absorption of the laser radiation by water initiates surface evaporation, shock-wave emission, and the ejection of fine droplets via phase explosion.\[20-22\] The first stage, i.e., surface evaporation, results in a relatively slow plume expansion.\[18, 22, 23\] Material removal due to this process is not very efficient. However, during phase explosion, i.e., when the target is superheated close to its critical temperature, a rapidly expanding plume of vapor, droplets, and particulate matter is ejected. For example, a 70 ns laser pulse with 2.94 µm wavelength and 5.4 J/cm² fluence induces phase explosion at ~120 ns.\[18\] After ~1 µs, the phase explosion induced recoil pressure results in an efficient secondary material ejection process.

In conventional LAESI, the ablation plume is free to expand in three dimensions. The interaction of the ablation products with the electrospray plume has been studied using fast imaging by flash shadowgraphy.\[12\] Images show a
radially expanding ablation plume in which only a small fraction of the droplets are intercepted by the electrospray plume. It has been estimated that droplets with an effective diameter of 5 µm result in the best interaction with the electrospray droplets which range from 5 to 10 µm in LAESI experiments.[24-25] Time-resolved imaging experiments show a dependence of droplet bouncing, coalescence and separation on the size ratio of the two droplets and the Weber number, a dimensionless quantity dependent on the ratio of kinetic to surface energy.[26]

During confined ablation, such as that in the plume collimation experiments, the radial expansion of the plume is hindered and the plume dynamics are altered. As a consequence, increased pressures and temperatures within the target are reached. The ablation by fiber-transmitted laser pulses in a liquid environment has been a topic of interest due to the implications for medical applications.[27-31] These studies found that the fiber tip generates acoustic radiation that causes significant tensile stress in the water and leads to explosive vaporization, cavitation, and bubble formation.[28-29] The collapse of the generated bubbles can result in the ejection of a high speed liquid jet, and the disruption of tissue in front of the tip.[30, 32-33]

To explore the sample ejection in the plume collimation experiments, images were captured at 5 frames/s during the ablation of water in a 750 µm inner diameter capillary by mid-IR laser pulses with 20 Hz repetition rate delivered by an etched optical fiber (see Figure 5.5). The captured images reveal bubble formation and collapse followed by the ejection of liquid jets and droplets. Figure 5.5A shows the formation of a bubble in the droplet of water after the laser is turned on. In Figure 5.5B, another bubble is visible and the initial bubble is starting to collapse. Figure 5.5C indicates that one of the large bubbles has collapsed and smaller bubbles have formed. This is followed by the ejection of
Figure 5.5. Images show the mid-IR ablation of water in a capillary with an inner diameter of 750 µm by an etched optical fiber. Figures 5.5A-D shows sequential images of bubble formation, collapse and the ejection of liquid from the capillary.
liquid jets, as shown in Figure 5.5D. In the right half of the image the contrast is increased so that the ejection of the liquid jets can be visualized. At other times during the ablation small droplets are expelled from the capillary (not shown). Side jets were also occasionally observed. Due to the low resolution and long exposure time of these images, it difficult to discern the diameter of the ejected droplets, but they are measured to be less than 10 µm. It is also important to note that only the largest droplets were captured in these images and it is assumed that smaller droplets are also being ejected. These large observable droplets are comparable in size to the 5 to 10 µm electrospray droplets, promoting efficient droplet coalescence.

5.5 CONCLUSIONS

Conventional LAESI had been successfully used to analyze large populations of mammalian cells and individual plant cells with volumes greater than 1 nL. However, because the ablation plume is free to expand in all three dimensions, conventional LAESI is characterized by significant sample losses and low ionization efficiencies. Here we show that by performing confined ablation within a capillary, we can achieve higher sensitivity for LAESI ionization. This resulted in lower limits of detection.

The applicability of this technique for the in situ analysis of small cell populations was demonstrated for a diverse group of cell types. In typical ambient ionization mass spectrometry experiments a large cell population (~10^6 cells) or a large cell volume (on the nanoliter scale) is required to obtain strong signal. In these experiments, we demonstrated the analysis of small cell populations, corresponding to ~50 pL of cells, and a large single cell by plume collimation in LAESI.
The improved analytical figures of merit are attributed to a better overlap between the collimated ablation plume and the electrospray. In confined liquid ablation, bubble formation and collapse leads to the ejection of sample related droplets and liquid jets. Future experiments using high-speed photography can shed light on the ablation mechanism in plume collimation and can also give insight into the interaction between the ablation plume and the electrospray. The laser energy deposited through the fiber may also have an effect on the produced droplet size, which in turn will impact the percentage of droplets that coalesce with the electrospray plume. Imaging experiments can be used to optimize the interaction between the two plumes for even higher ionization efficiency.

To produce a more confined plume, the capillary and fiber dimensions can be reduced. This will also enable the analysis of reduced sample volumes. A problem related to using a capillary to hold the sample is the adhesion of the sample to the inner wall. This problem is further exaggerated if smaller sample volumes are used, where even minimal sample losses are significant. Capillaries with different surface properties or derivatized capillaries can be used to decrease the interaction with the sample and ensure that all or most of the material is ablated from the capillary.

Our experiments show that higher ionization efficiencies are necessary to move toward the routine mass spectrometric analysis of single mammalian cells in the ambient environment. Plume collimation in LAESI shows promise in advancing this field. With further optimization, plume collimation in LAESI has the potential to be coupled with flow cytometry for high throughput single cell analysis.
5.6 ACKNOWLEDGEMENTS

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5.7 REFERENCES


CHAPTER 6

Direct Observation of Subcellular Metabolite Gradients in Single Cells by LAESI Mass Spectrometry


6.1 ABSTRACT

Cell microdissection and analysis by laser ablation electrospray ionization mass spectrometry is a promising method for studying the compartmentalization of metabolites within subcellular domains in the ambient environment. Experiments performed on plant epidermal cells imply that there are significant differences between the metabolite composition of the nucleus and the cytoplasm. This technique is applicable to other cell types and may give insight into the mechanisms of disease and drug delivery.

6.2 INTRODUCTION

In recent years an increasing number of methods have been exploited for the proteomic[1-4] and metabolomic[5-8] analysis of single cells and have provided new insight into cellular subtypes. Local analysis on a subcellular level, however, requires new approaches. Heterogeneity of metabolite distributions within a cell is attributed to functional organization, compartmentalization into organelles, macromolecular crowding, and metabolite channeling due to the colocalization of enzymes.[9-10] This results in metabolite gradients within a cell and compartmentalization of metabolites in particular organelles.[11] The intracellular production, reaction, and
redistribution of metabolites do not always follow the kinetics established in vitro at low concentrations.[12] Subcellular trafficking between compartments often relies on active transport facilitated by transporter proteins.[13-14] For example, secondary metabolites can accumulate in the vacuole by the help of ABC transporters.[15-17] Determining the subcellular distributions of metabolites is challenging due to their high diffusion rates and rapid turnover.

Most techniques for the subcellular analysis of eukaryotic cells rely on the isolation of organelles by non-aqueous fractionation and require extensive sample preparation prior to chemical analysis.[18] Using tagging or labeling techniques, the distribution of some preselected metabolites can be followed by fluorescence resonance energy transfer.[19] More recently, cell membrane lipid distributions have been analyzed by secondary ion mass spectrometry (SIMS)[20-21] and selected metabolite levels have been determined in the cytoplasm, cytosolic lipid droplets, vacuole, granule and nucleus by nano-electrospray ionization (ESI) mass spectrometry (MS).[22-25] There are, however, few label-free multispecies methods that capture the spatial localization of diverse metabolites within a cell.

Femtosecond laser pulses have been used for disrupting and dissecting subcellular organelles, such as mitochondria and nuclei, in living mammalian cells.[26-28] This nanosurgery technique, however, is typically performed without opening the cell, therefore the resulting ablation products are not available for analysis.[29-31] In laser ablation electrospray ionization (LAESI) of biological samples, a mid-infrared (IR) laser generates a plume in the ambient environment by bursting the cells open. The ejected material is ionized by an electrospray and analyzed by a mass spectrometer.[32-35] The ablation and analysis of metabolites in single cells has been achieved by delivering the mid-IR laser pulses with an etched optical fiber for LAESI analysis.[8, 36-37] Herein we report the in-situ chemical analysis of metabolites localized in subcellular compartments by the combination of
microdissection and LAESI MS. We demonstrate the direct multispecies molecular analysis of subcellular compartments by this ambient ionization method. Large metabolite gradients between the cytoplasm and nucleus of *Allium cepa* epidermal cells are observed using this novel technique.

### 6.3 EXPERIMENTAL

#### 6.3.1 Laser Ablation Electrospray Ionization Mass Spectrometry. All experiments were carried out with an orthogonal acceleration time-of-flight mass spectrometer (QTOF Premier, Waters Co., Milford, MA) with a mass resolution of 8,000 (fwhm) equipped with a custom built electrospray source. A low noise syringe pump (Physio 22, Harvard Apparatus, Holliston, MA) operating at 300 nL/min was used to pump the electrospray solution, 50% aqueous methanol with 0.1% acetic acid (v/v), through a stainless steel emitter. A regulated power supply (PS350, Stanford Research Systems, Sunnyvale, CA) is used to apply ~ 3,100 V to the electrospray emitter to generate a steady electrospray. Laser radiation at 2.94 µm wavelength and 5 ns pulse length was provided by a Nd:YAG laser driven optical parametric oscillator (Opolette 100, Opotek, Carlsbad, CA) and was focused by a planoconvex calcium fluoride lens (Infrared Optical Products, Farmingdale, NY) into a GeO$_2$ fiber (450 µm core diameter, Infrared Fiber Systems, Inc., Silver Spring, MD). The fiber end used for ablation was etched in a 2% HNO$_3$ solution to produce a tip of ~15-25 µm. The fiber was held by a bare fiber chuck (BFC300, Siskiyou Corporation, Grants Pass, OR) that was attached to a micromanipulator (NMN-21, Narishige, Tokyo, Japan).

#### 6.3.2 Cell Preparation. Purple *Allium cepa* (*A. cepa*) bulbs were purchased from a local store in Washington, DC, and stored at 4° C prior to the analysis. A monolayer
of the epidermal cells from the *A. cepa* bulb was directly removed from the intact parenchyma tissue and mounted onto a precleaned microscope glass slide for the experiments. Optionally, the removed epidermis was stained to enhance the visibility of the nuclei. In this case, the wet surface of the epidermis was immersed in an aqueous solution of 0.05% toluidine blue water for one minute, rinsed with distilled water, and then mounted onto a precleaned microscope glass slide.

**6.3.3 Cell Microdissection.** Tungsten microdissecting needles with 1 µm tip diameter (RS-6065, Roboz Surgical Instrument Co., Gaithersburg, MD) or 5 µm tip diameter (72-0424, Harvard Apparatus, Holliston, MA) were placed in a microdissecting needle holder (RS6060 or RS6061, Roboz Surgical Instrument Co., Gaithersburg, MD) that was attached to a micromanipulator (MN-151, Narishige, Tokyo, Japan). To visualize the cells during the microdissection, a long distance video microscope with a 7× precision zoom optic (Edmund Optics, Barrington, NJ), a 2× infinity-corrected objective lens (M Plan Apo 2×, Mitutoyo Co., Kanagawa, Japan), and a CCD camera (Marlin F131, Allied Vision Technologies, Stadtroda, Germany) was placed orthogonal to the prepared epidermal tissue. An additional long distance video microscope with a 7× precision zoom optic (Edmund Optics, Barrington, NJ), a 5× infinity-corrected objective lens (M Plan Apo 5×, Mitutoyo Co., Kanagawa, Japan), and a CCD camera (Marlin F131, Allied Vision Technologies, Stadtroda, Germany) was placed at a shallow angle to the sample surface to monitor the distance between the fiber tip and the cell.

**6.3.4 Data Collection and Analysis.** An orthogonal acceleration time-of-flight mass spectrometer was used to collect and analyze the positive ions produced by LAESI after microdissection. No sample related ions were observed when the laser was off. The electrospray solvent mass spectra were subtracted from the LAESI mass
spectra in the MassLynx 4.1 software (Waters Co., Milford, MA). For the quantitative comparison of mass spectra from the nucleus and the cytoplasm, they were normalized by the sum of all ion intensities. EZinfo software (Version 2.0.0.0, Umetrics AB, Sweden) within the MarkerLynx application manager was used to perform orthogonal projections to latent structures discriminant analysis (OPLS-DA) multivariate statistical treatment with Pareto scaling.

6.3.5 Organelle Extraction and Electrospray Ionization. Additional experiments were performed using a nanoelectrospray emitter (Picotip, New Objective, Woburn, MA) with a 15 µm inner tip diameter to extract the nucleus or cytoplasm from the epidermal cells. First, the cells were stained with a 1% aqueous solution of methylene blue chloride, which was diluted in 50% methanol solution (v/v). In this case, the wet surface of the epidermis was immersed in the dye solution for one minute, rinsed with distilled water, and then mounted onto a precleaned microscope glass slide. The extraction of the nucleus or cytoplasm was performed under visualization by an upright microscope (BX51, Olympus America Inc., Center Valley, PA) equipped with long working distance objective lenses. The emitter was held by a micromanipulator (MN-151, Narishige, Tokyo, Japan) and used to pierce the cell. The targeted cell contents were extracted into the emitter by capillary action. Subsequently, the tip was connected to an electrospray apparatus and the contents in the emitter were electrosprayed. The electrospray solution (50% methanol/water and 0.1% acetic acid) was pumped through the system at 300 nL/min by a syringe pump (Physio 22, Harvard Apparatus, Holliston, MA) and 1800-2200 V high voltage was applied to the emitter by a power supply (PS350, Stanford Research Systems, Sunnyvale, CA). An orthogonal acceleration time-of-flight mass spectrometer (Q-TOF Premier, Waters Co., Milford, MA) with a mass resolution of 8,000 (fwhm) was used to collect and analyze the positive ions produced from the
extracted samples. The electrospray solvent spectra were subtracted from the sample-related spectra in the MassLynx 4.1 software (Waters Co., Milford, MA).

6.4 RESULTS AND DISCUSSION

6.4.1 Cell Microdissection. The schematic in Figure 6.1 shows the essential features of the experimental setup labeled with the critical dimensions. Epidermal cells from the A. cepa bulb were used as a model system in the form of an intact monolayer. The cell nuclei were visible without histological staining (see Figure 6.2A). A cellular monolayer of epidermal tissue was mounted onto a glass slide and a micromanipulator equipped with a microdissection needle with a tip diameter of ~1 µm was used for the microdissection. The tip was lowered to the cell wall to pierce and cut it along the inner edge, and peel it back to expose the cytoplasm and the organelles. The neighboring cells were not disrupted during the microdissection, and the nucleus remained intact (see Figure 6.2B). Immediately after the microdissection, a germanium oxide based optical fiber with a tip diameter of ~15 µm was brought adjacent to the nucleus, as shown in Figure 6.2C, to deliver mid-IR laser pulses at a wavelength of 2940 nm. The microdissection and ablation were visualized with a top-view long distance microscope in order to pinpoint the targeted cellular component. A side-view microscope was used to monitor the distance between the cell surface and the needle or the fiber. The ablation products were ionized by charged droplets from an electrospray that was on axis with the inlet orifice of a mass spectrometer.

6.4.2 Metabolite Gradients. To establish the spectra corresponding to the entire cell, initially intact epidermal cells were ablated and analyzed (see Figure 6.3A). Subsequently, microdissection was performed on an epidermal cell to expose the subcellular components. When targeting the cytoplasm away from the nucleus with
**Figure 6.1** Schematic of the subcellular LAESI setup with microdissection. Sample (Sample) is mounted on an x-y-z translation stage. Microdissection is performed by a sharp tungsten needle (µtip) followed by mid-IR laser ablation of the subcellular compartment using an etched optical fiber (Fiber). Mass spectrometer orifice (MSO) and electrospray emitter (ES) are on the same axis at a distance of \( d_2 = 12 \text{ mm} \). Sample is positioned at \( h = 15 \text{ mm} \) below this axis. The projection of the point of dissection to this axis is at \( d_1 = 7 \text{ mm} \) away from the MSO. The polar angles of the microdissection tip and the fiber are \( \theta_T = 45\text{-}60^\circ \) and \( \theta_F = 45\text{-}60^\circ \), respectively. The corresponding azimuthal angles are \( \theta_{Txz} = 120\text{-}135^\circ \) and \( \theta_{Fxz} = 45\text{-}60^\circ \), respectively. Reprinted with permission from ref. [38]. Copyright [2012], John Wiley and Sons.
Figure 6.2. Microscope images of *A. cepa* epidermal cells show (A) the intact cells and the targeted nucleus prior to microdissection in the dotted white circle; (B) the microdissection tip as it peels back the cell wall exposing the nucleus; and (C) the etched optical fiber tip as it is brought adjacent to the nucleus prior to ablation. Reprinted with permission from ref. [38]. Copyright [2012], John Wiley and Sons.
**Figure 6.3.** Mass spectra obtained from (A) a single intact *A. cepa* epidermal cell, (B) the cytoplasm of a microdissected cell, and (C) the nucleus of a microdissected cell. Distinct differences can be observed between the spectra from the cytoplasm and the nucleus. Reprinted with permission from ref. [38]. Copyright [2012], John Wiley and Sons.
a) single cell

b) cytoplasm

c) nucleus
the fiber tip, LAESI MS yielded a feature rich spectrum (see Figure 6.3B), with many of the same peaks that were detected for the intact single cell. Mass spectra from the cell cytoplasm contained peaks primarily corresponding to singly protonated molecules, quasimolecular (sodiated and potassiated) ions, and a few dimers. Tentative peak assignments were based on accurate mass measurements, information found in databases, such as the Plant Metabolic Network database (http://plantcyc.org/), and the related literature, as well as previous experimental results from LAESI analysis of A. cepa cells, which included tandem MS measurements. The tentative identification of selected peaks is shown in Table 6.1. For example, highly abundant hexose, alliin, and oligosaccharides were among the putatively assigned metabolites detected in the cell cytoplasm.

As the sharpened tip of the optical fiber was comparable in size to the ~20 µm diameter of the cell nucleus, selective ablation of the nuclear region was possible. A significant numbers of peaks, corresponding to over thirty metabolites, were detected from the nucleus (see Figure 6.3C). Due to the lower volume of material in a nucleus than that of the cytoplasm or a single cell, the mass spectra obtained from it exhibited lower signal intensities. As expected, some common metabolites were detected between the nucleus and cytoplasm since some cross-contamination by the cytoplasm surrounding the nucleus is inevitable. However, the relative intensities of metabolites varied. For example, the peak at m/z 219 (potassiated hexose) was one of the strongest peaks from the cytoplasm and, in most cases, had a relative intensity less than 40% in the nucleus. Comparisons between the normalized intensities of ions in the nucleus and cytoplasm can be found in Figure 6.4. The ratios of the peak intensities between the nucleus and cytoplasm are also noted in Table 6.1.

To identify metabolites with strong variance between the nucleus and the cytoplasm, multivariate statistical analysis, in particular orthogonal projections to
Table 6.1. Tentative metabolite assignments for a select number of peaks detected from the nucleus and cytoplasm after microdissection. The ID corresponds to the metabolite ID shown in Figure 6.4. The ratio of the normalized peak intensities in the nucleus and cytoplasm, $I_{\text{nuc/cyt}}$, is also given. Reprinted with permission from ref. [38]. Copyright [2012], John Wiley and Sons.
<table>
<thead>
<tr>
<th>ID</th>
<th>m/z meas.</th>
<th>m/z calc.</th>
<th>Δm (mDa)</th>
<th>Metabolite</th>
<th>Formula</th>
<th>I nuc/cyt</th>
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<td>C_3H_5NO_2 (+H^+)</td>
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<td>127.0508</td>
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<td>C_5H_6N_2O_2 (+H^+)</td>
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<td>147.0770</td>
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<td>C_5H_10N_2O_3 (+H^+)</td>
<td>4.4</td>
</tr>
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<td>152.0348</td>
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<td>C_9H_6NO (+H^+)</td>
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<td>175.1195</td>
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<td>C_6H_14N_2O_2 (+H^+)</td>
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<td>9</td>
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<td>203.0532</td>
<td>-2.0</td>
<td></td>
<td>C_6H_12O_6 (+Na^+)</td>
<td>0.8</td>
</tr>
<tr>
<td>10</td>
<td>219.0271</td>
<td>219.0271</td>
<td>0.0</td>
<td></td>
<td>C_6H_12O_6 (+K^+)</td>
<td>0.5</td>
</tr>
<tr>
<td>11</td>
<td>399.0896</td>
<td>399.0899</td>
<td>0.3</td>
<td>N-Formyl-L-methionine</td>
<td>C_6H_14NO_3S (+K^+)</td>
<td>0.4</td>
</tr>
<tr>
<td>12</td>
<td>216.0129</td>
<td>216.0097</td>
<td>-3.2</td>
<td></td>
<td>C_6H_13NO_5 (+H^+)</td>
<td>1.0</td>
</tr>
<tr>
<td>13</td>
<td>290.0855</td>
<td>290.0785</td>
<td>-7.0</td>
<td>Procyanidin</td>
<td>C_{30}H_{26}O_{12} (+H^+ + H^+)</td>
<td>0.5</td>
</tr>
<tr>
<td>14</td>
<td>317.1053</td>
<td>317.1113</td>
<td>6.0</td>
<td>Glutamylphenylalanine</td>
<td>C_{14}H_{18}N_2O_5 (+Na^+)</td>
<td>0.6</td>
</tr>
<tr>
<td>15</td>
<td>325.1154</td>
<td>325.1135</td>
<td>-1.9</td>
<td>Glucosan or dextrin unit</td>
<td>C_6H_12O_5 (2M+H^+)</td>
<td>2.3</td>
</tr>
<tr>
<td>16</td>
<td>343.1250</td>
<td>343.1235</td>
<td>-1.5</td>
<td>Disaccharide (2 hexose units)</td>
<td>C_{12}H_{22}O_{11} (+H^+)</td>
<td>1.3</td>
</tr>
<tr>
<td>17</td>
<td>360.1471</td>
<td>360.1500</td>
<td>2.9</td>
<td></td>
<td>C_{12}H_{22}O_{11} (+NH_4^+)</td>
<td>0.5</td>
</tr>
<tr>
<td>18</td>
<td>365.1055</td>
<td>365.1054</td>
<td>-0.1</td>
<td></td>
<td>C_{12}H_{22}O_{11} (+Na^+)</td>
<td>0.6</td>
</tr>
<tr>
<td>19</td>
<td>381.0793</td>
<td>381.0799</td>
<td>0.6</td>
<td></td>
<td>C_{12}H_{22}O_{11} (+K^+)</td>
<td>0.5</td>
</tr>
<tr>
<td>20</td>
<td>358.1198</td>
<td>358.1226</td>
<td>2.8</td>
<td>Aspartylglycosamine Tetrasaccharide (4 hexose units)</td>
<td>C_{12}H_{21}N_2O_8 (+H^+)</td>
<td>0.2</td>
</tr>
<tr>
<td>21</td>
<td>362.0968</td>
<td>362.1020</td>
<td>5.2</td>
<td></td>
<td>C_{24}H_{42}O_{21} (H_2O+K^+ + H^+)</td>
<td>2.8</td>
</tr>
<tr>
<td>22</td>
<td>443.1128</td>
<td>443.1284</td>
<td>15.6</td>
<td>Pentasaccharide (5 hexose units)</td>
<td>C_{30}H_{52}O_{26} (H_2O+K^+ + H^+)</td>
<td>n/a^a</td>
</tr>
<tr>
<td>23</td>
<td>543.1440</td>
<td>543.1328</td>
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<td>Trisaccharide (3 hexose units)</td>
<td>C_{18}H_{32}O_{16} (+K^+)</td>
<td>4.8</td>
</tr>
<tr>
<td>24</td>
<td>571.2153</td>
<td></td>
<td></td>
<td>Unknown</td>
<td></td>
<td>n/a^a</td>
</tr>
</tbody>
</table>

^a This ion was not detected in the cell cytoplasm.
**Figure 6.4.** Comparison of the normalized intensities of some metabolites detected in the nucleus (black) and cytoplasm (gray). The metabolite ID corresponds to the tentative metabolite assignments shown in Table 6.1. Reprinted with permission from ref. [38]. Copyright [2012], John Wiley and Sons.
latent structures discriminant analysis (OPLS-DA), was performed on the mass spectra. This method enabled us to separate predictive components, i.e., those responsible for the differences between the two organelles, from non-predictive variations, i.e., those describing the differences between one nucleus and another. The resulting S-plot, shown in Figure 6.5, highlights the metabolites with high correlation and covariance. For example, metabolites that are more specific to the cytoplasm, such as hexose (m/z 203 and 219) and alliin (m/z 178), are in upper right corner, whereas those that are more characteristic of the nucleus, such as arginine (m/z 175) and glutamine (m/z 147) are in the lower left corner. The points located near the center represent metabolites that are not statistically different in the two regions. The putative assignments of the statistically different metabolites (labeled with their m/z values) are listed in Table 6.2.

These results demonstrate that hexose and some of the secondary metabolites, e.g., alliin, primarily accumulate in the cytoplasm. Indeed, hexose is known to be more abundant in the vacuoles, and it has been suggested that this is the result of an active uptake mechanism.[39-40] In contrast, some amino acids were more readily detected in the nuclei. Although there have been very few studies on the localization of metabolites in plant nuclei, other small metabolites, such as flavonoids, have been found to be localized in them.[41-42] Furthermore, the enzyme involved in the metabolism of arginine, arginine decarboxylase, has been found to be localized to the nuclei of non-photosynthetic tissues, which may suggest the presence of arginine in the studied nuclei.[43] As the compartmentalization of metabolites in the nucleus is relatively unexplored, questions remain as to whether these metabolites are locally produced, consumed, or actively transported to them.

6.4.3 Organelle Extraction and Electrospray Ionization. To validate our
Figure 6.5. Metabolites that account for most of the variance in the spectra between the cytoplasm and the nucleus are found by the S-plot. Peaks with high covariance are correlated either to the cytoplasm (top right wing) or to the nucleus (bottom left wing). See Table 6.2 for the putative identification of these metabolites. Reprinted with permission from ref. [38]. Copyright [2012], John Wiley and Sons.
Table 6.2. Data analysis by OPLS-DA identified metabolites with the most variance between the nucleus and cytoplasm. These metabolites are labeled with $m/z_{meas}$. in Figure 6.5. Reprinted with permission from ref. [38]. Copyright [2012], John Wiley and Sons.
<table>
<thead>
<tr>
<th>Compartiment</th>
<th>$m/z_{\text{meas.}}$</th>
<th>Metabolites</th>
<th>Formula</th>
<th>$I_{\text{nuc/cyt}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nucleus</td>
<td>443.1128</td>
<td>Pentasaccharide (5 hexose units)</td>
<td>$C_{30}H_{52}O_{26}(\text{H}_2\text{O}+\text{K}^++\text{H}^+)$</td>
<td>n/a$^a$</td>
</tr>
<tr>
<td>Nucleus</td>
<td>362.0968</td>
<td>Tetrasaccharide (4 hexose units)</td>
<td>$C_{24}H_{42}O_{21}(\text{H}_2\text{O}+\text{K}^++\text{H}^+)$</td>
<td>2.8</td>
</tr>
<tr>
<td>Nucleus</td>
<td>147.0817</td>
<td>Glutamine</td>
<td>$C_6H_{10}N_2O_3(+\text{H}^+)$</td>
<td>4.4</td>
</tr>
<tr>
<td>Nucleus</td>
<td>175.1205</td>
<td>Arginine</td>
<td>$C_6H_{14}N_4O_2(+\text{H}^+)$</td>
<td>13.7</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>203.0552</td>
<td>Hexose</td>
<td>$C_6H_{12}O_6(+\text{Na}^+)$</td>
<td>0.8</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>178.0575</td>
<td>Alliin</td>
<td>$C_6H_{11}NO_3S(+\text{H}^+)$</td>
<td>0.8</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>358.1198</td>
<td>Aspartylglycosamine</td>
<td>$C_{12}H_{21}N_3O_8(+\text{H}^+)$</td>
<td>0.2</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>216.0129</td>
<td>N-formyl-L-methionine</td>
<td>$C_6H_{11}NO_3S(+\text{K}^+)$</td>
<td>0.4</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>219.0271</td>
<td>Hexose</td>
<td>$C_6H_{12}O_6(+\text{K}^+)$</td>
<td>0.5</td>
</tr>
<tr>
<td>Cytoplasm</td>
<td>88.0423</td>
<td>Aminoacrylic acid</td>
<td>$C_3H_3NO_2(+\text{H}^+)$</td>
<td>0.3</td>
</tr>
</tbody>
</table>

$^a$ This ion was not detected in the cell cytoplasm.
findings on the large metabolite gradients between the nucleus and the cytoplasm with independent methods, additional experiments were performed. A cationic dye, toluidine blue, with an affinity to the nucleus was introduced to stain the sample. Optical microscope images confirmed that the stain molecules preferentially localized in the nuclei. In-situ analysis of dissected stained cells by LAESI also detected significantly higher abundance of the dye molecules in the nucleus than in the cytoplasm resulting in an intensity ratio of $I_{\text{nuc/cyt}} = 3.0$. Further confirmation was obtained by separately extracting the nucleus and the cytoplasm from the cells stained by methylene blue using a nanoelectrospray emitter.[44] Images of a cell before and after the extraction of the nucleus confirm its successful removal with minimal damage to the cell (see Figure 6.6A and 6.6B). The captured nucleus is clearly visible at the tip of the emitter (see the inset in Figure 6.6C). Comparison of the mass spectra obtained for the directly electrosprayed nucleus and cytoplasm in the vicinity of the methylene blue molecular ion show significantly stronger peaks for the stain in the nucleus (see Figure 6.6C). Indeed, multiple experiments show an intensity ratio of $I_{\text{nuc/cyt}} = 2.5$. Thus both microdissection combined with LAESI and organelle extraction followed by electrospray ionization indicate that for staining agents our results are consistent with the optically discernible gradient. It is therefore expected that the abundance differences observed for metabolites also reflect existing gradients.

6.5 CONCLUSIONS

In summary, we have demonstrated in-situ ambient analysis of a large number of metabolites from subcellular regions by performing cell microdissection, selective ablation, and LAESI mass spectrometry. This method provides insight into the
Figure 6.6. (A) Microscope image prior to extraction shows stained A. cepa cells with the nuclei clearly visible. The targeted nucleus is emphasized with a black dashed circle. (B) After extraction of the nucleus with the emitter all of the surrounding cells remain intact and there is minimal damage to the targeted cell. (C) Positive ion electrospray mass spectra in the vicinity of the methylene blue ion from the nucleus (top) and cytoplasm (bottom) are compared. The inset shows a nucleus captured inside the emitter before being electrosprayed. Reprinted with permission from ref. [38]. Copyright [2012], John Wiley and Sons.
Methylene blue
284.122
distribution of metabolites on a subcellular level with minimal change to the integrity of the compartments. The results show that the metabolic makeup of the nucleus and cytoplasm are significantly different. The often large concentration gradients may result from the compartmentalization of metabolites, metabolic channeling, and the active uptake of metabolites for, e.g., energy storage or detoxification. Furthermore, local production or consumption of metabolites may also contribute to the differences in the metabolite composition. Additional studies may shed light on the different mechanisms behind producing these metabolite gradients.

Although we used relatively large cells, this technique can be extended to explore subcellular heterogeneity in smaller cells. The current analytical challenge lies in the sensitivity of the mass spectrometer. As single cell technologies advance, we expect fast developments in subcellular analysis. Microdissection combined with LAESI mass spectrometry has the potential to address important biological questions arising from subcellular heterogeneity and give insight into the subcellular variations of metabolic pathways affected by diseases and drug delivery.

6.6 ACKNOWLEDGEMENTS

Financial support from the U.S. National Science Foundation (Grant CHE-1152302), and the George Washington University Selective Excellence Fund is acknowledged. Infrared Fiber systems, Silver Spring, MD, generously provided the GeO\textsubscript{2}-based glass fibers.
6.7 REFERENCES


CHAPTER 7

CONCLUSIONS AND OUTLOOK

Based in part on material published by
J.A. Stolee, B.N. Walker, V. Zorba, R.E. Russo, and A. Vertes in


7.1 PHOTONIC ION SOURCES

7.1.1 Photonic Interactions. Pulsed laser excitation of nanostructures above a certain fluence can give rise to ion production from organic and biomolecular adsorbates. In many cases, the associated phenomena rely on modified optical, electronic, and thermal properties of the substrate due to its nanoscopic structuring. Enhancement of the excitation field in the proximity of the surface, various confinement effects, plasmon resonances, in-plume reactions, and adsorbate-solvent and adsorbate-surface interactions may all affect the mechanism of desorption, ionization, and fragmentation.

Many of these phenomena can be derived from photonic interactions. They result in a new method of producing ions that we have termed photonic ionization. The unique properties associated with the corresponding devices, i.e., photonic ion sources, include polarization and fluence dependent fragmentation, as well as ion yield resonances at particular aspect ratios of the nanoscopic features.

Accumulating evidence points to the importance of the relationship between the characteristic lengths of transport processes and the critical dimensions of the nanostructures plays in ion production, e.g., the aspect ratio for LISMA and NAPA. The confinement of energy, charge or plume material that occurs when a
characteristic length exceeds the critical dimension of the structure can all be important in the underlying ionization mechanisms. With a growing understanding of the mechanisms, there is an increasing possibility of rational nanostructure design for the production of ions.

The ability to control ion decomposition levels from no or little fragmentation to the production of structure-specific fragments through adjusting the laser fluence or polarization is a feature unique to photonic ion sources. This property offers a yet unutilized approach to structure exploration in mass spectrometry. Currently, complex methods and extra instrumentation are required to induce controllable fragmentation in organic and biomolecules. Manipulating the fluence and the plane of polarization in photonic ion sources offers a simpler approach, potentially coupled with a single stage mass spectrometer.

7.1.2 Improvements to NAPA. Chemical modification of the surface to either selectively capture certain adsorbate types or to enhance the desorption ionization yields can also have a major impact on the performance of photonic ion sources. The modification of the substrate, via chemical derivatization, can decrease the adsorbate-surface interaction thus increasing the rate of desorbing and ionizing intact molecules. Introducing protons from the surface or from the solvent trapped in the nanostructure can result in more efficient ionization. Ultimately, ion production from large adsorbates (m/z > 10,000) is very efficient through conventional MALDI, so there is less motivation for the use of nanostructures in that mass range. Therefore, it is more likely that surface modification will find a use in lowering the limit of detection and controlling the internal energy of the produced ions.

Analytical applications of nanostructured ionization platforms benefit from ultra-low limits of detection with a wide dynamic range. Tailoring the dimensions of these structures through nanofabrication has shown that major improvements can be
achieved in the ion yields due to resonant behavior, e.g., at certain aspect ratios for NAPA. A greater understanding of the essential factors, contributing to the laser desorption and ionization mechanisms in these systems, will allow for the optimization of the nanostructure dimensions for efficient ion production. As a result, the application of nanostructured ionization platforms could be extended from metabolomics to other classes of biomolecules.

7.1.3 Future Directions. Primary metabolites in single cells are present at the low femtomole levels. With certain nanostructures exhibiting limits of detection in the zeptomoles to yoctomoles range, they are prime candidates for the panoramic metabolic analysis of single cells. This fast growing field promises new insights into cellular heterogeneity and related biological phenomena. Integration of the culturing and manipulation of cells on these nanostructured platforms can also be achieved by common nanofabrication procedures.[1]

Combining separation with ion production in nanostructured systems promises enhanced capabilities. For example, due to the unique adsorbate–surface interactions on silicon nanowires, molecules in a complex mixture can be separated to different regions of the structure followed by laser desorption ionization of the separated components.[2] Other strategies include substrate-selective binding for the capture of a target molecule. Selective capture by nanostructured platforms is a promising tool for the analysis of the enzymatic digest products of proteins and for the cleanup of complex samples.[3-5] Altering the surface chemistry of the nanostructures can promote selective capture of certain molecules and improve the sensitivity by their accumulation on the surface.

With the advent of ambient ion sources, there is growing interest in shifting the use of nanostructured laser desorption ionization platforms from vacuum to atmospheric pressure.[6, 7] With a large surface area in these systems exposed to
laser excitation, oxygen and humidity in the ambient environment, chemical stability is expected to be a significant issue. Despite the technical difficulties in creating a sufficiently stable laser desorption ionization substrate, the promise of minimal sample preparation and the ability to directly analyze functioning cells can provide ample incentive to pursue their development.

Utilizing the interactions of photonic structures with laser radiation for ion production is beginning to take off. The small size of these versatile multifunctional ionization platforms makes them good candidates for the integration with miniaturized mass spectrometers. Understanding the underlying principles of ion production by nanostructures paves the way for their rational design and integration into complex analytical systems.

7.2 AMBIENT IONIZATION

Ambient ion sources have revolutionized biological mass spectrometry by enabling in vivo analysis with minimal sample preparation. They provide a much more accurate snapshot of the biochemistry within a biological system than ion sources that require vacuum environment. In addition to the wide range of potential metabolic applications, ambient methods have also enabled 10 µm spatial resolution in imaging experiments [8], depth profiling [9], and even tissue analysis during surgical procedures.[10] Due to the losses introduced by the atmospheric pressure interface, however, they suffer from lower sensitivity and a more limited range of quantitation than techniques performed in vacuum.

We have demonstrated that LAESI shows great promise for single cell and subcellular analysis. Higher sensitivity in LAESI was achieved using a collimated ablation plume (see Chapter 5). The feasibility of subcellular analysis by combining cell microdissection with LAESI was established in Chapter 6. To extend the real-world applicability of single cell and subcellular analysis, improvements to these
methods are necessary. Improved atmospheric pressure interfaces and the use of more sensitive mass spectrometers will also aid in the analysis of these volume-limited samples.

7.2.1 Enhanced Sensitivity in LAESI. In addition to providing higher sensitivity in LAESI experiments, plume collimation enabled the analysis of single cells in their native environment. Further optimization of this technique can result in improved plume collimation and ionization efficiencies for routine single cell analysis.

In ESI experiments, the size and velocity of the produced droplets depend on the liquid flow rate and the applied voltage. It has been observed that the cone-jet mode produces droplets with diameters below 3 µm and it is the most efficient regime for ionization.[11] As recent studies indicate that the coalescence between two droplets is dependent on their relative sizes and velocities, systematic adjustment of the electrospray droplet properties can be carried out to ensure efficient coalescence with the ablation generated particles in LAESI.[12] To do so, the plumes can be visualized in fast imaging experiments and the sizes and velocities of the electrospray droplets measured by phase Doppler anemometry.[11, 13-15] These investigations will enable precise optimization of the ESI and laser ablation conditions to minimize losses due to inefficient droplet coalescence.

Fast imaging [16] and fluid dynamics modeling [17, 18] of the ablation plume can also give insight into the plume dynamics and the parameters that control the expansion and particle properties in the ablation plume. The dynamics of a freely expanding ablation plume in the ambient environment is strongly affected by the deposited laser energy.[16] It is expected that the energy per pulse and the repetition rate of the laser will also impact the plume dynamics during confined ablation. When the laser pulse is delivered by an optical fiber, energy deposition into the sample will also be affected by the tip shape of the fiber.
In plume collimation experiments involving cell samples, chemical modification of the capillaries will alter the surface properties of the inner walls enabling either enhanced or decreased interaction of the cells with the capillary. For example, the capillary can be made more hydrophobic to minimize the adherence of cells to the inner walls and facilitate their ejection. The surface can be tailored for different sample types for the most efficient ablation.

In another approach, plume collimation and ablation can take place in a hollow waveguide, eliminating the need for a separate capillary and fiber. The utilization of a hollow waveguide provides a simple means for the retrieval of untreated biological fluids, cells, and tissue contents from the ambient environment and the direct ablation of the extracted sample. Sample ablation in a hollow waveguide is expected to benefit from efficient ESI by producing a collimated ablation plume.

Although the plume collimation experiments are based on ionization by an electrospray plume, other ionization techniques such as photoionization could also be integrated with this system. Atmospheric pressure photoionization has already been successfully combined with infrared laser ablation to ionize not only polar molecules but also nonpolar compounds which tend to be more difficult to ionize by ESI.[19] By introducing new ionization modalities, higher metabolite coverage can be achieved for biological samples.

7.2.2 Subcellular Analysis. One of the most challenging steps in the chemical analysis of subcellular organelles is their isolation. During this step, sampling-related perturbations must be minimized so that the metabolite distributions within the cell and organelles are preserved. In Chapter 6 we described a method in which cell microdissection is performed with a manual micromanipulator to open the cell wall and expose the nucleus to laser ablation prior to analysis by mass spectrometry. This
Microdissection technique is rather crude and manual operation is not amenable to smaller cells and organelles.

Microdissection with micromanipulators controlled via stepper motors enables more precise positioning and may aid in the analysis of other large organelles. For smaller organelles, more sophisticated isolation methodologies are being developed that feature nanometer precision. For example, optical traps and tweezers have had success in isolating labeled organelles, such as mammalian cell lysosomes and mitochondria, with sizes smaller than 1 µm.[20, 21] Resolution as fine as 400 nm is achieved with laser capture microdissection.[22] When coupled with matrix-assisted laser desorption ionization, laser-capture microdissection enabled the isolation and analysis of a small number of cells.[23] However, shifting from the mass spectral analysis of small cell populations to single mammalian cells and organelles requires significant improvements in analytical sensitivity. Although subcellular analysis by mass spectrometry has many challenges ahead, the payoff will be a clearer picture of the metabolic processes in their natural environment, a fully functioning cell.

7.3 HIGH-THROUGHPUT ANALYSIS

In addition to high sensitivity and selectivity, many techniques that rely on mass spectrometry also benefit from high-throughput capabilities. High-throughput analysis is particularly essential for metabolomics studies in which a large number of samples need to be analyzed rapidly.

In current NAPA experiments, the chips are simply taped onto a MALDI target plate prior to analysis. As their dimensions are only 500 µm x 500 µm, which is smaller than the well diameter on our MALDI plate, the size of the MALDI plate will determine how many chips can be analyzed in a session (typically 384 wells per plate). When coupled with automated sample deposition using robotic spotters, the limitation in data acquisition time will be determined by the number of laser shots
per sample. Each experiment typically takes less than a minute per sample, thus enabling high-throughput analysis.

Currently, the LAESI experiments with plume collimation do not allow for high-throughput analysis. It takes time to acquire a sample (particularly for single cell analysis), deposit it into the capillary and insert the optical fiber from the other end, and this needs to be repeated for each new sample. The capillary used for plume collimation in LAESI can potentially be integrated with a flow cytometry system for high-throughput single cell analysis. It is envisioned that a hydrodynamically focused stream of single cells traveling through a flow through capillary would be detected by the scattering or deflection of a continuous laser beam irradiating the capillary. The detection of a cell can be used to trigger a delay generator and activate the mid-infrared laser when the sample reaches the point of ablation at the end of the capillary. Here, conventional focusing optics or an optical fiber can be used to ablate the cell at the open end of the capillary. Ionization of the ablated material can be achieved using electrospray ionization or other techniques such as photoionization.

The growing trend in mass spectrometry is moving towards the analysis of increasingly smaller scales. In order to fully understand the biochemistry occurring within a cell, highly sensitive techniques are needed. These novel photonic and ablation-based ion sources have the potential to make a significant impact in the field of mass spectrometry and they hold great promise for groundbreaking developments in metabolomics.
7.4 REFERENCES


Jessica A. Stolee graduated with cum laude from the George Washington University, Washington, D.C. in May of 2007 with a Bachelor of Science degree in Chemistry. She conducted undergraduate research under the supervision of Professor Akos Vertes investigating the use of mesostructured silicon for laser desorption ionization in mass spectrometry.

In September of 2007, Ms. Stolee enrolled in the Ph.D. program of the Department of Chemistry at the George Washington University and continued working under the direction of Professor Akos Vertes. Her work is focused on the development and fundamental understanding of novel ionization techniques for the direct analysis of metabolites and small molecules by mass spectrometry. Research she conducted at the George Washington University and Oak Ridge National Laboratory led to the development of silicon nanopost arrays (NAPA) as matrix-free nanophotonic ion sources. Another multidisciplinary project aimed at discovering novel ambient ionization methods, primarily based on laser ablation electrospay ionization (LAESI), for the in-situ analysis of small cell populations, single cells, and subcellular organelles.

Ms. Stolee has co-authored eight publications (including four cover page articles) and another one has been submitted for review, she is the co-inventor of three filed patents, and her work has been presented 18 times at national and international conferences. As a result, she has been recognized by 5 national and international awards, including the prestigious Dimitris N. Chorafas Foundation Prize.


AWARDS

2012  Dimitris N. Chorafas Foundation Prize

2012  Washington-Baltimore Mass Spectrometry Discussion Group Young Investigator Travel Award

2010  Washington-Baltimore Mass Spectrometry Discussion Group Young Investigator Travel Award

2010  Achievement Rewards for College Scientists, Endowment Fellow

2009  Achievement Rewards for College Scientists, Scholar

2007  American Institute of Chemists Prize

2007  A.D. Britt Scholarship
CONFERENCE PROCEEDINGS


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