FINKEL, NANCY HAWKINS. Surface-Assisted Laser Desorption/Ionization-Mass Spectrometry (SALDI-MS) of Controlled Nanopore Cavities and the Associated Thermal Properties. (Under the direction of Lin He)

Surface-Assisted Laser Desorption/Ionization-Mass Spectrometry (SALDI-MS) utilizes a solid surface as an energy transfer medium for desorption/ionization. The purpose of this research was to generate controlled feature size and distribution so that the proposed mechanisms of desorption/ionization could each be studied in turn. The thermal contribution to desorption/ionization and how it relates to MS response was studied in this segment.

In this study, substrates with feature changes from 20 to 60 percent porosity. Peptides, sugars and low molecular weight proteins are used to determine MS performance of the substrates. Pore size and distribution of 100 nm each on Si (0.002-0.005 Ohm/cm) shows the best SALDI-MS signal/noise among those tested. The thermal properties of the substrates were evaluated using thermography in which the temperature changes from different substrates under the same conditions were measured. This measured temperature change was found to be directly related to substrate porosity and MS response. These results confirmed our hypothesis that SALDI-MS is thermally induced and that the increased density of pores on the surface aid in the thermal desorption of the analyte.

The optimized substrate generated was used to profile metabolites in a complex biological plant system. *Arabidopsis thaliana* was transgenically modified with a human inositol polyphosphate-5-phosphatase enzyme and used as a model to test SALDI-MS performance. The small molecules commonly found in the SALDI-MS data include amino acids, organic acids and steroids. Simple sugars required derivatization to increase ionization
efficiency. Suppression effects did not allow for the observation of sugar alcohols, even after derivatization.
Surface-Assisted Laser Desorption/Ionization-Mass Spectrometry (SALDI-MS) of Controlled Nanopore Cavities and the Associated Thermal Properties

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FABRICATION OF NANOMETER ORDERED ARRAYS

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CHAPTER 1

1. INTRODUCTION

The goals of this project were 1) fabrication of ordered nanofeature arrays on silicon to be used as SALDI-MS substrates 2) correlation of surface features and the corresponding thermal properties with MS response 3) application of the SALDI-MS substrate to metabolic profiling. This work demonstrates the first application of SALDI-MS with ordered array fabrication. The results reported herein show that surface porosity is the key morphological feature to enhancing the MS response. The increased porosity leads to increased heating, which then leads to increased MS response. Chapter 3 describes the fabrication of ordered nanocavity structure. The thermal and surface feature contributions to SALDI-MS are examined in Chapter 4. Chapter 5 explores the feasibility of using SALDI-MS for metabolic profiling.
CHAPTER 2

2. LITERATURE REVIEW

2.1 Organic Matrix and Surface Based Desorption/Ionization MS.

The applications of Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) and Electrospray Ionization (ESI) have revolutionized mass spectrometry in the biological sciences, in particular forensics, DNA sequencing of the human genome, proteomics and most recently metabolomics.\textsuperscript{3, 4}

The typical MALDI-MS matrices are organic acids, such as dihydrороxybenzoic acid or $\alpha$-cyano-4-hydroxycinnamic acid. Most matrices have a strong absorption coefficient at wavelength of 337 nm. When the analyte is mixed with the matrix, the matrix serves as an energy transfer medium from the laser to the analyte. This soft desorption technique allows the sample to desorb from the surface with little degradation. MALDI-MS analysis is relatively simple; the challenge is in determining the type of matrix needed for the desired analyte and the sample preparation. The presence of matrix introduces MS peaks in the low mass range that makes the detection of small molecules difficult and the matrix forms clusters and fragments that complicate spectra interpretation. In recent years, several approaches have been undertaken to clean up the low mass region, including: adjusting the matrix to analyte ratio, using low concentration of surfactant as additives and using larger and more stable matrix molecules.\textsuperscript{5-7} While effective to a certain extent, these type of methods require prior knowledge on the nature and the concentration of the analytes to be detected; therefore are not applicable to detect unknowns in complex samples.

Organic matrix-assisted laser desorption ionization technique was developed in parallel with surface-based laser desorption ionization technique, both as soft-ionization
techniques. The earliest attempt of surface-based analysis used a suspension of 30-nm cobalt particles in glycerol as the matrix.\textsuperscript{8} This research resulted in the ionization of proteins with masses greater than 20,000 Daltons. The authors suggested that desorption in this setup was primarily thermal.\textsuperscript{8} The metal Co nanoparticles were heated from laser irradiation. This heat was conductively transferred to the surrounding glycerol in which analytes were dissolved.\textsuperscript{8} The importance of laser induced heating in SALDI-MS was demonstrated further when the desorption/ionization was observed from laser heated frozen glycerol sample because it suggests that the temperature may not be that important.\textsuperscript{9} Many different materials have been tested as potential substrates, including graphite, carbon nanotubes, Ag, Au, W, WO\textsubscript{3}, Sn, Zn, ZnO, Si, Mo, TiO\textsubscript{2}, and Al.\textsuperscript{10-14} Unlike the solid Au substrates previously tested, Au clusters implanted into the biological material, as well as Au nanoparticles in the 2-10 nm range have been shown to be effective matrices.\textsuperscript{15, 16} While most SALDI-MS is believed to be a thermal-induced process, the quantum confinement effect is believed to play the main role in small particle assisted desorption. The authors of the Au nanoparticle paper argue that the enhanced ionization of the 2 nm particles as opposed to the larger 5 and 10 nm particles is due to the quantum confinement effect.\textsuperscript{17} The ionization observed using 5 and 10 nm particles was attributed to the rapid and evenly distributed thermal energy transfer through vibrational excitation.\textsuperscript{15} The quantum confinement effect occurs when the diameter of the particle becomes smaller than the Exciton Bohr Radius.\textsuperscript{18} At this point the energy levels in the conduction and valence band spread out into separate entities where they can no longer be assumed to be continuous. When the energy levels spread out into discrete levels the molecule can then be excited to multiple states. The energy transfer is thought to be electronic excitation which results in enhanced ionization compared to the larger
nanoparticles tested. The quantum confinement effect occurs in the 2-10 nm range when a few additional atoms change the absorption and emission wavelength of the optical excitation. The smaller particles have an increased absorption coefficient at UV range than larger ones so the energy transfer from laser to particle is more efficient. Siuzdak et al. recently introduced Si nanowires capped with Au catalyst at the tips of the wires. The authors suggests the added sensitivity of the nanowires is due to field desorption in the high electric field. Neither of these theories has been substantiated.

In 1999, Siuzdak discovered desorption/ionization on porous silicon (DIOS) as an effective substrate for SALDI-MS analysis. Porous silicon and/or silicon nanowires appear to be the most versatile solid substrate introduced to date, mainly due to sensitivity and to the ability to selectively analyze classes of compounds by derivatizing the silicon. The fabrication method to generate the porous silicon of electrochemical etching is relatively straightforward, although reproducibility has hindered the technique. The optimal mass range using porous silicon is < 3000 amu. The porous silicon structure has a random distribution of nanometer sized pores. By altering etching parameters the optimum DIOS substrate was found to have pore size of 100 nm and inter-particle distance of 100 nm. Because of its superior performance, the DIOS substrate was used as the baseline comparison to the nanocavity ordered arrays prepared in this study.

2.2 Desorption/Ionization Mechanisms

Many theories about the laser desorption/ionization mechanisms have been proposed, but the exact energy pathways are not fully understood. Knowledge of the mechanism will ultimately reveal how to best optimize the laser desorption/ionization system to maximize ion yield, and therefore enhance sensitivity. Research has indicated that desorption/ionization is
most likely an accumulation of many different mechanisms that combine to result in the overall effect.\textsuperscript{25} It is generally accepted that the photons from the laser are first absorbed by the matrix molecules or silicon in the form of heat. The absorbed thermal energy then either causes the sudden temperature jump in matrix and leads to plume formation or partially transfers heat to the analyte and desorbs it from the surface. The wavelength of the laser, laser fluence and pulse width all directly influence the transfer of heat to matrix.\textsuperscript{26} Laser fluence is the amount of energy per unit area. For most matrices, the larger the fluence the more intense the ion signals until it reaches a threshold limit above which the analyte molecule is broken into fragments and the overall signal to noise decreases.\textsuperscript{26}

The possible analyte ionization mechanisms is divided into two categories, primary and secondary ionization, based on timescale.\textsuperscript{27} Primary ionization refers to preformed ions or ions generated during the first few nanoseconds. The possible pathways for primary ion formation discussed to date include: 1) multiple photon absorption 2) energy pooling 3) excited state proton transfer 4) thermal ionization 5) pressure pulses and 6) spallation. Multiple photon absorption involves matrix molecules absorbing multiple photons to enter an excited electronic state. The ionization potential of a typical matrix is 8-9 eV.\textsuperscript{26} The photon energy from the 337 nm nitrogen laser is 3.68 eV.\textsuperscript{26} At least two photons are required to ionize a matrix molecule. After the matrix molecules are excited they transfer energy to the analyte molecules resulting in ionization.

The ions generated during the first few nanoseconds are thought to be preformed ions that desorb from the analyte/matrix mixture.\textsuperscript{27} This theory was substantiated by two sets of experiments involving front and back-side illumination of Au substrates, generate ions but the possibility of ionization from photochemistry is removed. The detection of ions
suggested that the ions observed ions are preformed and thermally desorbed since the backside irradiation served as a heater. Preformed ions desorb during primary ionization have also been demonstrated using an IR laser with direct front-side illumination, which only has enough energy to thermally desorb the ion from the surface.

During secondary ionization, proton or cation transfer from the matrix to the analytes in the plume has been shown to be the dominant cause of ionization. The high concentration of molecules and ions in the plume cause collisions. The collisions result in charge transfer. The charge transfer will depend on the concentration of each species (collision probability) and the proton affinity (charge transfer probability). The collective result of these factors results in charged analytes. The dominant species in the plume is the neutral matrix molecule, with the ion to neutral ratio as $10^{-4}$ to $10^{-7}$. The suppression effect between matrix and analyte molecules also demonstrates the importance of the matrix/analyte interactions. It is more thermodynamically favorable for multiply charge molecules in the plume to be reduced, while singly charged species would not be neutralized. The proton affinity for neutral analytes and proteins (~ 900 kJ/mol) are higher than the matrix (~ 850 kJ/mol), so the neutral analytes and proteins become charged in the plume and the matrix is deprotonated. When protons are not readily available the less favorable transfer of cations to the analytes occurs.

The surface assisted laser desorption ionization (SALDI-MS) desorption/ionization mechanism is more limited to the thermal and electronic properties of the substrate. The porous Si absorbs the UV light from the N$_2$ laser and transfers the energy to the analytes which are desorbed. In the study of porous silicon three main factors that affect desorption/ionization include: 1) large surface area 2) a large absorption coefficient at 337
nm 3) high thermal conductivity.\textsuperscript{31} As the substrate's surface becomes more porous the thermal conductivity decreases. This results in a higher local temperature where analytes are thermally desorbed. In addition, the increased conductivity after etching could enhance a localized field desorption effect increasing ion yield.

The most accepted desorption mechanism of the solid matrix is the thermal component.\textsuperscript{11, 12, 25, 31-33} In SALDI-MS analysis if the substrate heats up very quickly and that heat is contained to one area there is a thermal vaporization of the compounds from the surface. One experiment using 35 nanometer and 1 micrometer particles of TiN/glycerol excluded all but the heating of the particles for desorption/ionization.\textsuperscript{32} The authors showed that metal and non-metal particles showed identical results using the same laser fluence. The glycerol was transparent to the laser light so photo-reactions were excluded. The analytes in the glycerol were vaporized by this heat. The change in temperature due to size of the particle was calculated to be 7500 K for the nanoparticles and 600 K for the microparticles. This corresponded with the fact that a larger laser fluence was required to desorb/ionize the analytes in the microparticle suspension.\textsuperscript{32} In one experiment that used an IR and UV light source the laser fluence differed, but the calculated laser surface temperatures was found to be 1000 K for both light sources.\textsuperscript{25}

The other possible contributor to the desorption/ionization mechanism in SALDI-MS is the electronic component. It has been suggested that the pointed edges of the anodically etched porous substrate are sites of electron emission in the presence of the high electric field. This field is only applied initially during the initiation of desorption. After the analyte is in the time of flight tube this field is no longer present. These electrons are believed to serve as a source of ionization by hitting the analyte molecules and ejecting an electron. The
problem with this theory is that the lifetime of the electron in the plume is low and a large
source of electrons has been shown to neutralize rather than enhance ionization.\textsuperscript{26} It has also
been suggested that the porous Si network serves to separate the charges of analyte ions and
their counter ions, which increases the amount of preformed ions that are thermally
desorbed.\textsuperscript{25} The increased ion/nanoparticle ratio using the Au nanoparticle of 5 nm suggests
that the analytes are electronically excited which facilitates desorption/ionization. The
substrate electronic state is excited and passes that energy to the analyte resulting in
excitation. Photoluminescence, which is thought to occur due to the quantum confinement
effect, has been shown to not be an important factor in the desorption/ionization mechanism
of SALDI-MS. Photoluminescence of the substrate occurs when the electronically excited
state decays to the ground state through fluorescence or phosphorescence. Fluorescence
occurs from an excited singlet state, while phosphorescence occurs from an excited triplet
state. This inconsistency indicates that the electronic state may not be critical to the
desorption/ionization efficiency.\textsuperscript{31}

The SALDI-MS ionization mechanism differs from MALDI-MS in a few distinct
ways. First there are no matrix molecules in the plume from which protons can be plucked.
Although there are still sources of protons from water vapor and solvents the ratio of
available protons is significantly lower. Secondly there are also no matrix particles that can
combine and ionize the analytes in the plume. The desorption/ionization mechanism occurs
through a different route by the removal of the organic matrix, which may be the reason why
SALDI-MS is less sensitive than MALDI-MS.

In the study of porous silicon the structure/function relationship has been highly
debated. The science required to generate the features of systematically different sizes
existed; however, the complex fabrication protocol and the cost associated do not allow an extensive study to elucidate such a relationship. This work introduces a method of fabrication that examines how the patterned structure of the silicon contributes to the MS response and how the thermal properties of the substrate change with the surface features. The electronic properties were briefly explored, but further research on this component will not be discussed in this paper.

2.3 Physical and Thermal Properties of Porous Silicon

The properties of porous silicon have been exhaustively studied due to the discovery of luminescence at room temperature in the early 1990s and the possible optoelectronic applications in the semiconductor industry. Thermal conductivity increases with doping level and decreases with porosity. For example, the thermal conductivity of bulk Si 150 W/m·K drops to 1.2-2 W/m·K after etching to nanoporous structure. Little difference in thermal conductivity has been found due to the thickness of the porous silicon structure because the depth of heating is not significant. The higher porosity leads to lower conductivity, lower conductivity leads to higher local temperature, higher local temperature leads to higher conductivity. The thermal diffusion ($\alpha$) is dependent on the thermal conductivity ($k$). The thermal conductivity cannot be measured, so thermal diffusion is measured and thermal conductivity is calculated. The density of the silicon ($p$) and specific heat ($c$) also affect the thermal conductivity. The relationship of thermal conductivity and thermal diffusion is:

$$k = pc\alpha$$

The electronic properties of porous Si are largely dependent on the surface conditions of the porous silicon. The presence of surface states and band bending determine the
direction and rate of current change during exposure to air. Adsorbates on the surface of Si can cause the surface electron conductivity to increase or decrease. The large surface area of porous silicon serves as the surface conduction channel for electrons. For example water is an electron donor. If the porous Si is p-type then the hole depletion layer is initially formed on the surface. When exposed to water vapor or air the Si adsorbs the electron donor and shifts the Fermi level towards the middle of the band gap and decreases the current. If the porous Si is n-type then the hole depletion is in the conduction band. This moves the Fermi level closer to the conduction band and an increase in current is observed. Both n and p-type Si have been tested as SALDI-MS substrates. SALDI-MS substrates prepared with n-type Si give a better MS response. Although the literature has suggested that this is merely due to the size of the pores that can be generated, it could also be the direct result of increased electrical conductivity of the substrate. The electrical conductance of porous Si has also been found to be temperature dependent. The electrical conductance increases after the critical temperature is reached, which can be used to explain the combined increase in MS response due to both the electronic and thermal components. Increased electrical conductance enhances field desorption of ions from the substrate resulting in higher ion yield in SALDI-MS. It is also a common occurrence for Si to oxidize when exposed to air. If this same logic is applied, then the conductivity of both n-type and p-type porous Si decreases when oxidized because the silicon dioxide acts as an insulator.

In addition to affecting the surface electronic state, a thin layer of water absorbed on the surface of the porous silicon is also thought to facilitate analyte solvation and aid desorption. Water is a natural contaminant of vacuum systems so the presence of a thin water layer on the porous Si after insertion into the SALDI-MS system is possible.
Research has shown that ionization is based on aqueous phase basicities instead of gas phase basicities. This indicates that the analytes were solvated first before competing for protons in the pseudo-aqueous phase. Further evidences come from the study of ionizing cooled or frozen samples where the ionization was increased by the presence of water. In a different experiment water vapor was injected into the vacuum chamber and ion yield was increased. It is believed that the solvent layer stabilizes the ions by counter balancing charge. The solvation and desorption of ions from a thin water layer on a field emitter tip has also been observed, and the water cluster ions have been observed simultaneously in both positive and negative mode, supporting the current speculation. If the SALDI-MS response is enhanced by field desorption, these cluster ions may play a role in desorption/ionization by providing the ionization in the plume.

2.4 Nanometer Feature Fabrication

2.4.1 Nanofabrication Techniques

Some techniques used to generate ordered nanometer-sized features are electron beam lithography, x-ray lithography and nanosphere lithography. E-beam lithography utilizes an electron beam, as found in scanning electron microscope (SEM) systems, to raster across the surface of the substrate causing either a physical or chemical change to the surface and leaving the pattern. If the substrate being patterned is not susceptible to the electron beam then a mask layer that can be patterned can be added on top of the substrate. Then other etching methods can be used to transfer the pattern. With a high electric field (>100 keV) and field emission electron source, e-beam lithography can generate features as small as 1-2 nm with great resolution. The limitation to e-beam lithography is that it is a serial process. This means that to generate features in an array on the order of millimeters in size
would require ~24 hours of instrument time. The generation of ordered arrays large enough to use for SALDI-MS analysis would not be a time or cost-efficient approach. It should also be noted that this large investment of instrument time would only generate one substrate. E-beam could however be used once to generate a mask to be used with other methods to repeatedly generate ordered arrays.

X-ray lithography is a multi-step process which involves the transfer of a pattern using radiation. The x-ray photons are irradiated through an Au patterned mask which absorbs the radiation with little scattering. The minimal light scattering enhances the density of features that can be fabricated with this process. The pattern is transferred to a polymer resist that breaks down in chemical etching solution after exposure to the radiation. X-ray lithography is limited to features of 20-50 nm in size. The cost of this process as well as limited access to the proper instrumentation is a deterrent for using this method for fabrication of ordered arrays on a millimeter size scale. It also should be noted that the fabrication of the Au mask would be expensive and would probably require e-beam lithography.

The introduction of natural nanosphere lithography led to a cheap and inexpensive way to generate nanometer size features using a nanosphere mask, although not on the scale of 1 cm² we required in order for multiple samples to be analyzed using the same substrate. A monolayer of nanometer or micrometer sized beads that are hexagonally close-packed serves as an etching or deposition mask. Dip coating and nanosphere lithography are some of the early methods of monolayer deposition. Nanosphere lithography spreads nanosphere beads over oxidized silicon using spin coating. Optimization of the centrifugal force with bead size is required. The main limitation to this method is the relatively small area (20 µm²)
of achievable uniform monolayers. There is also a considerable loss of expensive bead suspension. The dip-coating method involves dipping a substrate in a concentrated bead suspension. As the substrate is slowly withdrawn the solvent evaporates. Crystal domains form as the solvent evaporates due to Alder phase transitions and crystallizations. This process is usually lengthy and requires excessive amounts of expensive bead suspensions. The thickness of the film is also not easy to control with a liquid interface.

Convective assembly is the natural assembly of spheres in hexagonally closest packing that occurs during evaporation after spreading of the beads. Convective assembly takes advantage of the confinement effect between two surfaces which allows controlled deposition of the nanospheres. It is a quick and easy way to generate large areas of monolayer coated substrates. A 10-25 µL aliquot of concentrated bead solution (5-10% by weight) is deposited between the two surfaces. The top plate moves along the surface at a rate of µm/s dragging the rectangular plug of sample. The crystal domains of beads form as the solvent evaporates (Figure 2.4.1).

The proposed equation attempts to explain how the factors that control convective assembly are related. The $\epsilon$ and $h$ represent the porosity and the height of the nanoparticle.
\[ v_c = \frac{\beta J_p \ell \phi}{h (\ell - \epsilon)(\ell - \varphi)} = \frac{K \phi}{h (\ell - \epsilon)(\ell - \varphi)} \]

The volume fraction of the beads in solution is represented by \( \varphi \), while \( \beta \) relates the average solvent velocity with the average particle speed before entering the drying region. The \( \ell \) parameter is the length of the colloidal crystal. The \( \beta \) parameter is difficult to determine and is assumed to be approximately equal to one for nanospheres. The beads are spread on a wafer at a velocity \( (v_c) \) and at a thickness of \( h \). The volumetric fluxes for the particle, solvent, and solvent evaporation are represented by \( J_p, J_s \) and \( J_e \). It should be noted that the spreading rate \( (v_w) \) is not the same as the growth rate of the monolayer of beads \( (v_c) \). The evaporative flux, the drying length and parameter \( \beta \) are lumped into one term, \( K \), because none of them can be determined experimentally. It has been shown that although the \( K \) term will be dependent of the rate of evaporation it should remain constant in a temperature and humidity controlled environment. Experimentally it was determined that higher concentration of bead permitted faster deposition rates and that changing the humidity during bead deposition doesn’t change the assembly of the beads into large crystal domains. When the spreading rate and the rate of crystal growth were equal the monolayer of beads resulted. Increasing the spreading rate introduced voids in the crystal structure. If the spreading rate was decreased multiple layers of beads would result. Increasing the concentration of the beads also resulted in multilayer bead formation. Increasing the size of the beads required the spreading rate to decrease in order to generate a monolayer of close-packed beads.

By optimizing the concentration of beads and spreading rate a monolayer of hexagonally close-packed beads results. Adjusting these factors can also produce a bilayer of beads. Centimeter regions of hexagonally closest packed beads can be generated in a matter
of minutes, although some local defects are present. The equipment required to setup convective assembly includes: a syringe pump which is used to move the glass slide over the Si wafer and an attachment to stabilize the Si wafer. The benefits of the convective assembly technique are that the technique is inexpensive, simple, fast and reproducible on a large scale. The size and distribution of the features are limited only by the size of beads that can be made with high reproducibility. The smallest polystyrene-COOH beads made reproducibly are 170 nm.

2.4.2 Reactive Ion Etching

Once the pattern is generated by convective assembly the pattern is transferred to the silicon using reactive ion etching (RIE). RIE uses reactive radicals generated from the breakdown of halogenated gases in a plasma to selectively react with the Si substrate. Halogenated gases in the presence of a plasma generates electrons that break down a mixture of SF$_6$ and O$_2$ gases into a radical and ion mixture. An electric field is applied to direct the reactive radicals and ions towards the silicon. The resulting product is gaseous SiH$_4$ which desorbs from the surface and is removed by a pump. The plasma consists of only 1% radicals and 0.01% ions, but even at this low concentration etching occurs. The mixture of SF$_6$ and O$_2$ as well as Cl$_2$ and O$_2$ generate radicals that attack silicon. The Cl$_2$ gas is better at etching in one direction and is more commonly used in deep reactive ion etching (DRIE).

The etching mechanism in reactive ion etching is diagramed in the following equations:

\[
\begin{align*}
SF_6 + O_2 + \text{plasma} & \rightarrow SF_x^{y^-} + SOF_x^{y^-} + F^- \\
SF_x^{y^-} + SiO_2 & \rightarrow SiF_x^{y^-} + O_2 + SOF_x^{y^-} \\
4F^- + Si & \rightarrow SiF_4
\end{align*}
\]
Radical SiF$_x$ species are generated and the gaseous radicals such as SiF$_4$ are pumped out of the chamber. The fluoride anions attack the Si where it etches Si at a rate of 20 times faster than SiO$_2$.$^{58}$ The reactive ion etch is directional but a degree of isotropic etching also occurs, especially as the feature depth increases so does the degree of isotropic etching. The presence of isotropic etching using SF$_6$ and O$_2$ limited the depth of feature fabrication in the data presented herein. In addition the etching depth using polymer beads has shown in our research to be less than SiO$_2$ beads. It is probably because the polymer acts as a sponge where the reactive fluorinated species react with the conjugated double bonds resulting in a much slower etch.$^{60}$ The etching rate has to be slowed down in order to favor anisotropic etching. Several parameters including power of the applied potential and chamber pressure can alter final feature fabrication. Increasing the power setting increases the rate of the fluoride ions and radical that are directed towards the surface and result in a deeper etch.$^{58}$ Changing the chamber pressure affects the equilibrium of the reaction. Highly isotropic etched features will result if the reacted SiF$_4$ molecules are allowed to remain in the chamber due to high chamber pressure.$^{61}$

2.5 Metabolite Profiling

2.5.1 Metabolic Profiling Methodology

MALDI-MS has been instrumental in the analysis of proteomics and genomics. The latest push in understanding biological systems has been the analysis of the metabolites in plant and biological systems on a large scale. The standard analytical method of metabolites has been gas chromatography mass spectrometry (GC/MS), although electrospray mass spec is gaining popularity.$^{62, 63}$ Fourier transform ion cyclotron mass spectrometry (FT-ICR) is the most promising area of compound identification. The high resolving power of FT-ICR
allows metabolites to be identified using the elemental compositions. The FT-ICR method is growing very quickly, only slowed by the expense of the instrument. In GC/MS analysis the derivatization of the small molecules with a silylating agent volatilizes the non-volatile small molecules by disrupting hydrogen bonding. The limitations of this method include: uncertain degree of derivatization and limited classes of chemical compounds and mass range. One advantage of the GC/MS method is the established database for identification. Analysis of the metabolites by SALDI-MS using the ordered array substrate provides complementary information to the GC/MS method. SALDI-MS offers a quick screening process to identify the compounds affected by the genetic or environmental stresses to the system on the metabolic level. The identification of compounds using SALDI-MS is complicated by the fact that there are 10,000 possible unidentified metabolites many of which are isomers. Collision induced decay (CID) can be used with SALDI-MS to break the parent compound into daughter ions. It should be noted that suppression effects as well as the high salt concentration within the samples limit the screening process.

2.5.2 Biological Plant Sample Model

The biological model system in this research was transgenically modified Arabidopsis thaliana. Genetic research on Arabidopsis thaliana is plentiful because the genome of the small plant has been known for many years. The wildtype strain in this research was ecotype Col-O. More specifically, the inositol phosphate pathway was interrupted by transforming the gene for human inositol 5 phosphate into the Arabidopsis thaliana plant. Metabolic profiling using SALDI-MS will examine the osmotic-induced fluctuation of metabolites in the inositol phosphate pathway by comparing wildtype versus transgenic plants.
The inositol phosphate signaling pathway involves the production of two secondary messengers from the plasma membrane phospholipid phosphatidylinositol 4,5 bisphosphate (PIP$_2$). The successive phosphorylations of the phosphatidylinositol by kinases result in the production of PIP$_2$. The enzyme, phospholipase C, is activated by G protein signaling to breakdown PIP$_2$. The PIP$_2$ is broken down into two secondary messengers: 1) diacylglycerol (DAG) which remains in the membrane and 2) inositol phosphate 3 (IP$_3$) which is water soluble. IP$_3$ serves to activate the Ca$^+$ channels in the cytosol while DAG activates a protein kinase. The IP$_3$ diffuses through the cytosol and binds to the receptor sites on the endoplasmic reticulum, which opens up the Ca$^+$ channels and increases the

![Diagram of the phosphatidylinositol signaling pathway](image)

**Figure 2.5.1** The phosphatidyl inositol signaling pathway was transgenically modified to include a human inositol-5-phosphatase which specifically breaks down IP$_3$.
concentration inside the cytosol very quickly. In addition the DAG remains in the membrane and activates protein kinase C. Protein kinase C is only activated when phosphatidylserine and Ca\(^ {+} \) are bound. Under normal circumstances protein kinase C’s affinity for Ca\(^ {+} \) is low, however when DAG binds to the kinase the affinity increases. When protein kinase C is activated it translocates from its position as peripheral membrane protein to inside the plasma membrane. Protein kinase C phosphorylates many target proteins, generating a cellular response.

**Figure 2.5.2** Proline generation via the glutamate pathway in response to osmotic stress in plant systems.\(^ {1} \)
The PI pathway has been shown to be involved in the response of plants to abiotic stress such as salinity, cold, flooding and drought. The IP₃ concentration dramatically increases in response to stress. The turnover of PI is also stimulated. These two factors indicate that the IP pathway is up-regulated in response to stress.

Several compounds have been identified as important osmoregulants. These include proline, sugars, and sugar alcohols and betaines. Proline concentration increase due to osmotic stress has been found in many different plant systems. There are many theories as to how proline acts as a osmoregulator including: 1) membrane stabilizer 2) hydroxyl radical scavenger 3) source of nitrogen and carbon and 4) energy sink. There are two different pathways through which proline concentrations can increase: the ornithine-dependent pathway and glutamate-dependent pathway. The glutamate-dependent pathway is believed to be activated under stress conditions, which involves converting glutamic acid to proline.

The increased sugars and sugar alcohols in response to osmotic stress is due to starch hydrolysis. The sugars are believed to be involved with protecting macromolecules inside the cell or maintaining membrane integrity by interacting with the polar headgroups. This interaction is thought to prevent membrane fusion. None of the theories about how increased sugar concentrations act as osmoprotectants have been substantiated. The sugar alcohol, mannitol, is not normally found in Arabidopsis plants. However, in transgenic plants that increased levels of mannitol have been found in the seeds germinated in 4X higher concentration of sugar alcohol.

Glycine betaine is also an extensively studied osmoregulant. Transforming plants with the genetic information to make glycine betaine and external application of glycine
betaine to plants have aided in the resistance to osmotic stress. However, glycine betaine has not been shown to increase in *Arabidopsis thaliana*. Glycine betaine can be generated by two different pathways: dehydrogenation of choline or N-methylation of glycine. The limitation to the uptake of glycine betaine is the concentration of choline available and the ability to transport choline inside the chloroplast where synthesis occurs. Other betaines such as proline betaine and alanine betaine also serve as osmoregulatory agents.

The plant system used in the profiling experiments had the inositol phosphate pathway altered by transforming the wildtype (ecotype Col-O) strain of *Arabidopsis thaliana* with the human inositol-5-phosphatase enzyme. The phenotypic changes between the wildtype and transgenic plant were indiscriminant. The concentration of IP3 was shown to increase in the transgenic sample. The transgenic strain was found to be more resistant to drought stress. However, the pathways affected are unknown.

![Glycine betaine production pathways in plant systems: A) choline dehydrogenation/oxidation pathway and B) glycine methylation pathway.](image)
2.6 References

CHAPTER 3

3. FABRICATION OF ORDERED ARRAYS

3.1 Introduction

The introduction of desorption/ionization using surface based technology has allowed for analysis of small molecules using SALDI-MS.\textsuperscript{1-3} In the traditional MALDI-MS technique the organic matrix clouds the low mass region so that distinction between sample peaks and matrix peaks is difficult to discern.\textsuperscript{3} A number of inorganic materials have been tested as SALDI-MS substrates with different degrees of success.\textsuperscript{4-9} The most prominent example in this class of approaches was reported by Siuzdak \textit{et al.} in which porous silicon is used in Desorption/Ionization On porous Silicon (DIOS).\textsuperscript{1, 3, 10}

Various methods have been reported to generate the porous Si structures, including the use of conventional electrochemical etching\textsuperscript{10}, plasma-enhanced chemical vapor deposition (PECVD)\textsuperscript{4}, hyperthermal reactive atom etching\textsuperscript{7}, and direct nanoparticle mixing. etc.\textsuperscript{11} The reproducible preparation of a high-performance DIOS substrate using any of these methods is not trivial. Most methods yield a broad range of sizes, depths and densities on the same surface that cause spot-to-spot and batch-to-batch variations in DIOS performance. Developing methods to reproducibly generate porous Si substrates with optimal porosity and thickness is thus critical to broader applications of DIOS. In addition, the complexities of the analyte desorption/ionization process makes it a great challenge for both theorists and experimentalists to elucidate the exact energy and proton transferring pathways in DIOS. The ability to systematically tune the surface features and independently tune one influential component at a time is imperative for in-depth understanding of surface morphological contributions to desorption and ionization of adsorbed analytes.
The world of nanofabrication has exploded over the past few years. The ability to generate highly ordered features on the nanoscale has been shown by several groups recently.\textsuperscript{12, 13} Nanosphere lithography has resulted in the successful deposition of large areas of hexagonally packed two-dimensional crystals of nanospheres on solid substrates.\textsuperscript{14-16} The combination of reactive ion etching (RIE) with these nanosphere masks has resulted in the fabrication of ordered porous arrays that are highly reproducible and a fraction of the cost of the more traditional lithography methods.\textsuperscript{15, 17} The more traditional fabrication methods of x-ray lithography and e-beam lithography of large scale generation of ordered arrays are limited. The serial nature of e-beam lithography to generate nanometer scale features on a millimeter area would require a lot of instrument time and would be very costly. The application of e-beam lithography for the fabrication of nanocavity arrays would not be an efficient methodology. X-ray lithography, although faster, is still limited by the proximity of features. X-ray lithography is generally limited to how small of a mask that could be generated. This process was too costly with limited rewards.

Nanosphere lithography in combination with reactive ion etching was quick and relatively low cost with the generation of features in the ideal sub-nanometer scale. The mask size, and therefore the resulting feature sizes could be systematically altered using different size beads. The size range of beads used in our experiments ranged from 50 nm to 970 nm leading to features in the range of 30-900 nm. The spreading of beads could also be controlled to generate a monolayer and bilayer of beads. This allowed the density of the pores to be compared.

In this work we demonstrate that this simple, yet reproducible method can be used to generate ordered nanostructures on a Si wafer. Variation in the etching parameters and the
resulting feature morphology will be explored. The reproducibility of the patterned substrate versus DIOS in SALDI-MS analysis will also be discussed. A more detailed look at how the generated ordered arrays affect MS response will occur in Chapter 4.

3.2 Experimental Section

3.2.1 Materials Used

SiO₂ beads of 50, 330, 570 and 970-nm diameter and polystyrene-COOH beads of 90, 170 and 300-nm diameter were purchased from Bangs Lab (Fisher, IN) and used as received (10% weight content). Phosphorus-doped (100) single-crystalline silicon wafer at 0.05-0.2 Ω/cm and Antimony-doped (100) single-crystalline silicon wafer at 0.002-0.005 Ω/cm was purchased from Silicon Sense, Inc. (Nashua, NH). The wafers were stored under vacuum until needed. HF(49%), H₂SO₄, H₂O₂ (30%), CH₃OH (HPLC grade), and H₂O (HPLC grade) were purchased from Fisher Scientific (Pittsburgh, PA). CH₃CH₂OH was purchased from Aaper Alcohol (Shelbyville, KY). Angiotensin I, bradykinin, Ala-Leu-Ala-Leu, Val-Met, glucose, glucose-d₁ and trifluoroacetic acid (TFA) were purchased from Sigma Aldrich (St. Louis, MO). DI H₂O of 18 MΩ (Millipore, PO) was used throughout the experiments.

3.2.2 DIOS Fabrication

The standard anodic etching of Si for DIOS substrate preparation was needed for comparison. A Teflon coated etching cell was used with a Au working electrode, and two platinum wires as the counter and reference electrodes (Figure 3.3.1). The etching solution was 1.5 mL of 25% hydrofluoric acid (HF)/ethanol. White light from a 50 W tungsten lamp was used. Prior to etching the silicon was cleaned for 1 minute in 5% hydrofluoric acid/ethanol to remove SiO₂ layer. The silicon wafer was then rinsed with ethanol and dried with nitrogen. Etching parameters were dependent on the resistivity of the Si. The silicon
wafer of 0.05 to 0.2 Ohm/cm resistivity was etched with a current density of 4 mA/cm² for 4 min. The silicon wafer of 0.005 to 0.02 Ohm/cm resistivity was etched with a current density of 5 mA/cm² for 1 min. The post-etching processing of the silicon involves a 1 min soak in 15% H₂O₂/ethanol followed by a 1 min soak in 5% hydrofluoric acid/ethanol. The substrate was stored in ethanol in a plastic Petri dish until SALDI-MS analysis.

### 3.2.3 Fabrication of Nanometer Ordered Arrays

The microscope slides used as bead spreaders and the Si wafer were cleaned in freshly prepared piranha (3:1 H₂SO₄/H₂O₂) for 2 h. The peroxide serves to oxidize the silicon to improve spreading of the hydrophilic beads as well as clean the substrate. The substrates were then rinsed in copious amounts of water and dried in an oven at 70°C for 2 h. Then 10-15 µL of concentrated bead solution (10% by weight) was deposited by pipet under the cleaned microscope slide and spread on the oxidized silicon. The typical spreading rate was 10-45 µm/s. The amount of beads and the spreading rate were optimized for the size of beads being spread. The surfaces were dry by the time the spreading was completed. The substrates were stored in plastic Petri dishes in ethanol until etched. The beads were spread on two different types of silicon: 1) 0.05-0.2 Ohm/cm n-type P doped (100) single crystalline silicon 2) 0.002-0.005 Ohm/cm n-type Sb doped (100) single crystalline silicon. The sizes of the beads ranged from 50-970 nm and were either SiO₂ or polystyrene-COOH.

The mask was transferred to silicon using the RIE (SEMIGroup Reactive Ion Etching System 1000P). The radio frequency power was set to 100 W to generate the plasma. The chamber pressure was 50 mTorr. The etching gases SF₆ and O₂ were used at flow rates of 15 and 5 standard centimeters cubed per minute (SCCM). The etching time varied between 0.5 and 10 min depending on desired final features. After etching the substrates were sonicated in
ethanol to remove the beads. The required sonication time for SiO₂ beads were 30 min, while the polystyrene beads were removed after a 10 min sonication. The substrates were stored under ethanol in glass vials until SALDI-MS analysis.

A FEI XL30 SEM-FEG field emission scanning electron microscope (FE-SEM) was used to examine the surface features. The working voltage was 5 kV with a working distance of ~5 mm. To measure the pore depths generated, the Si wafer was broken in the middle and attached to an aluminum block with conductive tape to examine the cross-section of the etched features. Approximately 20 pores and posts from each surface were randomly chosen to measure the size distribution of surface features.

3.2.4 Electrospray parameters

An electrospray device was built in-house. Sample solution was pushed through a 10 μL syringe using a Harvard syringe pump at a flow rate of 250 nL/min. The solution passed through a 100 μm internal diameter Nanopore™ capillary that had been pulled to a 5 μm tip. The distal end of the capillary had a small region of conductive coating. An 8 kV potential was applied using a Spellman high voltage source between the capillary and grounded stainless steel SALDI-MS plate. The porous silicon substrate was mounted on the SALDI-MS plate. As the solution passed through the electric field, very fine positively charged droplets formed and a thin homogenous layer of sample was sprayed on the porous Si. The voltage and distance between the capillary and the ground were optimized to achieve a stable electrospray.

3.2.5 SALDI-MS Parameters

SALDI-MS analysis was completed using an Applied Biosystem Voyager STR time of flight. The samples were analyzed in the linear mode using an accelerating voltage of 20
kV scanning in the 50-500 amu range. Prior to spotting of the analyte solution the oxide layer on the silicon was removed by dipping in 5% HF/ethanol for 1 min. The surface was then rinsed with ethanol and dried under nitrogen. The substrates were spotted with 250 pmol of glucose and glucose-d\textsubscript{1} as MS standards. The samples were analyzed in triplicate, with an average of 50 shots/spectrum. The MS conditions were optimized at an extraction voltage of 20 kV with grid of 96% and a delay time of 150 ns.

3.3 Results and Discussion

3.3.1 DIOS Fabrication

The standard methodology for producing porous silicon, termed DIOS substrates, uses photoanodic etching.\textsuperscript{18} There are two types of silicon: n-type and p-type which are classified according to dopant. The n-type Si is doped with Group 3 elements such as boron, while p-type Si is doped with Group 4 elements such as phosphorus. The impurities introduced with doping either add (n-type) or detract (p-type) electrons from the network.

For electrochemical etching of n-type Si a light source is required to generate an electron hole on the surface of the Si. In the acidic hydrofluoric acid dissolution solution the six fluoride anions add to the Si until the formation of the final product silicon hexafluoride dissociates form the surface. The overall reaction mechanism is:

$$\text{Si} + 6\text{HF} + h\nu \rightarrow \text{SiF}_6^{2-} + 2\text{H}^+ + 2\text{H}_2$$

In p-type Si no light source is required to create holes on the surface.

The anodic etching setup is shown in Figure 3.3.1. Etching occurs by applying a current in an electrochemical cell containing 25% hydrofluoric acid/ethanol where the Si substrate is in contact with the anode. The resulting Si surface is terminated by silicon
The nanometer sized features are randomly distributed. The pore geometry is variegated as can be observed in the cross-section FE-SEM images in Figure 3.3.1 panels A and B. Two resistivities of Si were etched (0.002-0.005 Ohm/cm and 0.02-0.5 Ohm/cm), with the lower resistivity Si DIOS substrate showing better DIOS performance. The average feature size of the lower resistivity Si for 1 min etching was: pore size 129 +/- 26 nm; post size 86 +/- 28 nm; and pore depth 1.19 +/- 0.16 μm.

### 3.3.2 Nanocavity Ordered Array Fabrication

The general scheme for nanocavity feature fabrication is shown in Scheme 3.3.1. The beads were spread in a monolayer on oxidized Si. Then the substrates were place in a RIE chamber where the exposed Si in between the beads was selectively etched by a combination of SF₆ and O₂ gas. The substrates were sonicated to remove the beads. Then the oxide layer was removed with a diluted hydrofluoric acid solution. The sample analyzed was spotted on the substrate and then analyzed by SALDI-MS.
Panels A and B of Figure 3.3.2 illustrate representative field emission scanning electron microscopic (FE-SEM) images of a monolayer of hexagonally packed SiO₂ nanospheres assembled on a Si wafer (0.02-0.5 Ohm/cm) at different scales. Local defects are observed due to the formation of crystal domains during the deposition. However, the laser beam in the MS experiments was ~100 times larger than the defects, so the analyzed surface area during data collection was expected to be statistically consistent. The preparation of the substrates using nanosphere lithography and RIE resulted in ordered porous features on the surface that can be clearly observed as triangle-shaped nanopores in the FE-SEM images (Figure 3.3.2, panels C and D). Note that the posts left behind (the Si portions that were
protected from the etching gas by the nanoparticles) were hexagonally shaped instead of circular. This is a result of isotropic etching due to ion scattering from the contoured bead surface.

In theory, the geometry of the nanocavities on the surface is a function of wafer resistivity, crystal orientation, the geometry of the mask opening and the duration of etching. The in-plane width of pores on a (100) single crystalline Si wafer is expected to be controlled by the size of beads and the RIE etching conditions. However, when the mask topology is at the same length scale as the mask thickness, the ion bombardment on the mask particles scatters the etching into multiple directions (Figure 3.3.3 panel B). This finding was

![Figure 3.3.3](image)

**Figure 3.3.3** Schematic drawings of top view (A) and side view (B) of SiO₂ nanoparticle-masked surface during RIE. The dotted line in panel A indicates the cross-sectioning position for the side view scheme; the arrows in panel B represent etching molecules. Corresponding FE-SEM images are shown in panels C and D, respectively. The surface was prepared with 1 min etching.

![Figure 3.3.4](image)

**Figure 3.3.4** Top-down FE-SEM Images of Nanocavity Si surfaces etched for A) 0.5 min B) 1 min C) 1.5 min and D) 5 min. The images were adjusted to show the same magnification scale for comparison.
consistent with the initially generated features being smaller than expected. At longer etching times the isotropic scattering of the bombarding ions led to etching under the bead mask and the widening of the pores (Figure 3.3.3). It should also be noted that the SiO$_2$ beads are susceptible to etching by the SF$_6$ and O$_2$ radical mixture but at a rate 20 times slower than the etching of Si. The erosion of the SiO$_2$ mask results in the generation of larger pores during extended etching times. The combination of the eroding mask and isotropic etching increased the in-plane width with extended etching times (Figure 3.3.4).

The targeted size range of features is 50-300 nm pores based on previous DIOS research. Table 3.3.1 containing the bead patterned substrates used and their respective features are listed below. Twenty randomly selected pores and posts were measured using FE-SEM. Please note that the 50 and 90 nm PS-COOH beads were not uniform so no close packed patterns can be formed. This data is omitted in the table. The substrates prepared using the 570 and 970 nm SiO$_2$ beads did not show significant MS response to justify the cost of the FE-SEM analysis. At extended etching times of 5 and 10 minutes of the low resistivity 170 nm PS-COOH coated substrates were left with only a roughened surface due to the extensive isotropic etching. The isotropic etching effect is evident in the 1.5 min etching of the same low resistivity 170 nm PS-COOH coated substrates by the decreased pore depth. The percentage porosity was calculated by selecting a 1x10$^6$ nm$^2$ area of FE-SEM image and counting the number of pores in the area. The area of the pores are calculated and multiplied by the number of pores present, then divided by the total area. The calculated value is multiplied by 100 and reported as a percentage. The depth of the pores was not taken into account in the calculation.
Table 3.3.1 Nanocavity feature and size distribution.

<table>
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<th>Size of Bead (nm)</th>
<th>Material of Bead</th>
<th>Silicon Resistivity (Ohm/cm)</th>
<th>Etching time (min)</th>
<th>Pore Width (nm)</th>
<th>Pore Width SD (nm)</th>
<th>Post Width (nm)</th>
<th>Post Width SD (nm)</th>
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The polystyrene-COOH patterns spread in a monolayer in a larger overall area than the silicon dioxide particles. The polystyrene-COOH beads are also resistant to etching by the plasma so the pattern was preserved under similar etching conditions. The overall etching process using the polystyrene was not as deep as the silicon dioxide. This was probably due to the polystyrene becoming coated with fluorine and thus lessening the concentration of the reactive fluoride species to etch the silicon. The lower resistivity silicon was etched to a greater depth than the higher resistivity silicon, although the pore size and post size varied little (Figure 3.3.5).

Figure 3.3.5 The etching depths for the high resistivity Si (0.02-0.5 Ohm/cm) resulted in a shallower etch than the lower resistivity Si (0.002-0.005 Ohm/cm), panels A and B respectively. The Si was etched for 1 min using a 300 nm PS-COOH bead mask.
The spreading rate can be adjusted so that a bilayer of beads can be generated. This bilayer generated substrates with half the number of pores as the monolayer bead mask. The features are not as triangular in shape as the monolayer generated features due to the overlap of the second layer of beads onto the triangle (Figure 3.3.6).

As previously discussed, anisotropic versus isotropic etching is affected by the etching rate, consequently variation in etching parameters, such as the chamber pressure and power also greatly change the generated feature shape and size. In our typical etching experiments the chamber pressure is set to 50 mTorr. Decreasing the chamber pressure to 40 mTorr causes the features to become elongated and

**Figure 3.3.6** Bilayer of beads generated pores of similar size but half the density. The cross-section (A) and top-down (B) FE-SEM images show the different pattern.

**Figure 3.3.7** The RIE parameters influence the feature topography. FE-SEM images show the features generated by a 40 (A) and 60 (B) mTorr in chamber pressure. Images of the monolayer and bilayer of beads are shown when the power is increased to 150 W (panels C and D). The depth of features is markedly larger for the bilayer protected region of the substrate.
pointed tips were generated (Figure 3.3.7, panel A). Increasing the chamber pressure to 60 mTorr increases the isotropic etching of the surface. This is due to a buildup of SiH₄ due to increased ion bombardment and insufficient removal of SiH₄ from the chamber slowing the etching process. The isotropic etching almost completely removes the features from the substrate surface. The typical setting used for the power in the RIE system is 100 W. At 150 W there is a marked difference between the surface feature depth for the monolayer and bilayer regions (Figure 3.3.7, Panel D). The bilayer surface is more sufficiently covered and therefore protected from the bombarding fluoride ions. The monolayer is not protected so the features are eroded as they are being generated.

3.3.3 Reproducibility of patterned substrate versus DIOS

Reproducibility is important for everyday application of SALDI-MS analysis. This includes spot to spot and substrate to substrate reproducibility. Spot to spot variation in SALDI-MS response can be reduced using electrospray deposition instead of the standard dried droplet method. The dried droplet method consists of pipetting a 0.5 – 1 µL spot onto the SALDI-MS substrate. The dried droplet method has “coffee stain rings” around the outside of the spot where the analyte concentrates. In order to determine if the nanocavity ordered array was more reproducible than the DIOS substrate electrospray deposition was used to minimize the variation due to sample drying.²⁰⁻²²

The most popular substrate used in SALDI-MS analysis is DIOS, porous silicon prepared via anodic etching. The commercial availability of the substrate has made it easily accessible for general use. The generation of the porous silicon is not a consistent process, with a wide variation of pores generated under the same conditions from substrate to
substrate. However, in the combined nanosphere lithography and convective assembly method applied in this research the pore size and distributions were more consistent.

In order to determine if the reproducibility between substrates, we compared our best reactive ion etched substrate with the DIOS substrate to see if the controlled feature generation improved reproducibility. Glucose and glucose-d$_1$ (250 and 125 pmol) were deposited using electrospray deposition due to the increased variability of the traditional dried-droplet method (Figure 3.3.8). The inconsistency of the SALDI-MS response necessitated a deuterated internal standard for ratio calculations, because the analyte concentration is changing in addition to the substrate variation, i.e. the internal standard takes out the MS variation caused by the instruments/substrates. In Figure 3.3.8 the FE-SEM

Figure 3.3.8 The nanocavity ordered array (A) MS performance was compared to DIOS (B). Sample SALDI-MS spectra of glucose and glucose-d$_1$ shows similar signal intensity. The FE-SEM images of the substrate are shown in panels C and D.
images of the substrates and the MS spectra are shown. Note that the packing is not completely uniform due to the crystal domains and some point defects. The laser beam profile diameter is 250 µm so the defects found in the surfaces will be minimized on this scale.

Three replicates of each sample were analyzed with an average of 50 shots per spectrum. The ratio of the peak areas of the glucose/glucose-d₁ were compared. The ratio of the glucose/glucose-d₁ on DIOS was 2.07 +/- 0.31 and RIE was 2.00 +/- 0.26. The reproducibility of each was found to be relatively consistent at the percent coefficient of variation (CV) of 13.1 and 15.2% for the RIE versus DIOS substrate. Therefore making an ordered array system does not dramatically improve the SALDI-MS reproducibility.

3.4 Conclusions

The fabrication of nanometer size features in a variety of distributions and depths were prepared using nanosphere lithography. Substrates with porosities ranging from 4-60% were prepared with features ranging from 40 to 250 nm. The best performing substrate had a porosity of 45% with pores of approximately 100 nm width and 100 nm distribution. The improved substrate reproducibility showed similar performance as the DIOS substrate, but the variation was not significantly improved. The variation in pore width, depth and interpore spacing were demonstrated and will be used to understand the mechanism and determine the optimum MS desorption/ionization performance. The correlation of the surface feature properties and corresponding thermal properties will be studied in more detail in Chapter 4 of this work.
3.5 References

4. THE THERMAL AND FEATURE CONTRIBUTION TO SALDI-MS

4.1 Introduction

Surface assisted laser desorption/ionization (SALDI-MS) is a thermal and electronic process. Although a number of inorganic substrates have been shown as effective SALDI-MS substrates, porous Si has been the most popular. A plethora of information is available on how the thermal and electronic properties of Si change when the porosity increases. The thermal properties were focused on in this work, although this should not detract from the importance of the electronic properties.

In order to study the thermal properties of the porous silicon several methods were explored before settling on the infrared imaging. When light is shown on to the surface of a sample there are several factors that detract from an accurate temperature measurement including radiative relaxation, energy trapped in the metastable state and energy consumed in chemical reactions (Figure 4.1.1). As the substrate heats there is a change in temperature, density and pressure immediately in front of the substrate as it interacts with its environment. The method used in this work to measure temperature change was infrared imaging. Although we also explored the complementary technique of pressure change using photoacoustic spectroscopy that data is too preliminary to include in this discussion.

Thermal conductivity is a measure of the ability of a solid or liquid to transfer heat. The thermal conductivity of Si drops from 150 W/m K to 1.2-2 W/m K after etching to a pore size of 2-5 nm. This is not unexpected considering the low thermal conductivity of air and the introduction of local crystalline defects as electron traps. The thermal
conductivity drop with increasing porosity suggests that the local temperature of porous Si upon laser irradiation will be much higher than the unetched surface due to the slow heat dissipation through the airpockets. Consequently, it is plausible to suspect that rapidly increased local temperature leads to a rapid evaporation of the analyte from the porous Si in a thermal-induced desorption fashion in SALDI-MS response. The temperature dependent phenomenon is supported by the experimental observation that a threshold temperature must be met for adequate desorption/ionization efficiency. In addition, a study using an IR and UV laser generated the same temperature changes on the surface even though a higher laser fluence using the IR laser was required.

The study of how the surface properties relate to SALDI-MS analysis has been limited due to the difficulty in generating nanocavity ordered arrays to study how the pore geometry contributes to MS response. The generation of the nanocavity ordered arrays has allowed for a closer investigation into how the pore size and distribution correlate to SALDI-MS response. This data suggests that percentage porosity of Si is the key factor in the
desorption/ionization efficiency in SALDI-MS analysis. Above the threshold percentage porosity MS response is significantly improved (6-fold). The temperature change of the substrates is linearly correlated with the increased porosity.

4.2 Experimental Section

4.2.1 Surface Measurements

A FEI XL30 SEM-FEG field emission scanning electron microscope (FE-SEM) was used to examine the surface features. The working voltage was 5 kV with a working distance of ~5 mm. To measure the pore depths generated, the Si wafer was broken in the middle and attached to an aluminum block with conductive tape to examine the cross-section of the etched features. Approximately 20 pores and posts from each surface were randomly chosen to measure the size distribution of surface features. The triangular pore sizes were measured as the bisected length of the triangle.

4.2.2. SALDI-MS Measurements

SALDI-MS analysis was completed using an Applied Biosystem Voyager STR. The samples were analyzed in the linear mode using an accelerating voltage of 20 kV scanning in the 50-500 amu range. Prior to spotting of the analyte solution the oxide layer on the silicon was removed by soaking in 5% hydrofluoric acid/ethanol for 1 minute. The surface was then rinsed with ethanol and dried under nitrogen. Several small peptides, proteins and sugars were used to determine MS response. One or more of the three sets of standards was used in the analysis of the substrates: 1) 20 pmol of small peptides Val-Met (248.1 m/z) and Ala-Leu-Ala-Leu (386.3 m/z) 2) 10 pmol of angiotensin (1296.7 m/z) and bradykinin (1060.6 m/z) and/or 3) 250 pmol of glucose (203.05 m/z, sodium adduct) and 125 pmol glucose-d$_1$ (204.05 m/z, sodium adduct). A signal to noise of 50:1 was the minimum response threshold
for acceptable data unless otherwise stated. The laser fluence was measured using a laser power meter outside of the focal lenses and converted to mJ/cm$^2$ units.

4.2.3 Thermal Measurements

The ThermaCAM SC3000 instrument (FLIR Systems, North Billerica, MA) was used to measure the surface temperature of the substrates. Scheme 4.3.1 shows the typical sample setup: an Ar laser of 514 nm was used as the irradiation source for surface heating. The continuous wave laser was used because the pulsed 337 nm Nd/YAG surface temperatures were not measurable using the infrared camera setup. The laser power was measured at 1 W, and the incident beam was at a 45 degree angle. The infrared camera was oriented orthogonal to the substrate to reduce the possible surface reflection. The camera was stabilized on a x-z translational stage to optimize camera-surface distance. The substrates were heated for 5-30 sec, followed by the manual blocking of the laser beam. The temperature changes as a function of time were recorded by the connected computer. The temperature changes recorded in three different positions on the substrate were determined from the base temperature and the temperature at 5 seconds. The areas selected were at the same positions on each of the substrate and each were located within the incident laser beam profile. The results presented herein notes the temperature changes due to changes in feature size and distribution.

4.3 Results and Discussion

4.3.1 Surface temperature of patterned Si and MS response due to feature changes

Initially 170 nm PS-COOH substrates were prepared at etching times of 0.5, 1, 1.5 and 5 minutes. The resulting features were listed in Table 2.3.1. The temperature change of each of these substrates was measured using infrared imaging (Scheme 4.3.1). The substrate
was placed approximately 10 cm from the infrared camera. The laser beam was a continuous wave Ar laser at 514 nm wavelength. The incident angle was 45 degrees and was reflected into a beam block. The temperature change was recorded during heating and then cooling back to room temperature. The substrate was exposed to the laser beam for approximately 5-30 seconds. The highest temperature change was observed from the substrate with pore size of 100 nm (Figure 4.3.2). As the pore size increases above 100 nm the surface temperature starts to decrease, which is believed to be due to the isotropic etching of the surface to a roughened state without a distinct pattern. The observed thermal trends confirm the suggested decrease in thermal diffusivity and conductivity with increased surface defects. Note that the absolute temperatures measured were much lower than what were expected inside of the MS chamber because a continuous laser with less irradiation

**Scheme 4.3.1** Thermography setup used in thermal measurements.

**Figure 4.3.2** Infrared temperature profiles of 170 nm PS-COOH patterned substrate’s etched with resulting pore sizes of 41, 99, 759 and >750 nm pores.
power was used in the thermal measurements. In addition, Si surfaces had lower absorption coefficient at the 514 nm irradiation wavelength. However, the general trend of the temperature response was expected to be representative of the thermal changes of the different patterned substrates.

The temperature changes were calculated as the difference between the temperatures of the substrate before the irradiation and after the substrates were heated for 5 s by the laser. Figure 4.3.3 shows the temperature changes of 170 and 300 nm PS-COOH patterned substrates at different etching times versus the MS performance. In particular, as the relative temperature change increased upon heating the signal-to-noise ratio of glucose also increased. It is important to note that a drastic MS signal increases after it reaches a value of 48ºC. Although the absolute temperature is not an accurate reflection of actual temperature, the data does indicate that above a threshold temperature the signal significantly improves.

Substrates were fabricated using 170 and 300 nm PS-COOH beads on low resistivity Si (0.002-0.005 Ohm/cm). The best substrate performance in terms of signal to noise was generally observed from the 170 nm mask etched for 1 to 1.5 min. In general this corresponds to a pore size of approximately 100 nm with a distance 100 nm apart. It should be noted that the nanosphere lithography generated substrates’ performance was comparable
to DIOS. The MS signal was calculated for glucose and glucose-d₁ using the automated method on the MALDI instrument and plotted against surface geometry. The pore size and distribution was combined into a measure of percent porosity. The percentage porosity was calculated by determining the total porous area/total area. The number of pores (80 using 170 nm bead mask and 25 using 300 nm bead mask) in a 1 x 10⁶ nm² were counted using FE-SEM images. The pore area was calculated as a triangle, with the reported pore diameter as the bisected length of the triangle. It was then observed that when the percentage porosity reaches a threshold of 20% the MS response jumps six-fold (Figure 4.3.4). This MS jump and the corresponding temperature change indicate that above the laser induced heating above the porosity of 20% is sufficient to desorb a significant number of analytes.

The percent porosity appears to have a linear correlation with the change in temperature (Figure 4.3.5). The highest temperature change was observed from the substrate of highest porosity. This observation confirmed the suggested decrease in thermal conductivity with increased surface defects. The existence of the direct correlation of percent porosity and temperature change confirmed the critical role of laser-induced heating in
improving analyte desorption efficiency. The laser source used in the thermal measurements is continuous wave and allowed to heat the substrate for 5 s. The SALDI-MS laser is pulsed wave where the shots are 5 ns. The pulse peak power is higher in the pulsed laser the averaged power is less than the continuous wave laser. However, the temperature changes using the pulsed laser would be much larger, but would cool faster because of the short pulse duration.

In addition, the MS signal to noise over the laser fluence 4-22 mJ/cm² was monitored for each of the substrates (Figure 4.3.6). These data demonstrate that the laser flux needed to desorb glucose decreases with increasing porosity. It should also be noted that the DIOS substrate requires much less energy (4.3 mJ/cm²) to desorb off the surface and indicates that the temperature change of the DIOS substrate is more significant. This is probably due to the fact that the DIOS substrates porosity

**Figure 4.3.5** The linear correlation between percent porosity and temperature change.

**Figure 4.3.6** The SALDI-MS laser threshold value decreased with increasing porosity.
is 50-60%. Although the DIOS data is included in Figure 4.3.6 the percentage porosity for the DIOS substrate was estimated.

The thermal trends of low (0.002-0.005 Ohm/cm) and high resistivity (0.02-0.5 Ohm/cm) Si are plotted versus the percent porosity in Figure 4.3.7. The slope for the low and high resistivity substrates is significantly different. The higher doping and thus lower resistivity Si has the higher temperature change with increasing porosity. Although previous data indicates that the lower resistivity Si has higher MS response as a DIOS substrate this has been credited only to the pore size. These data indicate that surface temperature change is also a contributor to MS response differences due Si resistivity.

4.4 Conclusions

The temperature difference of the porous Si is a direct result of surface porosity. The temperature change on the surface of the porous Si structure correlates with the MS response, which was demonstrated by the increased MS response and decreased laser threshold required to desorb/ionize analytes with increasing porosity. There is a temperature threshold above which the analytes are desorbed from the SALDI-MS substrate. However, extensive
heating causes the analytes to degrade and signal to noise drop. Samples with similar porosities, and therefore thermal properties, were compared using variations in etching parameters. The feature shape was not shown to influence the MS response.
4.5 References

CHAPTER 5

5. METABOLITE PROFILING

5.1 Introduction

Metabolites as a group are highly diverse with wide variations in their chemical and physical properties.\textsuperscript{1} The exact number of metabolites is unknown though is expected to be around 200,000 in plants.\textsuperscript{2} The concentrations of metabolites span 7-9 orders of magnitude.\textsuperscript{2} As secondary messengers in signaling pathways, their functions are interrelated and overlap.\textsuperscript{3} The biological system can adapt to change so that when a required compound is limited in one pathway changes occur to compensate. This shifting in pathways makes it difficult to study the effects of changing a pathway. There may be other metabolites that are upregulated to account for this change in pathways. Typically the signaling pathways are altered by either knocking out a gene or adding genetic information to change the system.\textsuperscript{3} The changes can reveal how the pathway works, although by no means do they provide the complete picture. Metabolic profiling by far has been the most effective method of identifying and providing a comprehensive picture of the changes in the biological system when there is limited knowledge of what changes are expected.\textsuperscript{1-4}

No analytical technique can monitor all of the metabolites simultaneously. Therefore a combination of methods will be required for comprehensive metabolite profiling. The application of SALDI-MS in metabolic profiling has become another possible method to study metabolites. In order to test the application of SALDI-MS for metabolic profiling an ordered nanocavity porous Si substrate was used to profile biochemical changes in a complex biological plant system.\textsuperscript{5, 6} Although the SALDI-MS technique is limited by suppression effects and dynamic range, the quick profiling can be used to pinpoint large fluctuations in
metabolites. Once these changes are identified more sophisticated techniques can be applied in structural identification and quantitation.

The model system used in this experiment was *Arabidopsis thaliana* (ecotype Col-O) plants provided by Dr. Perera and Dr. Boss’s group. The transgenic plant was genetically transformed with the human gene encoding inositol polyphosphate-5-phosphatase in order to modify the inositol phosphate signaling pathway. The resulting plant was found to have decreased quantity of inositol (1,4,5) triphosphate (Ins(1,4,5)P₃), but no phenotypical changes were observed under optimal growth conditions. The transgenic plants were more tolerant of drought and were slower to regain an upright position (gravitrope) when placed horizontally. Plant samples were examined using SALDI-MS to profile significant deviation in compounds between the two plant lines. Proline, glycine betaine, sugars, and sugar alcohols were the targeted compounds due to research indicating their involvement in metabolic responses to dehydration. Compounds such as choline, betaine aldehyde and glutamic acid are also monitored because of their roles in the pathway to generate glycine betaine and proline. GC/MS was used for comparison and identification of metabolites.

Ionization of sugars using traditional MALDI-MS is somewhat limited in sensitivity compared to proteins, because most sugars lack basic sites for protonation. In most cases the sugar is ionized at low efficiency as a sodium or potassium adduct. In order to examine the smaller quantity sugars and sugar alcohols in the plant extracts it was necessary to derivatize the extract with glycidyltrimethylammonium (GTMA) to enhance ionization. This method has been shown to enhance detection sensitivity by 1000 times.

5.2 Experimental Section

5.2.1 Fabrication of Nanocavity Ordered Arrays
The SALDI-MS substrates were prepared using the method described in Chapter 3. The beads used were 170 nm PS-COOH and etched for 1.5 min.

5.2.2 Extraction of Metabolites from Plants

Approximately 100 wildtype (Wt) and transgenic (Tr) plant seeds were plated on half strength Murashige and Skoog growth media and grown in the dark for 7 days before harvest. The seedlings provided the root and shoot samples. For the drought experiments, plants were grown for 6 weeks, stressed by withholding water and then allowed to recover. Leaves were harvested at 8 days and frozen in liquid nitrogen in order to explore the ability of the SALDI-MS to monitor biological changes when exposed to dehydration. In Experiment 2 the plants were grown for 6 weeks in a temperature/humidity controlled plant chamber with light, were withheld water for 5 days, then the plants were watered and allowed to recover. Samples at 4

<table>
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Table 5.2.1 Plant material used in the first metabolite experiment included the roots, shoots and leaves. The second experiment included larger amounts of leaves only.
timepoints were tested: 1) Day 0 as a control before water was withheld 2) Day 3 after abiotic stress 3) Day 4 after abiotic stress 4) Day 8 after the plant was re-hydrated for 3 days and allowed to recover.

Different parts of the plant material was harvested and immediately frozen in liquid nitrogen. The frozen plant material was ground with a mortar and pestle. The amount of plant material used in each extraction is listed in Table 5.1.1. The amount of extraction solution and derivatizing agents used for leaf extracts were exactly twice the amounts listed below for the shoot and root extracts in both Experiment 1 and 2. To the vial containing the shoots/roots 1 mL of 2.5:1:1 MeOH/Chloroform/H$_2$O @ -20ºC was added. The samples were mixed at 4ºC for 30 min then centrifuged at 9000 g for 10 min. The supernatant was then transferred to a glass vial. The pellet was further extracted with 0.5 mL 1:1 MeOH/chloroform @ -20ºC. The samples were mixed at 4ºC for 15 additional minutes then centrifuged at 9000 g for 10 min. The supernatant was then transferred to the original vial. Then 250 µL of water was added to the supernatant to separate the water/methanol from the chloroform. The chloroform and water/methanol fractions were transferred to separate eppendorf tubes. The water/methanol (W/M) fraction contained predominantly: sugars, amino acids and organic acids. The chloroform fraction contained: lipophilic compounds, lipids, chlorophylls and waxes. The samples were then speed vacuum dried overnight. The samples then had to be split so that one portion could be used for the SALDI-MS analysis while the other was further derivatized so that it could be analyzed by GC/MS. To each dried sample 65 µL water/methanol was added to W/M fraction of root and shoot samples. The sample was then split sample into two 32.5 µL aliquots. The same procedure was followed for the leaves except the total volume was 130 µL split into two 65 µL fractions. Then 65 µL
2.5:1:1 methanol/chloroform/water was added to the chloroform fraction of root and shoot samples. The sample was split into two 32.5 µL aliquots. The same procedure was followed for the leaves except the total volume was 130 µL split into two 65 µL fractions. The SALDI-MS samples were stored at -20ºC until analysis. The GC/MS fractions were dried in a speed vac. Then 12.5 µL of methoxyamine hydrochloride (20 mg/mL) in pyridine was added to the shoots/roots, while 25 µL was added to the leaves, to protect the carbonyl groups. The samples were transferred to silanized glass inserts. The samples were mixed on a shaker for 90 min at 28.8ºC. Then 20 µL of MSTFA (N-methyl-N-trimethylsilyl trifluoroacetamide) was added to the shoots/roots. The acidic protons in the sample were then exchanged for trimethylsilyl groups. Then the inserts containing the sample was transferred to eppendorf tubes to heat at 37ºC for 30 min. The GC/MS samples were removed and stored at -20ºC until sample analysis.

5.2.3 SALDI-MS Parameters

SALDI-MS analysis was completed using an Applied Biosystem Voyager STR. The samples were analyzed in the linear mode using an accelerating voltage of 20 kV scanning in the 50-500 amu range. Prior to spotting of the analyte solution the oxide layer on the silicon was removed by soaking in 5% hydrofluoric acid/ethanol for 1 minute. The surface was then rinsed with ethanol and dried under nitrogen. The extract was diluted 10X with 1:1 water/methanol 0.1% trifluoroacetic acid. The extract was spiked with 75 pmol/µL of diethylamine (75.01 m/z) and 25 pmol/µL Ala-Leu-Ala-Leu (386.30 m/z) as calibration standards. The (Ala-Leu-Ala-Leu+K)⁺ (425.22 m/z) peak was also used as an internal standard to account for fluctuations in desorption/ionization efficiency.
5.2.4 Derivatization of Sugars and Sugar Alcohols

A 10 µL aliquot of leaf extract was added to an eppendorf tube and dried using a speed vac. Then 5 µL of 5 M NaOH and 1.4 µL of glycidyltrimethylammonium (GTMA) were added to the eppendorf tube. The mixture was vortexed and derivatized for 1 hour at 60°C. The extract was then diluted 100X with deionized water. The sample spotting solution was prepared by diluting 10X with water.

5.2.5 GC/MS parameters

The GC instrument used was a HP 5890 electron impact ionization at 70 eV. The mass range is 50-450 amu. A 30 meter 0.25 mm DB17 column was used at an injection temperature of 325°C. The ion source and interface temperature was set to 280°C. The helium flow was 1 mL/min. After a 5 min solvent delay at 70°C, the oven temperature was increased in increments of 5 degrees/min until a final 280°C was established. The 280°C temperature was held for 1 min. The samples were analyzed in splitless mode with a 1 µL injection volume. Identification of some of the metabolites was achieved using a downloadable library available online.3

5.3 Results and Discussion

The baseline study of the roots, shoots and leaves of the wildtype and the transgenic Arabidopsis thaliana plant were tested in Experiment 1. The primary objective was to determine what compounds could be identified by both the SALDI-MS and GC/MS methods. The plant extract was split into water/methanol and chloroform fractions. The water/methanol fraction was expected to contain organic acids, sugars and amino acids. The chloroform fraction should be composed of lipids, lipophilic compounds, chlorophylls and waxes. However, if the concentration of the compounds in one fraction was considerably
high the partitioning into the other solvent is not unexpected. This was the case with some of
the compounds in this research.

The data from both the water/methanol and the chloroform fractions were analyzed
by SALDI-MS and GC/MS. The SALDI-MS spectra were complicated by sodium and
potassium adducts, due to the high concentration of sodium and potassium ions in the
extracts. The data were de-convoluted before comparing with the GC/MS results. In

![Diagram of GC, MS/MS, and SALDI-MS spectra]

**Figure 5.3.1** Detection and Identification of asparagine using GC/MS and SALDI-MS.

addition, the peak positions in SALDI-MS spectra were calibrated using diethylamine and
Ala-Leu-Ala-Leu. The raw SALDI-MS data was then processed by normalizing the data to
the internal standard peak (Ala-Leu-Ala-Leu+K)⁺. The area ratio was then normalized to
remove the differences in weight. If a compound was identified in both fractions then the totals from each fraction were combined.

The results of the GC/MS data were as expected in that the largest number of amino acids was found in the water/methanol fractions and the steroids were only found in the chloroform fraction. Similar chemical distribution was observed using SALDI-MS, but the detection sensitivity was much lower. There were a large number of metabolites whose molecular weights were identical. The only way to distinguish between the ambiguities was by using a hard ionization technique to fragment the molecule into daughter ions. Although SALDI-MS is capable of fragmentation using collision induced decay (CID), it was not used due to the inconsistency of the method. The SALDI-MS technique was capable of profiling and identifying fluctuating metabolites that could be targeted in further analysis. GC/MS was used to help identify and quantitate these changes. Despite the limitations of SALDI-MS, the benefit of high throughput analysis outweighs the quantitation concerns.

An example of the comparison of the SALDI-MS and GC/MS data in the identification of asparagine is shown in Figure 5.3.1. It should be noted that identification of compounds in SALDI-MS analysis is purely based on molecular weight. The compounds were first identified by GC/MS then through fragment analysis a SALDI-MS peak corresponding to the same molecular weight would be considered a potential match. The fluctuation in the data reported below is assumed to be due to concentration change, although we cannot account for variation in extraction efficiency. The error bars on the graph account for MS response fluctuation.

The resulting summaries of metabolites identified by both methods are listed in Table 5.3.1 and Table 5.3.2. The highlighted compounds cannot be distinguished using the
SALDI-MS method alone and has been listed for all possibilities. Some isomers were differentiated using GC/MS.

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Table 5.3.1 Identification of metabolites by GC/MS and SALDI-MS in water/methanol fractions. A positive response indicates that the compound was present at or above limits of detection for the analysis method used.

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Table 5.3.2 Identification of metabolites by GC/MS and SALDI-MS in chloroform fractions. A positive response indicates that the compound was present at or above limits of detection for the analysis method used.

Although many compounds were monitored, three compounds in each of the wildtype and transgenic water/methanol and chloroform extracts are shown in following examples as representative peaks of interest. The SALDI-MS spectra are shown in Figure 5.3.2. The compounds focused on in the plant samples were (proline+H)+ (116.07 m/z), (sucrose+K)+ (381.08 m/z), (glutamic acid+H)+ (148.06 m/z), (choline+H)+ (103.10 m/z), (betaine
aldehyde+H)\(^+\) (105.11 m/z) and (\(\beta\)-sitosterol+K)\(^+\) (453.35 m/z). The \(\beta\)-sitosterol concentration was not expected to fluctuate due to the osmotic stress.\(^{10}\)

Based on the literature, proline and glutamic acid were expected to increase in concentration when an osmotic stress was applied.\(^{17,18}\) In our analysis, however, proline in wildtype samples does not increase due to abiotic stress (Figure 5.3.3).\(^{9,13}\) The decrease in proline concentration was observed 3 days after the stress, where increased proline concentrations were found after 10 minutes of dehydration of other plant systems.\(^{13}\) In contrast, the proline data in transgenic system was increased consistent with previous research. In addition the glutamic acid concentration also decreased during abiotic stress. This compound is found in the proline production pathway and corroborates the decrease in proline was possibly a biological change and not a method error/limitation.\(^{9}\)

The increased sucrose concentration under dehydration conditions is consistent with the research that shows an increased breakdown of sugars due to dehydration.\(^{8,19}\) The ratio

![Figure 5.3.2] SALDI-MS spectra of leaf extract fractions from Experiment 1 from water/methanol(left) and chloroform (right). The wildtype is in black and the transgenic is in red.
of sucrose in the transgenic plants is significantly less than in the wildtype, which would indicate that the degradation pathway of starches may be avoided in this system.

Glycine betaine, proline betaine, nor alanine betaine were observed in either the wildtype or transgenic plant systems. In some plant species glycine betaine concentrations have been shown to increase due to osmotic stress.\textsuperscript{10, 11} Two compounds that are in the glycine betaine synthesis pathway, choline and betaine aldehyde, are found in the plant system in high concentrations (Figure 5.3.4). The ratio of choline and betaine aldehyde were scaled to fit into the range of the other data. The actual ratio is 3X that the ratio in Figure 5.3.3. The concentration of betaine aldehyde increases faster in the transgenic plant system than the wildtype which may indicate a faster response to stress than the wildtype.
conjugate amino acid-betaine product may be present but it is not observed in the SALDI-MS spectra.

The sugar alcohols and some simple sugars were not observed consistently or at all in the SALDI-MS spectra. This is not highly unexpected since carbohydrates do not ionize well with the traditional MALDI technique. In order to improve ionization, the sugars and sugar alcohols were derivatized with a tertiary amine compound, glycidyltrimethyl-ammonium (GTMA), to enhance ionization. In this established MALDI-MS method the same derivatization was used except the matrix used was surface based. The compounds of interest included: ananitol, xylitol, mannitol, sorbitol, glucose, fructose and inositol. The derivatized compounds should result in the mass shift of 93 amu. After derivatization one peak appeared corresponding the sugars glucose, fructose and inositol. An increase in the sugar concentration was found in response to dehydration (Figure 5.3.4). One limit to using SALDI-MS is that sugar alcohols cannot be observed. The GC/MS data confirms their presence.

**Figure 5.3.4** Increased C6 sugar concentration due to abiotic stress. The water/methanol fractions of A) Day 0 as a control before water was withheld B) Day 3 after abiotic stress C) Day 4 after abiotic stress D) Day 8 after the plant was re-hydrated for 3 days and allowed to recover.
5.4 Conclusion

This work has demonstrated that SALDI-MS can be used to profile biological samples. This method allows for selection of peaks for further investigation by more sophisticated methods. The changes observed in the SALDI-MS spectra are from only one experiment and one sample set. To confirm that these biological changes are truly indicative of the system more samples would have to be analyzed. The biological significance of these changes is hard to predict because identification with absolute certainty of the compounds is not possible. The identities need to be further confirmed by possibly coupling a separation method with the SALDI-MS analysis and CID of the peaks to determine the daughter peaks of the compounds. It would also be informative if proteomic and genetic data could be combined to elucidate how the metabolic data is relevant.
5.6 References