A microfluidic bacteria culturing device with MALDI mass spectrometry detection

Pratap reddy machavaram siva
Louisiana State University and Agricultural and Mechanical College, pmacha1@lsu.edu

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_theses

Part of the Chemistry Commons

Recommended Citation
machavaram siva, Pratap reddy, 'A microfluidic bacteria culturing device with MALDI mass spectrometry detection' (2013). LSU Master's Theses. 3115.
https://digitalcommons.lsu.edu/gradschool_theses/3115

This Thesis is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Master’s Theses by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.
A MICROFLUIDIC BACTERIA CULTURING DEVICE WITH MALDI MASS SPECTROMETRY DETECTION

A Thesis

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirement for the degree of Master of Science

in

The Department of Chemistry

by

Machavaram Siva Pratap Reddy
M.S., McNeese State University, 2010
B.S., Sri Ramachandra University, 2007
August 2013
This thesis is dedicated to my loving parents, my teachers, my sister, brother in-law and my friends.
ACKNOWLEDGMENTS

First and foremost, I would like to thank my advisors Dr. Kermit K Murray and Dr. Steven A Soper, for their support, advice and help during my time here in Louisiana State University. I would like to express my sincere gratitude to Dr. Murray for sharing his knowledge and encouragement to complete this project. Dr. Soper your quest for knowledge and the wisdom you provided will serve me for the lifetime. Special thanks to Dr. Pojman for his help and advises in all the matters concerning my personal and academic life. I will never forget the advices you gave me Dr. Pojman and I will never let you down for the hope you have on me.

I am grateful to all my colleagues in Murray research group, Soper research group and in the chemistry department for their valuable advice, friendship and good humor. I am very much thankful to god for letting me to work with such a talented people at LSU. Special thanks to my friends Venkatesulu Salla, Dinesh Bhupathiraju, Swathi Pullagurla, Udara dharmasiri, and Samuel for their wonderful support in both my personal life and professional career at LSU. Last but not least I would like to thank my parents Chenna Reddy Machavaram, Dhanalakshmi Machavaram for their support and sacrifice that had made me complete this work. Special thanks to my sister, Jyothi Machavaram and my brother in-law, Anjith Kumar Gaddam for their valuable suggestions, encouragement and love.
# TABLE OF CONTENTS

ACKNOWLEDGMENTS .................................................................................. iii

LIST OF TABLES ......................................................................................... vi

LIST OF FIGURES ...................................................................................... vii

ABSTRACT ................................................................................................... ix

CHAPTER 1. INTRODUCTION ....................................................................... 1
  1.1 PROTEOMICS .................................................................................. 1
  1.2 CELL CULTURING .......................................................................... 2
  1.3 MICROFLUIDICS ............................................................................ 4
  1.4 FABRICATION MATERIALS ............................................................... 4
  1.5 MASS SPECTROMETRY .................................................................. 7
  1.6 MATRIX-ASSISTED LASER DESORPTION IONIZATION MASS SPECTROMETRY ................................................................. 9
  1.7 MICROFLUIDICS-MASS SPECTROMETER INTERFACING ..................... 11
  1.8 MALDI ANALYSIS OF BACTERIA ..................................................... 15
  1.9 AFFINITY CAPTURE ....................................................................... 17
  1.10 MICROFLUIDIC CELL CULTURING ............................................... 19
  1.11 RESEARCH OBJECTIVES ................................................................ 21

CHAPTER 2. EXPERIMENTAL ..................................................................... 22
  2.1 MICROFLUIDIC DEVICE AND DESCRIPTION ..................................... 22
  2.2 FABRICATION AND ASSEMBLY ...................................................... 23
  2.3 BONDING TWO DIFFERENT POLYMERS ....................................... 25
  2.4 CELL ISOLATION ........................................................................... 26
  2.5 REAGENTS .................................................................................... 26
  2.6 ANTIBODY IMMOBILIZATION ......................................................... 27
  2.7 CELL CULTURING ......................................................................... 28
  2.8 DIGESTION ................................................................................... 29
  2.9 SAMPLE HANDLING ....................................................................... 31
  2.10 MICROSCOPY ............................................................................. 31
  2.11 MALDI-TOF MS ........................................................................... 32

CHAPTER 3. RESULTS AND DISCUSSION ............................................... 34
  3.1 OVERVIEW ................................................................................... 34
  3.2 CELL ISOLATION ........................................................................... 34
  3.3 *E. coli* RELEASE FROM CHANNEL SURFACE ................................ 35
  3.4 CELL CULTURING ......................................................................... 36
  3.5 DIGESTION ................................................................................... 37
  3.6 MALDI-TOF MS ............................................................................. 38

CHAPTER 4. CONCLUSION .......................................................................... 43
LIST OF TABLES

Table 1: Physical properties of commonly used polymer materials. ........................................... 5
LIST OF FIGURES

Figure 1: Schematic of a linear time-of-flight mass spectrometer.................................................. 10

Figure 2: Schematic of a reflectron time-of-flight mass spectrometer........................................... 10

Figure 3: a) Schematic representation of the integrated microfluidic device: b) multiple processing units on a single polymer surface and c) a single processing unit showing isolation module, culturing chamber, and micro-post bioreactor)................................................................. 24

Figure 4: SEM images of the cell capture module showing the curvilinear isolation channels..... 26

Figure 5: Schematic representation of antibody immobilization and E. coli captured on the PMMS substrate................................................................................................................................. 27

Figure 6: The top image shows a schematic of adding culture medium and E. coli cells (shown as black dots) to the culturing chamber and the bottom image shows the microfluidic cell-culturing component. The schematic shows the three-step process: culture chamber before introducing growth medium, filling with nutrient medium from nutrient inlet, and sample introduction. The microfluidic cell-culturing module consists of analyte inlet A, analyte outlet B, nutrient inlet C, and outlet D.................................................................................................................................................... 29

Figure 7: Components of the micropost bioreactor: a) schematic of the micropost bioreactor, b) photograph of the bioreactor for digesting using immobilized trypsin on to the microposts. The image shows three reservoirs: two inlets for sample and reagents and one outlet for both reagents and processed sample...................................................................................................................................................... 30

Figure 8: Schematic representation of trypsin immobilization on a PMMA substrate............... 31

Figure 9: A) Inverted optical microscope, B) image showing the syringe pump connected to the microscope for monitoring experimental steps................................................................................................................................................ 32

Figure 10: Hemocytometer slide showing E. coli cells marked with black circles. E. coli cells/mL were quantified by counting cells on a hemocytometer slide................................................................. 34

Figure 11: Inverted optical microscope images showing cells captured on the antibody immobilized PMMA surface. The black circle shows the cells attached to the walls of the curvilinear channels........................................ 35

Figure 12: Optical microscope images of the microfluidic culture chamber from top right to left (a, b) culturing chamber before infusion of nutrients and sample, and image collected after 4 hours of culturing. Images c and d shows cell culturing for 10 and 20 h respectively.......... 38

Figure 13: Laser desorption ionization mass spectrum of CHCA matrix........................................ 39

Figure 14: Mass spectrum of blank cell culture medium............................................................... 39
Figure 15: Mass spectrum of *E. coli* cells cultured for 10 hours and digested with trypsin in the microfluidic chip.

Figure 16: Mass spectrum of *E. coli* cells cultured for 4 hours and digested with trypsin in the microfluidic chip.

Figure 17: Mass spectrum of *E. coli* cells cultured and digested off-chip.
ABSTRACT

A novel microfluidic device was developed for bacterial cell culturing using mass spectrometry as the detector. One of the challenges in proteomics is to achieve high sensitivity in the identification of proteins in complex samples with widely varying concentrations. The main limitations for proteomic studies are relatively slow and labor-intensive steps such as cell culturing and protein digestion of small sample quantities. Microfluidics is a promising approach to increase throughput and to reduce the time-consuming steps that are necessary for proteomics. When an analytical detection method is combined with microfluidics it can overcome limitations that are important in the analysis of biological samples. In this work a microfluidic device was constructed from poly(methyl methacrylate) PMMA using hot embossing from a brass metal mold prepared from micro-milling and combined with off-line matrix assisted laser desorption-mass spectrometry mass spectrometry (MALDI-MS) for analysis. In this work, E. coli K12 strain was selected as a model for performing the analysis. Microfluidic devices were used to process the sample and mass spectrometry was used as detection method. The microfluidic device used in this study consists of three modules, capture, culture, and digestion chamber, integrated onto a single platform. The cells are captured on the microfluidic chip using polyclonal goat antibody on a modified PMMA surface, and are released using 0.25% trypsin, and transferred to the culture cell, which is filled with the growth medium. The temperature of the culture cell is maintained at 37°C using a heater and a PDMS cover slip was used for air perfusion. Samples collected at different culturing durations (4 h, and 10 h) are transferred to a micro-post bioreactor, which contains immobilized trypsin. The effluent from the microfluidic device was spotted onto a MALDI target and analyzed using MALDI time-of-flight mass spectrometry.
CHAPTER 1. INTRODUCTION

The goal of the research described in this thesis was to develop a microfluidic device with off-line matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) for bacterial culturing and detection of bacterial proteins. The system has potential applications in the detection of bacteria and in medical diagnostics in the detection of circulating tumor cells (CTC) and other rare cells. We designed and fabricated a microfluidic device with isolation, culturing, and digestion components integrated on a single poly(methylmethacrylate) (PMMA) surface with micromilling and hot embossing. E. coli K12 strain were used. E. coli cells are first captured in curvilinear channels immobilized with polyclonal goat antibody. Cells are released and perfused into culturing chamber, which is the important component of this device. Cell culturing was performed on a PMMA chip with PDMS cover slip. This microfluidic-culturing format was equipped with heater and PDMS cover slip in place of an incubator to maintain temperature and air perfusion. After incubation of E. coli in the microfluidic culturing device at 37°C for 4 h, 10 h, and 20 h, the cultured cells were analyzed with MALDI-TOF MS.

This device integrates steps for proteomic research with MALDI-TOF MS such as isolation, culturing and digestion, which reduce the total processing time.

1.1 PROTEOMICS

Proteomics is the large-scale study of proteins, particularly their structure and function.\(^1\) Proteomics plays a vital role in areas such as chemistry, biology and medicine. The study of proteins has been a scientific focus for decades because it generates insight on how proteins affect cells and how cell processes affect proteins. Protein analysis also helps in investigating the relationship between disease states and the protein compliment for clinical diagnostics. A goal of
proteomics is to analyze the varying proteome in an organism at different times, for example; protein content of a healthy cell varies from diseased (e.g. cancerous) cells. Identification of these different proteins (biomarkers) helps in targeting for treatment. Both purification and identification of proteins plays an important role in achieving this goal.

There are two general strategies for proteomic analysis; bottom-up and top-down. Bottom-up strategies involve cleaving the parent protein into peptide fragments that are smaller but sufficiently distinctive to allow protein identification. This approach is based upon mass measurement of peptide fragments. In this method, peptide fragments unique to each protein are created and the resulting peptides are analyzed by peptide mass fingerprinting or by tandem mass spectrometry for peptide sequence tags. A different approach to bottom-up sequencing is shotgun sequencing, which uses multi-dimensional separations such as strong cation exchange chromatography (SCX) and reverse phased liquid chromatography (RPLC) to separate the proteolytic fragments generated from mixture of intact proteins. On the other hand, an intact protein identification method, the top-down approach, can be employed in which proteins are separated first and then ionized with fragmentation occurring in the mass-spectrometer, without the need for prior chemical or enzymatic proteolysis. This approach helps in obtaining useful information on protein masses, structures and modifications.

1.2 CELL CULTURING

Cell culturing is an essential tool in biological science, clinical science, and biotechnology. This method is important in identification of bacteria, which is important for diagnosis. Proper care should be taken during cell culturing because cellular events mainly depend on extracellular stimuli from the surrounding environment. Cell-based assays are capable of providing valuable information on potential drug targets such as peptides and proteins as well as advancing cell
biology. Major drawbacks of large surface area vessel cell culture formats are variation in cell seeding densities, nutrient delivery, and waste removal. These drawbacks are the major sources of stress on cell cultures that introduces intracellular variations such as protein secretion and concentration of intracellular components.

Many research groups are working towards improving the throughput for culturing methods. Development of multiplexed devices such as microtiter plates and array bioreactors has been used for large scale screening applications and improved throughput.

The major limitations of these conventional cell culturing methods include, but not limited to, high cost, technical expertise, specialized facilities for harvesting, media exchanging, inability to continuously monitor cells over long period of time, and sub-culturing procedures (transferring some cells from primary culture into a fresh culture device/medium, which is done in case of culturing adherent cells and in also lyophilized cells). To overcome these limitations, a new technology, microfluidics, was introduced in the area of cell biology. Microfluidics has many advantages when compared to conventional cell culturing methods. First, microfluidics enables experiments on analyte cells in well-defined chemical and physical environments. Second, these devices can reduce the number of cells needed for experiments. Third, microfluidics can be used in experiments that require continuous monitoring. Last, these devices permit experiments to be performed parallel and with high throughput. Microfluidics is also important due to increased fluid control, ability to address cellular length scale, controlling cell culture environment and improved culture efficiency.

Due to these advantages, microfluidic technologies are used in a large number of areas with promising applications in cell-based biosensors and drug screenings. Many research groups have performed cell culture on different microfluidic array with different type of cells.
1.3 MICROFLUIDICS

Microfluidics is defined as the manipulation of fluids in channels with dimensions of tens of micrometers. Microfluidic devices are manufactured by fabricating microstructures on a planar substrate (glass or polymer), which are advantageous for manipulating small sample volumes, rapidly processing materials, and integrating sample pretreatment and separation strategies. The ease with which materials can be manipulated and the ability to fabricate structures with interconnecting channels that have low dead volume contributes to the high quality analysis done using these devices.

Over the past decade there has been an increased interest in the area of micro total analysis systems (µ-TAS), which is also known as microfluidics. Focusing on miniaturization of analytical chemical methods leads to increased interest in the area of microfabrication research. Application of microfabrication techniques has entered the life sciences field because of the increasing interest in the analysis of complex biological samples such as cells.

The use of microfluidic devices in the field of biomedical research has a number of significant advantages. First, because of the low volume of samples and reagents this is significant in cases of rare samples and expensive reagents. The fabrication materials and techniques used to construct microfluidics devices are relatively inexpensive and are very useful in mass production of multiplex devices.

1.4 FABRICATION MATERIALS

Microfluidic devices fabricated on polymer surfaces are advantageous for manipulating small sample volumes and rapid sample processing by integrating sample pretreatment and separation on a single device. Materials that are used as substrates also play a major role in the analysis.
Earlier microfluidic devices were fabricated on silicon,\textsuperscript{37} glass,\textsuperscript{38} and quartz.\textsuperscript{39} These materials are used because they are well established and easily fabricated. Microelectronic fabrication technologies such as lithography, wet chemical etching, and thermal bonding are used with these substrates.\textsuperscript{40} In the case of these devices, the cost of the substrate and fabrication process, which often involves harmful chemicals, increases the overall cost of the device. Surface properties can also be problematic for these substrates.

Mass production of disposable devices is important and useful, which includes low cost raw materials and development of suitable polymer fabrication technologies. The use of polymers increased rapidly in developing microfluidic devices due to their low cost, fair mechanical properties, good optical characteristics and good chemical resistance, which are advantages with respect to substrates such as glass and silicon.\textsuperscript{41} Some of the physical properties of polymers are shown in table below.\textsuperscript{42}

**Table 1:** Physical properties of commonly used polymer materials.

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Density (X 10(^3)Kg/m(^3))</th>
<th>Glass Temperature (T_g) (°C)</th>
<th>Thermal conductivity (\lambda) (W m(^{-1}) K(^{-1}))</th>
<th>Heat distortion temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMMA</td>
<td>1.18-1.19</td>
<td>106</td>
<td>0.186</td>
<td>80-110</td>
</tr>
<tr>
<td>PDMS</td>
<td>0.965</td>
<td>-149.85 – -123.25</td>
<td>0.002</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Some of the polymers used in fabricating microfluidic devices are PMMA, poly(methyl methacrylate); PDMS, poly(dimethyl siloxane); PC, poly(carbonate); COC, (cyclic olefin copolymer); PE, poly(ethylene); PS, poly(styrene); and PA, poly(amide). These polymers have different chemical, optical, and biological characteristics. When choosing polymers, care should be taken in selecting the properties, fabrication methods, and analysis types. PMMA, PC, and COC, are the most popular polymer substrates used for microfabrication by means of hot embossing.\textsuperscript{40,43}
The key goal in development of these devices is the ability to control and manipulate the surface chemistry of polymers for proteomic analysis. PMMA and PDMS are hydrophobic but hydrophobicity is not favorable for protein analysis due to protein absorption by hydrophobic interaction. In this case, both PMMA and PDMS have good surface properties, which can be modified either chemically or by UV or oxygen plasma. PMMA when exposed to UV or oxygen plasma creates carboxylic acid groups on the surface that make it hydrophilic. A PDMS surface can also be manipulated using chemical modification such as APTES, [(3-aminopropyl) triethoxysilane] or GPTES, (3-glycidoxypropyltriethoxysilane). In order to form a closed channel network, microfluidic devices are bonded with a cover slip. There are several different methods for bonding thermoplastics: thermal bonding, lamination, adhesives, solvent, and surface modification. All of these methods are based on the properties of the substrate and the size of the channels, for example thermal bonding is based on the transition glass temperature of the polymer, but this method is not suitable for bonding of structured microfluidic substrates with small channel sizes due to deformation of the channel during heating.

Bonding two different polymers (e.g. PMMA and PDMS) is not straightforward due to their surface properties. Chemical assisted bonding of thermoplastics and elastomers was described by Gu et al. In this method, a PMMA surface was treated with a corona discharge, which oxidizes the surface through the formation of polar groups on the reactive sites. The PDMS was prepared by spin coating onto a topaz film and curing thermally. Activated PMMA was suspended in 3-(trimethoxy silyl) propyl methacrylate (TMSPMA) for 20 minutes. Thermal annealing (90°C overnight) was then performed to facilitate the formation of chemical bonds. The bonding
strength of this method was measured by peeling force (the force required to peel the cover slip from the substrate), which was found to be $24 \pm 2$ N.

Tang et al.\textsuperscript{54} proposed chemical gluing of thermoplastics and elastomers at room temperature. In this method a PMMA substrate was treated with 3-amino propyltriethoxysilane (APTES) and the PDMS cover slip was treated with 3-glycidoxy propyltriethoxysilane (GPTES). Amine terminated silane on one substrate and epoxy terminated silane on the other surface were coupled via a silane coupling reaction followed by amino epoxy bond formation at room temperature. Bonding between these two polymers was achieved at room temperature ($25^\circ$C for 1 h).

1.5 MASS SPECTROMETRY

Mass spectrometry is an essential tool for characterization of biomolecules by revealing their mass and structure.\textsuperscript{55-57} Proteomics is the major field where mass spectrometry is extensively used. The principle involved in mass spectrometry is ionizing sample compounds to measure their mass to charge ratio ($m/z$). There are two general types of ionization techniques known as soft ionization and hard ionization. Hard ionization results in breaking of chemical bonds and the formation of fragment ions. In hard ionization techniques, the sample is vaporized and ions are generated from the volatile sample. The high energy used for ionization removes valence electrons, which is normally accompanied by fragmentation of the molecule. Hard ionization techniques, such as electron ionization (EI), spark ionization, thermal ionization, glow discharge, and inductively coupled plasma (ICP) are routinely used for small, volatile molecules. In case of soft ionization, ions are typically formed without breaking any chemical bonds. All the covalent interactions are kept intact because low energy is used for ionization, for example by adding a
proton to the analyte molecule. Soft ionization methods include chemical ionization, desorption ionization, and spray ionization.

Soft ionization techniques such as ESI (electrospray ionization), and MALDI (matrix assisted laser desorption ionization) are necessary methods for large, nonvolatile, polar molecules such as proteins, peptides, lipids, polymers, and oligonucleotides.58

In 1968 Dole recognize the generation of gas phase ions of macromolecules by spraying a solution from the tip of an electrically charged capillary. Fenn and co-workers developed electrospray as an interface for mass spectrometry.59 Electrospray is a method by which a liquid is dispersed into a small-charged droplet by applying a high electric potential between a capillary tip and a counter electrode. The resultant droplets are dried by a countercurrent flow of a drying gas.60 The mechanism of ESI includes three main steps; formation of charged droplets, solvent evaporation, and gas phase ion formation.61

Many proteins that are disease biomarkers are low abundance and are difficult to isolate from complex samples.62-64 Application of these technologies in bacterial analysis can be applied to clinical and food samples for high sensitivity and speed.65,66 MALDI is used in identification of bacteria based on expressed protein profiles.67,68 This is based on the ionization of high abundance proteins, which are often characteristic of different bacterial species. MALDI MS has been identified as a rapid high-throughput identification method.69-74 It has been extensively employed in the analysis of different microbial isolates due to the fact that it can be used for unpurified extracts and complex peptide mixtures.75,76
Matrix assisted laser desorption ionization mass spectrometry (MALDI MS) was introduced in 1985 by Karas and Hillenkamp and demonstrated for large molecule ionization in 1988.\textsuperscript{77,78} This technique involves mixing the sample with a matrix, which has a strong absorption at the laser wavelength. The energy from the laser ionizes the matrix; this spares sample molecules from being exposed to excessive energy, which would lead to decomposition. The laser irradiates the sample spot and leads to excitation of matrix molecules, which causes both the matrix and analyte to be desorbed from the surface. Protons are transferred to the analyte, forming analyte ions.\textsuperscript{79}

MALDI when combined with a TOF analyzer can be used in two different modes, linear and reflectron.\textsuperscript{60,80} These TOF analyzers are commonly associated with MALDI due to high data acquisition rate and sensitivity. The linear TOF analyzer is simple and basic in configuration (as shown in Figure 1), which makes it less expensive. Reflectron is the common term used for an ion mirror and was developed by Mamyrin and co-workers.\textsuperscript{80,81} A schematic of a reflectron is shown in Figure 2. In a time-of-flight mass spectrometer, ions are accelerated at a fixed kinetic energy with an electric potential and passed through field free region (drift region), where they are separated based on their mass-to-charge ratio.

Matrices are typically small organic acids that can form crystals and have strong absorption at the laser wavelength of choice.\textsuperscript{82} The matrix plays several roles in the process and must meet number of requirements such as absorbing energy from laser radiation, isolating analyte molecules, and (in positive ion mode) providing a proton for analyte ionization. The choices of matrix and sample preparation are important in obtaining spectra; nevertheless the choice of matrix is also important for the control of fragmentation.\textsuperscript{83}
The matrix should also be soluble with solvents compatible with the analyte and should be vacuum stable. Commonly used matrices for proteins and peptides are sinapinic acid (SA), 2,5 dihydroxy benzoic acid (DHB), α-cyano-4-hydroxy-cinnamic acid (CHCA).\textsuperscript{83, 84}

In the sample preparation step, both matrix and analyte are mixed prior to the deposition on to target surface or deposited individually and mixed on the surface, at a matrix to analyte molar ratio approximately 100 to 10,000.\textsuperscript{60} Some of the MALDI-MS sample preparation techniques are the dried droplet method,\textsuperscript{85} vacuum drying method,\textsuperscript{85} fast solvent evaporation,\textsuperscript{86} two-layer
method, and sublimation method. Of these, the most commonly used sample preparation method is dried droplet, which involves deposition of analyte and matrix onto the target plate and drying at room temperature. This lets the matrix to crystallize and incorporate analyte molecules thereby reducing aggregation of analyte molecules. It is also advantageous to use volatile solvents, as rapid evaporation increases the homogeneity of the crystallized sample.

1.7 MICROFLUIDICS-MASS SPECTROMETER INTERFACING

The precise and accurate determination of chemical or biological parameters has always been the major concern in scientific research. In the real world, an analyte of interest is present in smaller quantities of a complex mixture. This means discrimination of the analyte from critical interferences is a major concern. Typically an integrated microfluidic device can perform all major processes required for complete analysis of complex samples. Improvement in overall analytical performance can be achieved by minimizing the scale on which analysis is performed. The major factor that concerns the overall assay is the detection method. Some of the commonly used detection methods for microfluidics are fluorescence, amperometry, and mass spectrometry.

Optical detection is one of the prominent detection methods in microfluidics and fluorescence is a routine optical detection method. The ease and simplicity of microfluidic devices has led to coupling them to fluorescent detection schemes. Recent advances in optical detection methods are fluorescence lifetime imaging (FLIM), high throughput single molecule imaging, multicolor analysis, surface enhanced Raman spectroscopy, and surface-plasmon resonance detection.

Electrochemical methods have high sensitivity and ease of miniaturization. These methods are necessary for detecting low concentrations of analyte in complex samples. Amperometric
detection is a popular technique implemented in field-portable devices.\textsuperscript{96} Conductivity detection is also a common means of detecting inorganic ions in solution.\textsuperscript{97} Single and multi-walled carbon nanotubes are a new group of nanomaterial’s that have been widely used in electro analysis.\textsuperscript{98} Microelectrodes are easily integrated into portable microfluidic devices allowing in-field sample analysis.

Mass spectrometry is an essential tool for the characterization of biological samples, revealing mass and structure. Identification of low abundance species from limited sample requires a powerful analytical technique to obtain better resolution and detection sensitivity. Coupling microfluidics to MS is attractive because of the ease of parallel and multiple analyses. ESI and MALDI are the most commonly used methods for ionization of biomolecules.\textsuperscript{99-101}

Though the coupling of MS to microfluidics is difficult this analysis method can provide substantial information. In the early stages of this development, ESI MS is the most preferred method because ESI can be used with flowing liquids and its simple approach for interfacing microfluidics to MS, while not compromising sensitivity, mass accuracy and reproducibility. The first integration was demonstrated by the groups of Ramsey, Karger and Aebersold in 1997; ESI has become the choice of ionization for proteins and peptides.\textsuperscript{102,103-105} Various schemes for coupling microfluidics to MS have been developed.\textsuperscript{106,107} Coupling MS to online ESI is straightforward. In particular, the nanoliter and microliter per minute flow rate used in microfluidics is a good match to that used in nanoflow ESI sources. Many approaches for coupling ESI-MS to microfluidic chips have been developed. One of them is creating electrospray directly from chip.\textsuperscript{108} The spray can be done by either pressure driven flow or electrokinetically driven flow. An alternate approach is to attach a capillary to the microfluidic device.\textsuperscript{109} This method has difficulties in alignment and limited sample infusion into the
instrument. To overcome these difficulties sprayers can be directly fabricated in the fluidic device.\textsuperscript{110}

Several groups have developed multiple processing components on a single platform. Dahlin and coworkers\textsuperscript{111} developed and integrated microfluidic platform on a PDMS substrate. This device was fabricated in a two-level cross design consisting of solid-phase extraction (SPE) and capillary electrophoresis (CE) followed by ESI/TOF MS. The inner channels were cross-linked with polystyrene beads for (SPE) and the upper level channel performs CE. The device was equipped with an emitter tip coated with graphite to build electrical contact for ESI. A femtomole limit of detection was achieved with a mixture of six standard peptides. Gao and coworkers described an integrated microfluidic system for rapid and sensitive protein identification.\textsuperscript{112} In this work the device was coupled with capillary zone electrophoresis (CZE) and analyzed with ESI MS.

There are several advantages of using MALDI-MS as a detection source for low abundant analyte molecules.\textsuperscript{107} MALDI has higher tolerance for impurities such as salts, buffers, and other additives. MALDI predominantly generates single protonated analyte ions for simpler spectra. A major potential advantage of MALDI is multiplexing using laser desorption to read out multiple devices. Based on these advantages, integrating microfluidics with MALDI-MS is highly beneficial in the bioanalysis field. This integration reduces analysis time, contamination, and cost, and improves data acquisition. These platforms provide high sensitivity MS analysis from limited samples. Integration of all the steps required for proteomic analysis on a single device will improve process automation.

Compared to ESI, MALDI is more tolerant of salts, chemicals and other sample contaminants,\textsuperscript{113,114} which can be a limitation when coupling to microfluidics. Though MALDI is
not directly suitable for an on-line approach, several novel approaches have been described. For example, a rotating quartz wheel was used as a target plate. Liquid samples are transported into the mass spectrometer through a capillary and deposited onto a quartz wheel where the sample dries and is rotated into position for laser desorption. A similar approach was made using a rotating stainless steel ball. Solutions containing sample and matrix are deposited onto a rotating ball, which rotates several times per minute. In this case, the solutions are deposited on the ball and dried. After rotation the sample spot is set in position for laser desorption and analyzed using MALDI-TOF-MS. Off-line MALDI coupling to microfluidics can be accomplished by depositing the analyte directly onto a MALDI target plate. The rapid open access channel electrophoresis (ROACHE) technique was used for electrophoretic separation in an open channel. Another approach using a removable cover plate for MALDI analysis is a capillary isoelectric focusing with a removable resin tape was developed. Another group developed a direct coupling of thin layer chromatography to infrared MALDI for the analysis of gangliosides from cultured cells. Similarly a porous polymer monolith layer attached to a glass plate has been used for TLC separations of peptides and proteins as well as small molecules with subsequent direct detection by MALDI. A commercial approach such as compact disc (CD) microfluidic chip for parallel processing of protein digests for MALDI has also been developed.

Offline MALDI-MS analysis also provides a powerful method for coupling multiplexed parallel on chip analysis with MS detection, while multiple micro channels are not practically possible for ESI-MS. Samples that are processed through microfluidic devices are deposited onto MALDI target area matching to the laser spot size. Analyzing a large number and broad concentration of proteins present in a typical organism requires separation step before mass
spectrometry analysis. Xu et al. reported the fabrication and performance of a gel microfluidic chip interfaced to off-line IR MALDI MS. A poly(methylmethacrylate) chip along with a poly(dimethyl siloxane) cover slip was used in this study. The sieving gel was introduced into the microchannel and was polymerized. Peptide and protein samples were loaded into the microchannel by applying voltage across the separation channel. After electrophoresis the PDMS cover slip was peeled from PMMA chip and either the coverslip or the chip was analyzed using an IR MALDI-TOF mass spectrometer.

Lee et al. published a work in which a solid-phase bioreactor was coupled with off-line MALDI-TOF MS. The solid-phase bioreactor was immobilized with trypsin using EDC-NHS coupling chemistry. The effluent collected from the chip was continuously deposited onto a nitrocellulose coated MALDI target using a motor-driven xyz stage. Protein standards were used to test the digestion performance of the device. The results obtained from the continuous deposition interface were compared to spot deposition with a minimum sample volume of 1 nL. A minimum concentration of 300 pL and a better sequence coverage was reported for the continuous deposition interface.

Developing fully integrated multi-functional microfluidic system for the automated analysis of complex samples has significant importance in a number of application areas such as protein biology, biomarker discovery and diagnosis.

1.8 MALDI ANALYSIS OF BACTERIA

The minimal sample preparation, sample acquisition, and speed of the data acquisition combined with its potential for high throughput sample automation make MALDI-TOF-MS a valuable technique for screening and rapid identification of bacteria. Despite advantages of using MALDI for bacterial analysis there are few limitations that need to be addressed. In
order to produce a reproducible spectrum for the same species, care must be taken during selecting sample preparation methods, matrix solutions, organic solvents, acquisition methods and analysis methods. Using these parameters, species or strain specific information can be obtained from a MALDI mass spectrum. Many research groups have demonstrated different sample preparation methods for analyzing different bacterial samples.

Lindsay et al. reported a method for identification of bacteria from positive blood cultures by MALDI MS. Bacteria recovered from positive cultures from patient samples were analyzed using MALDI MS and the spectra were analyzed with MALDI fingerprinting software; 162 of 170 bacterial isolates were correctly identified.

Veloo et al. reported the identification of anaerobic bacteria using MALDI-TOF MS. This group compiled a database for the identification of gram-positive anaerobic bacteria. A database was created by well-characterized reference strains and sequenced clinical strains. This database was used to identify 107 unknown clinical strains of gram-positive anaerobic bacteria. The MALDI-TOF MS identification was compared with the genotyping identification, which was either 16S rRNA gene sequencing or fluorescent in situ hybridization. Of 107 tested strains, only 3 samples were unidentified. This group reported that mass spectra resembled the data obtained from the 16S rRNA sequencing. This might be due to the fact that many of the peaks in the spectra are derived from ribosomal proteins.

MALDI-TOF MS also plays an important role in the identification of bacteria that are difficult to culture or slow-growing bacteria, such as anaerobic and fastidious bacteria. Several groups reported the identification of anaerobic bacteria using MALDI-TOF MS. Recently the reliability of MALDI-TOF MS for the identification of anaerobic bacteria from clinical isolates was studied compared to 16S rRNA sequencing.
Ferreina et al. evaluated direct analysis of bacteria from urine samples.\textsuperscript{142} Out of 260 urine samples it was found that correct identification obtained at species and genus level by MALDI was comparable to cell cultured detection methods. This study suggests that a high bacterial count is required to obtain a reliable score from MALDI.

Effective identification of bacteria can be achieved by MALDI-TOF-MS. Several studies have shown that its use is not only limited to routine samples\textsuperscript{136, 143, 144} but can also be applicable to blood cultures.\textsuperscript{142, 145, 146} It is also currently recognized as the fastest technique to accurately identify microorganisms at genus and species level.\textsuperscript{147}

\textbf{1.9 AFFINITY CAPTURE}

Isolation and separation of a particular analyte from a large sample is important in rare cell analysis. Rare cells can be defined as cells that are less than 1000 /mL, such as circulating tumor cells, stem cells and in some cases bacterial cells. Several major \textit{E. coli} water-borne outbreaks have been reported in aquatic systems ranging from freshwater to marine.\textsuperscript{148, 149} Better understanding of \textit{E. coli} ecology and monitoring over large time scales can be enhanced by studying its proteomics. Understanding the changes in protein expression affected by the environment\textsuperscript{150} helps in protecting human populations from major \textit{E. coli} outbreaks.\textsuperscript{151-153} Monitoring techniques that have low detection thresholds for extremely rare cells and have rapid processing times are needed.

The U.S EPA allowable levels of \textit{E. coli} are 0, 200, and 1000 colony forming units (CFU) per 100 mL of drinking, swimming, and recreational waters respectively.\textsuperscript{154} In order to achieve these levels, pre-enrichment of cells is required. There are different isolation technologies for rare cells in heterogeneous sample volumes, such as fluorescence assisted cell sorting,\textsuperscript{155} flow-
through filtration, ELISA, and immuno-magnetic assisted cell sorting. The major drawbacks of these particular techniques are time-consuming protocols and expensive reagents.

Recent work has shown that cells can be accumulated using microfluidic devices. The surfaces of micro-channels or beads trapped within a micro channels are used for cell selection. Liu et al. generated a device for processing E. coli cells from input volumes of 1 mL with a limit of detection of 1 CFU/µL. Beyer et al. reported a microfluidic device that can process a large sample volume of approximately 50 µL for search of target analytes such as K-12 or O157:H7 with a limit of detection of 0.2 CFU/µL. These results are important in rare cell analysis due to the probability of securing minimal target cells. Therefore microfluidic devices with larger input volumes are required to provide higher confidence in selecting target cells.

Affinity-based separation mainly depends on certain molecules recognizable on the cells of interest that selectively bind to a substrate. Sorting of cells from a heterogeneous sample is achieved by binding the cells of interest to molecules that are immobilized on the microfluidic device. In such a situation, unwanted cells can be removed. This technique is highly specific to those cells that express the complementary group of molecules that has the ability to separate cells of similar size and density. In this technique, antibodies are immobilized onto the capture surface either by covalent bonds or absorption. An antibody recognizes different cell subpopulations, which have the ability to obtain high purity (when the concentration of the target cells in the suspension are low) in separating analyte cells from the mixed suspension. The sensitivity of the system is based on the binding at various shear rates. The major limitation of this technique is specificity of the biomarkers. For example CTCs are reported to be a good measure of cancer prognosis but, for instance, Nagrath et al. demonstrated the methodology for isolating CTCs from peripheral blood samples using anti EpCAM antibody, which is used for
separation of CTCs in breast cancer. Effective isolation was achieved in a single processing step. Optimization of parameters needs to be considered for achieving sufficient binding capacity. To achieve sufficient interaction time, flow should be maintained for the binding of targeted cells to occur. Two parameters that need to be considered in this technique are selecting suitable markers and surface area to volume ratio for optimum binding.

1.10 MICROFLUIDIC CELL CULTURING

Microfluidic systems have significant advantages and applications in cell biology and cell-based assays due to their automated and high throughput approaches. Cell culturing is a key step in cell biology, tissue engineering, and drug development. Conventional cell cultures are done either in vitro or in vivo. Due to inconsistencies between in vitro and in vivo environments such as cell-cell interactions and differences in microenvironment, a novel approach was established. It was found that microfluidics systems provide an in vivo like environment for cell culturing. This is because microfluidic systems can be used to supply nutrients, buffers, and even oxygen along with draining waste products by cellular activities in a way resembling human circulatory systems. Many researches had developed different patterned devices for different cell types.

One of the first studies on adherent cell culture in microfluidic channels was performed by Tilles, et al. The author developed a microsystem with a culturing area 25 mm wide and 75 mm long made from polycarbonate and glass. Primary rat hepatocytes were seeded in co-culture with fibroblasts (cells that synthesizes extra cellular matrix and collagen). Cell viability and hepatocyte function was observed and compared between the gas exchange membrane top and polycarbonate cover slip. The author also reported that an increased flow rate led to decreased viability.
Li et al. developed a microfluidic method to study the concentration of a single cardiac myocytes.\textsuperscript{177} This was an integrated device consisting of all necessary operations such as cell selection and retention, chemical stimulation, and a quantitative analysis of intracellular calcium concentration.

Walker et al. developed a device for ovary cells.\textsuperscript{178} This device consisted of microfluidic channels patterned on a PDMS surface, which were used as culture vessels. Hediger et al. developed a microsystem for epithelial cells.\textsuperscript{179} The main goal was to achieve cell culturing and potentiometric characterization of cells on a single platform. Heischkel et al. presented a microchip with buried microchannels for culture, stimulation, and recording of neural cells.\textsuperscript{180} Raty et al. used embryo cells in a microfluidic device and observed a higher proliferation rate with regular media changes compared to conventional culture methods.\textsuperscript{181}

It was found that the proliferation rate was higher in a microfluidic environment when compared to conventional methods. Microfluidic cell culturing has become a basic tool for many cell-based applications. Recent reports have shown that many novel microfluidic cell culturing systems are worthy of attention, because microfluidic cell culture studies are used in many cell-based applications including toxicological studies, drug discovery, cell, and tissue engineering.\textsuperscript{175,182,183}
1.11 RESEARCH OBJECTIVES

In our work a novel integrated microfluidic device fabricated on a PMMA substrate for bacterial cell culturing was designed and fabricated. This integrated chip consisted of components for isolation of analyte molecules from a complex sample using affinity selection; these isolated cells were amplified by on-chip cell culture and digested on a trypsin-immobilized bioreactor. This processed sample was deposited onto a MALDI target and analyzed using a MALDI TOF mass spectrometer.
CHAPTER 2. EXPERIMENTAL

In this work, an integrated microfluidic device was fabricated on a poly(methyl methacrylate) (PMMA) surface and matrix assisted laser desorption ionization (MALDI) time of flight mass-spectrometry (TOF-MS) was used as the detector. A detailed description of the microfluidic chip design, fabrication, and assembly with MALDI detection is presented.

2.1 MICROFLUIDIC DEVICE AND DESCRIPTION

In this work, a novel integrated microfluidic-MALDI device for bacterial cell culture was designed and tested. This device has three components fabricated on a single substrate as shown in Figure 3: a cell isolation component, where the cells of interest are isolated from the cell suspension using an antigen-antibody interaction; a cell culture chamber, where the isolated cells are cultured under suitable conditions (37°C temperature and air perfusion); and a micropost bioreactor for digestion, which contains trypsin immobilized on the micro-pillars. Cultured cells are digested in this latter component and deposited on a MALDI target.

The isolation chamber was 9.5 mm long and contained 16 curvilinear channels that were 15 µm in wide and 80 µm in depth with a radius of curvature of 120 µm as shown in Figure 6. The surface area of the 16 channels was 40 mm² wide with a volume of 250 nL. The chip output was directed into chamber B for culturing the isolated cells. Waste was collected through the waste reservoir using a syringe pump.

The culture chamber consisted of a 3 mm diameter and 300 µm deep circular microfluidic chamber with a volume of 2.1 µL. It comprised 2 reservoirs: one for the sample input and the other for nutrients. It also had two waste reservoirs one for sample waste and the other for nutrient waste. Cultured cells are transferred to bioreactor.

The bioreactor consisted of a 4 cm long, 200 µm wide and 50 µm deep microfluidic channel populated with an array of 50 µm in diameter micro-posts with a 50 µm inter post spacing. It had
2 reservoirs one for the sample input and the other for output. There were approximately 570 posts in the bioreactor, which gives a total volume of 340 nL with a 22 mm² surface area.

**2.2 FABRICATION AND ASSEMBLY**

Fabrication of the microfluidic device involved the following four steps: i) mold fabrication using high precession milling, ii) replication of the design on a PMMA substrate, iii) post processing including drilling of reservoirs, oxygen plasma and UV activation of fabricated device and cover plate assembly, and iv) immobilization of antibody and trypsin. Microstructures were designed using computer software Autocad (Autodesk, San Rafael, CA). The design was transferred to a micromilling machine (NMP 2522, KERN, Frankfurt, Germany), where high precision micromilling of a 6.3 mm thick brass metal plate (alloy 353 engravers brass, McMaster-Carr, Atlanta, GA, USA) was performed. PMMA was used as substrate and both PMMA (0.5 mm thickness, Good Fellow, Berwyn, PA) and PDMS (0.1 mm thickness) were used as cover slips for different portions of the chip.

The microstructures were transferred to the chip substrate using hot embossing via micro-replication from the brass mold master. Before the final assembly, the chips were washed with approximately 0.5 % isopropyl alcohol and ultrasonically cleaned for 10 minutes in deionized water. After cleaning, the chip was air-dried and stored it in oven for 30 minutes to remove the moisture from the polymer.

The PMMA substrate was masked with aluminum foil at the culture chamber and the rest, including PMMA cover slip, were exposed to UV radiation at 254 nm for 10 minutes at 15 mW/cm² irradiance, resulting in the formation of carboxylate moieties only in the UV exposed area. PMMA cover slips were co-axially arranged on the substrate and clamped between two borosilicate glass plates using binder clips, applying equal pressure throughout.
This setup was placed in a convection oven at 102°C, slightly above transition glass temperature of the UV modified materials. Cover slips were bonded thermally to the substrate. The temperature was increased from 50°C to 102°C at a rate of 20°C/min and held at 102°C for 15 minutes. The culture chamber was covered with PDMS cover slips made by thermal curing as described above. The chip assembly, after annealing cover slips to the substrate, was completed by gluing PEEK tubing to the inlet and outlet reservoirs, providing an interconnected chip assembly.
2.3 BONDING TWO DIFFERENT POLYMERS

In order to form a closed channel network, these devices were bonded with a cover slip. PMMA and PDMS are the two polymers that were used in this work. Bonding these two polymers is not straightforward due to their properties. In our work, we describe a simple method for bonding PMMA to PDMS at room temperature. The robust bonding method used chemical modification.

The PMMA microchip was fabricated by hot embossing microstructures onto the substrate from the mold master. The chip was then cleaned with distilled water followed by isopropyl alcohol and placed in an ultrasonic bath for 2 minutes. It was then dried in an oven at 50 °C for 30 minutes. The dried substrate was treated with a 60 W oxygen plasma for 1 minute and placed in an aqueous solution of 1% v/v APTES (amino propyl triethoxysilane) for 20 minutes. The PDMS cover slips were made by mixing PDMS base and curing agent (Dow Corning, Midland, MI) in a 10:1 w/w ratio. This pre-polymer solution was poured into a Petri dish and subjected to degasing by placing it in a desiccator connected to a vacuum pump. After removing the air bubbles from the pre polymer, it was cured thermally in an oven at 85 °C overnight. The thermally cured PDMS cover slips were treated with a 60 W oxygen plasma for 1 minute. The PMMA substrate, after suspension in APTES, was washed with water and dried under a stream of air. Both the thermoplastic and the PDMS were kept in contact at room temperature for 1 hour.
2.4 CELL ISOLATION

In our work, isolation of *E. coli* cells from sample volumes was demonstrated using high aspect ratio capture beds immobilized with a monoclonal antibody (mAbs) specific for the antigen membrane proteins expressed by *E. coli* K-12 (see Figure 4). The advantages of this method include pre-selectivity, which is important in case of a low number of pathogens of interest, cell purification, where cells are washed while attached to the surface, which is important for further processing and rapid analysis.

![SEM images of the cell capture module showing the curvilinear isolation channels.](image)

**Figure 4**: SEM images of the cell capture module showing the curvilinear isolation channels.

2.5 REAGENTS

*E. coli* ATCC 35218 (American Type Culture Collection, Manassas, VA, USA) was used. Goat anti *E. coli* antibody was purchased from US Biological (Swampscott, MA, USA). Reagents for cleaning and surface modification, including reagent grade isopropyl alcohol, 1-ethyl-3-[3-dimethyl amino propyl] carbodimide hydrochloride (EDC), N-hydroxy succinimide (NHS), 2-[4-morpholino]-ethane sulfonic acid (MES) and trypsin, were purchased from Sigma Aldrich (St. Louis, MO, USA).
2.6 ANTIBODY IMMOBILIZATION

Immobilization of the antibody was carried out in a 2-step process: EDC-NHS treatment and antibody attachment. The UV treated PMMA microchip was treated with a solution containing 60 mg EDC and 6 mg NHS (10:1) in a 500 mL of 0.1 M MES (2-(N-morpholino)ethanesulfonic acid) buffer (pH 5.05) and kept for 30 min at room temperature to obtain succinimidyl ester intermediate. After incubation, the excess EDC-NHS was removed by flushing with nuclease-free water. A solution of 100 µL of antibody mixed with 100 µL HEPS buffer (pH 7.2-7.4) was prepared. An aliquot of 10 µL was introduced into the channels and incubated at 4 °C overnight. The device was then rinsed with phosphate buffered saline (pH 7.4) to remove any non-specifically bound antibodies. A cell suspension of approximately 1000 cells/mL was prepared and approximately 500 µL of the cell suspension was introduced into the device using a syringe pump.

Figure 5: Schematic representation of antibody immobilization and *E. coli* captured on the PMMS substrate.
2.7 CELL CULTURING

Captured cells were washed with PBS then released from the channels by treating with 0.25 % trypsin in tris buffer. This solution helps in detaching the cells from the surface.

The culturing chamber was fabricated on a PMMA surface. The culturing bed consisted of 3 mm diameter and 300 µm deep circular chambers with an active volume of 2.1 µL. The device is shown in Figure 6.

Microchannel A was 100 µm deep and micro-channel C was 300 µm deep with the intent that the sample cells settle at the top of the nutrient medium. The 8 g/mL nutrient broth culture medium was prepared by autoclaving at 121 °C for 1 h. This culture medium was infused into the culturing bed through nutrient input channel C – D. when the culture bed is filled, waste is collected through reservoir D. Captured cells are released and passed through channel A–B. Since the depth of microchannel A is less than microchannel C, the cells float to the top and care should be taken not to disturb these cells. The procedure for filling the culture bed with nutrients and sample is shown in the Figure 6.

After introducing *E. coli* into the culture chamber, the temperature was maintained at 37 °C using a home-built heater. Using double-sided adhesive tape, the heating strip was attached to the polymer device. Since the culture bed was covered with the PDMS cover slip, perfusion of air can be achieved and no separate incubators are required. The setup was left at room temperature and monitored for 12 h using an inverted optical microscope.
Figure 6: The top image shows a schematic of adding culture medium and *E. coli* cells (shown as black dots) to the culturing chamber and the bottom image shows the microfluidic cell-culturing component. The schematic shows the three-step process: culture chamber before introducing growth medium, filling with nutrient medium from nutrient inlet, and sample introduction. The microfluidic cell-culturing module consists of analyte inlet A, analyte outlet B, nutrient inlet C, and outlet D.

### 2.8 DIGESTION

Efficient digestion of proteins is an indispensable component of the system and will ultimately be required for protein identification. Digestion efficiency in a solid phase micro-reactor depends on the bioreactor geometry. The higher surface to volume ratio, the greater the interaction between the cells and the immobilized enzymes.

The bioreactor (shown in Figure 7) consist of a 4 cm long, 200 µm wide and 50 µm deep channel populated with 50 µm diameter micro-posts with a 50 µm internal spacing between the posts. The UV activated PMMA device was annealed to a PMMA cover slip using a thermal bonding technique at 102 °C for 20 minutes. The microfluidic device was then rinsed with deionized water and air-dried. A solution of 60 mM EDC and 6 mM NHS was prepared in a 0.1 M MES buffer and infused into the bioreactor for 30 minutes, while the trypsin solution was
prepared by mixing 20 µM trypsin in a 100 mM PBS buffer (pH 7.0) and infused into the bioreactor using syringe pump for 2 h. The device was then covered with aluminum foil and stored at 4°C overnight for future use. A schematic representation of the process of trypsin immobilization is shown in Figure 8.

Figure 7: Components of the micropost bioreactor: a) Schematic of the micropost bioreactor, b) Photograph of the bioreactor for digesting using immobilized trypsin on to the microposts. The image shows three reservoirs: two inlets for sample and reagents and one outlet for both reagents and processed sample.
2.9 SAMPLE HANDLING

To control reagents and sample solutions in a microfluidic device, fluids are delivered through microchannels by positive displacement. In our experiments, the microfluidic device was operated by pressure driven flow to ensure completely laminar flow without any turbulence. A syringe pump (Model 11, Harvard Apparatus, Holliston, MA, USA) was used to infuse reagents and sample solutions into the device at various flow rates.

2.10 MICROSCOPY

Observation of cell capture and cell culturing in the microfluidic device was accomplished using an inverted optical microscope (Axiovert 200M, Zeiss, Oberkochen, Germany). The microscope was fitted with a JAI CV 252 monochrome video camera. Adobe premiere 6.0 (Adobe, San Jose, CA) was used for image acquisition and processing.

**Figure 8:** Schematic representation of trypsin immobilization on a PMMA substrate.
Figure 9: A) Inverted optical microscope, B) Image showing the syringe pump connected to the microscope for monitoring experimental steps.

2.11 MALDI-TOF MS

MALDI TOF MS analysis was carried out using a Bruker UltrafleXtreme MALDI TOF/TOF mass spectrometer. This instrument is designed for automated MS and MS/MS high throughput identification of proteins and peptides. This instrument is equipped with linear and reflectron detectors. It consists of a 4 GHz digitizer, a mass resolution of 40,000 from 700-5000 m/z, and a mass accuracy of 1 ppm and 5 ppm for internal and external calibration, respectively. This system is equipped with 1 KHz frequency tripled 335 nm Nd:YAG laser with a computer controlled laser spot size in the range of 10-100 µm.

Ions formed in the source region are accelerated by a delayed electrical field and focused by a lens system before they leave the source. The most commonly used MALDI target has 384 deposition spots for high-throughput analysis.

In this work, MALDI MS was calibrated with a peptide standard II (Bruker), which is a mix of nine standard peptides, m/z ranging from 757 (bradykinin 1-7) to 3147 (somatostatin). The matrix solution and external calibration mixture were mixed in a 1:1 (v/v) ratio. MALDI-TOF
analysis was performed in reflectron mode at 25 kV. Mass spectra were averaged over 100-200 individual laser shots and collected at a laser setting of approximately 40-50% of the maximum pulse energy of the laser.
CHAPTER 3. RESULTS AND DISCUSSION

3.1 OVERVIEW

Microfluidic and MALDI-TOF-MS experiments were performed on *E. coli* cells. The capture efficiency of the device was calculated and cell culture data at different culturing durations were compared. Cultured cells were digested on the microfluidic device and analyzed with MALDI-TOF-MS.

3.2 CELL ISLOATION

A stock solution was prepared by weighing approximately 30 µg of *E. coli* cells suspended in 1 mL of PBS buffer solution. The number of cells in the stock solution was calculated using a hemocytometer and found to be $6 \times 10^4$ cells/mL. Figure 10 shows a hemocytometer slide containing cells. After serial dilutions, approximately 1000 cells/mL solutions were prepared. Approximately 500 µL of input volume was infused into the capture channels and immobilized with monoclonal goat antibody (3 mg/mL) at a flow rate of 3 µL/min.

![Figure 10](image)

Figure 10: Hemocytometer slide showing *E. coli* cells marked with black circles. *E. coli* cells/mL were quantified by counting cells on a hemocytometer slide.

The capture efficiency was found to be 45 % ± 3% for the device. In our approach, immuno capture of *E. coli* cells followed by a wash and release from the capture bed shows sufficient efficiency for further sample processing steps. In addition, the use of a microfluidic device
provides high pre-concentrated factors due to the small selection bed volume. Figure 11 shows *E. coli* cells captured on the polymer surface. Captured cells were monitored using an inverted optical microscope. After capture, cells were washed with PBS solution at a flow rate of 5 µL/min to remove any impurities or non-specific binding materials.

![Figure 11: Inverted optical microscope images showing cells captured on the antibody immobilized PMMA surface. The black circle shows the cells attached to the walls of the curvilinear channels.](image)

3.3 **E. COLI RELEASE FROM CHANNEL SURFACE**

Following washing, the channels were treated with 0.25% trypsin solution at a flow rate of 1 µL/min to dislodge cells from the polymer surface. The process was monitored continuously under a microscope until the cells were detached. All of the perfusions were controlled using a programmable syringe pump.
3.4 CELL CULTURING

Cells released from the capture bed were moved onto the culture bed, which was filled with nutrients as indicated above. Approximately 224 cells that were captured were released and perfused into the culture device. Proper care should be taken to let the cells settle at the top of the nutrient medium. As mentioned earlier, this device consisted of a circular microfluidic chamber and four fluidic channels for introducing and collecting sample and nutrients into the chamber. As shown in Figure 6, A and B channels are used to input and collect cells, respectively, and are referred to as sample inlet and outlet reservoirs. Similarly C and D are nutrient inlet and waste reservoirs. The main stages of the device operation are loading cells into the device, perfusion of nutrients for cell culturing, and passage of cells. Figure 6 shows the schematic indicating adding nutrients and loading cells into the culture chamber. After filling the device with nutrients, cells released from the capture bed are loaded onto the nutrient bed.

Flow rate plays an important role in loading the cells and allowing them to settle at the top. Since trypsin is used in low concentration, it does not affect the cell growth. Initially the device was flushed with PBS to remove dead volume and air bubbles. In the case of off-chip cell culturing, the device was placed inside an incubator for humidity and temperature control. The chip-culturing device was equipped with a PDMS cover slip, which was chemically annealed. The PDMS is gas permeable and has a good diffusion of oxygen. From the preloading of the nutrient and to the final culture, the microfluidic device was monitored under the microscope. Data was collected at four different times, before loading the cells, four hours after loading *E. coli* cells on to the nutrient medium, after 10 hours of incubation, and after 20 hours of incubation. Images and video of the cell culturing were recorded using the inverted optical microscope. It was observed that initially, when the cells were loaded, it was difficult to focus
due to the low number of cells. After four hours of incubation, colony formation was clearly seen as shown in Figure 12b. The cells were seen alive, moving inside the circular fluidic chamber.

After continuous monitoring, data was collected at 10 hours as shown in Figure 12c. A major change in the cell density was observed after 10 hours of culturing: the entire fluidic chamber was populated with the *E. coli*. The viability of the cells after 10 hours viability started decreasing due to the decrease in area available to the cells, which in turn reduces the availability of nutrients for cell growth. With prolonged monitoring (20 hours) it was observed that the density of the cells didn’t change: comparing Figure 12c and Figure 12d, it was observed that the density of the cells didn’t change with time. The major change with prolonged culturing in the fluidic device is the viability: when observed after 20 hours, the population of cells in the microfluidic chamber was found to be static. There was no further growth found from 10-20 hours duration. The data collected at 20 hours was used only to monitor changes in the culturing chamber and the sample collected was not used further.

**3.5 DIGESTION**

Cultured samples, collected after 10 hours, were used for further processing. After 10 hours of incubation, all waste reservoirs were blocked and a pressure driven flow (syringe pump) was applied to remove cultured cells from the culturing chamber and the cells were then flowed into the bioreactor, which was immobilized with trypsin as described above. A flow rate of 15 µL/min was used initially for at least 15-20 seconds to remove cells from the dried nutrient bed followed by a 10 µL/min flow rate that was used to move the cultured material through the bioreactor. The cultured cells, when passed through the bioreactor, come in contact with the immobilized enzyme and underwent digestion.
Figure 12: Optical microscope images of the microfluidic culture chamber from top right to left (a, b) culturing chamber before infusion of nutrients and sample, and image collected after 4 hours of culturing. Images c and d shows cell culturing for 10 and 20 h respectively.

3.6 MALDI-TOF MS

Samples processed in the microfluidic chip were analyzed using MALDI-TOF MS. Samples collected from the microfluidic device were mixed with a CHCA matrix solution in a 1:1 (v/v) ratio. A 1 µL volume of the sample was placed on the MALDI target plate along with 1 µL of CHCA matrix which was prepared by mixing 10 mg of CHCA in 700 mL of acetonitrile and 300 mL of 0.1% TFA. The sample and matrix were mixed and air-dried. Analysis was carried out using MALDI-TOF MS and a mass range of 400 m/z to 2000 m/z was selected, which avoids low mass matrix peaks.
Figure 13: Laser desorption ionization mass spectrum of CHCA matrix.

Figure 14: Mass spectrum of blank cell culture medium.
Figure 15: Mass spectrum of *E. coli* cells cultured for 10 hours and digested with trypsin in the microfluidic chip.
Figure 16: Mass spectrum of *E. coli* cells cultured for 4 hours and digested with trypsin in the microfluidic chip.

Figure 17: Mass spectrum of *E. coli* cells cultured and digested off-chip.
Mass spectra were collected for blank matrix and culture medium (shown in Figure 13 and 14 respectively) without any sample. Mass spectra of *E. coli* cultured and digested on the chip at different culture times (4 hours and 10 hours) along with the *E. coli* cultured and digested off-chip (which is considered a reference) were also collected.

A mass spectrum of *E. coli* cells cultured for 10 hours and digested on the microfluidic device is shown in Figure 15. The spectra collected from different sample spots (n>10) are similar for all the samples (n>10). In each case the base peak was located at m/z 440. Some of the peaks in the spectra that are reproducible are labeled in Figure 15 and Figure 16. It is known that same species could give different spectra, due to different growth conditions or different chemicals that are used during sample processing techniques.\textsuperscript{186,187} For example use of TFA or formic acid creates different spectra with significant differences in relative intensities.\textsuperscript{128} Despite many variations in spectra due to experimental conditions certain peaks that are reproduced are labeled. These peaks might be peptides or lipids but cannot be assigned with confidence unless further processing such as separation or better mass accuracy (which can be achieved with an internal standard) are done. The mass spectrum of *E. coli* cells cultured for 4 hours and digested on chip is shown in Figure 16. Peaks greater than m/z 1000 showed a greater difference in the intensities, shown in inset spectrum of Figure 16. The mass spectrum of the sample processed off-chip by culturing the cells on a Petri dish and performing in-solution digestion is shown in Figure 17.

Since sensitivity and dynamic range limitations dictate the need for effective separation of protein or peptide species prior to analysis by mass spectrometry, we are still working on integrating a separation step on a microfluidic device. The data obtained from mass spectrometry cannot be used for bacterial identification until further processing is done.
CHAPTER 4. CONCLUSION

We have described the construction and operation of an integrated microfluidic chip for bacterial cell culturing with off-line MALDI MS for detection. This integrated system demonstrated cell isolation, cell culturing, and digestion of bacteria. The small surface area and volumes of the microfluidic device minimized the quantity of reagent and sample size. Incorporation of MALDI MS for identification reduces the overall analysis time and sample quantities. Isolation of analyte from complex samples can be achieved with our device and the culture chamber produced good results for culturing bacteria in a microenvironment. The capture efficiency of our device was found to be 45 ± 3% and the smallest time required to observe the growth of bacterial cells was found to be around 4 hours. This can confirm that microfluidic culturing devices can reduce the overall time compared to bench top culturing. The bioreactor requires less volume of analyte and duration for digestion. Peaks that are reproduced are labeled in the mass spectra obtained from on-chip and off-chip processing. Integration of on-chip separation will make this device a complete proteomic chip.

We are still working on integrating capillary electrophoretic separation on chip to get better information from the mass spectra data obtained from the complex samples such as bacterial cells.
REFERENCES


Pratap Reddy Machavaram Siva was born to Chenna Reddy Machavaram and Dhanalakshmi Machavaram in Guntur, India. He joined Sri Ramachandra Medical College and Research Institution, India, from 2003-2007. He obtained Bachelors in Pharmacy in July, 2007. After completing his bachelor’s he joined in Baxter India Pvt Ltd as a quality control assistant from July 2007- January 2008. Later he joined Medwin Hospitals as a clinical trial assistant and SAS programmer from January 2008- July 2008. After getting his visa to United States he joined McNeese State University at Lake Charles, La, for his master of science in chemistry from 2008-2010. In Fall 2010 he was accepted to graduate school doctoral program at Louisiana State University (LSU) in department of chemistry. Later in Fall 2012 he decided to terminate his doctoral program and currently he is a master’s candidate, which will be awarded in summer 2013.