Integrating Micro-Scale Separations to Matrix Assisted Laser Desorption and Ioniation Time of Flight Mass Spectrometry (MALDI-TOF-MS) for Protein Analysis

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INTEGRATING MICRO-SCALE SEPARATIONS TO MATRIX ASSISTED LASER DESORPTION AND IONIZATION TIME OF FLIGHT MASS SPECTROMETRY (MALDI-TOF-MS) FOR PROTEIN ANALYSIS

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In partial fulfillment of the
Requirements for the degree of
Doctor of Philosophy
In
The Department of Chemistry

By
Harrison K. Musyimi
B.S., Jomo Kenyatta University of Agriculture and Technology, 2000
December 2006
DEDICATION

Bernard and Georgina Musyimi Nthuli, Dad and Mom, I commend you for your love, continued prayers, and encouragement. Your godly guidance and principles are the foundations that have carried me throughout this challenging endeavor. I will forever be grateful to God for giving me parents like you who have been by side and a source of great strength and positive influence. Elizabeth, Rita, Martin and Wesley Musyimi, You have been to me more than a brother could ask for and I am glad to have you as my siblings. Your exhortations inspired me along way. Martin, you constantly believed in me and you were always there when I needed some one to talk to, thank you for being a friend. Mary W. Kamande and Nathan M. Musyimi, am very proud of you and grateful to have you in my life. We have come along way and there could have never been a better companion in life. Your prayers, confidence, and trust in me made my journey in graduate school much smoother. Dearly loved ones, I look forward to our future together. Nathan, you are truly a gift from God and may you grow up to be a man after God’s heart, I love you!
ACKNOWLEDGMENTS

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<th>Name</th>
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<tr>
<td>CE</td>
<td>capillary electrophoresis</td>
</tr>
<tr>
<td>CEC</td>
<td>capillary electrochromatography</td>
</tr>
<tr>
<td>CGE</td>
<td>capillary gel electrophoresis</td>
</tr>
<tr>
<td>CIEF</td>
<td>capillary isoelectric focusing</td>
</tr>
<tr>
<td>CITP</td>
<td>capillary isotachophoresis</td>
</tr>
<tr>
<td>CMC</td>
<td>critical micellar concentration</td>
</tr>
<tr>
<td>CZE</td>
<td>capillary zone electrophoresis</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ESI</td>
<td>electrospray ionization</td>
</tr>
<tr>
<td>EOF</td>
<td>electroosmotic flow</td>
</tr>
<tr>
<td>EWOD</td>
<td>electrowetting-on dielectric</td>
</tr>
<tr>
<td>FIA</td>
<td>flow injection analysis</td>
</tr>
<tr>
<td>GC</td>
<td>gas chromatography</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>high performance liquid chromatography</td>
</tr>
<tr>
<td>H$_3$PO$_4$</td>
<td>phosphoric acid</td>
</tr>
<tr>
<td>IMAC</td>
<td>immobilized metal affinity chromatography</td>
</tr>
<tr>
<td>LC</td>
<td>liquid chromatography</td>
</tr>
<tr>
<td>LCM</td>
<td>laser capture microdissection</td>
</tr>
<tr>
<td>LCST</td>
<td>liquid critical solution temperature</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix assisted laser desorption and ionization</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MEKC</td>
<td>micellar electrokinetic chromatography</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>µ-TAS</td>
<td>micro-total analysis systems</td>
</tr>
<tr>
<td>MS</td>
<td>mass spectrometry/mass spectrometer</td>
</tr>
<tr>
<td>Na₂B₄O₇</td>
<td>sodium borate</td>
</tr>
<tr>
<td>NaCl</td>
<td>sodium chloride</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>sodium phosphate</td>
</tr>
<tr>
<td>NaOH</td>
<td>sodium hydroxide</td>
</tr>
<tr>
<td>OT-CEC</td>
<td>open-tubular capillary electrochromatography</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PAGE</td>
<td>poly-acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PCT SPS</td>
<td>pressure cyclic technology sample preparation system</td>
</tr>
<tr>
<td>PMMA</td>
<td>poly-methyl methacrylate</td>
</tr>
<tr>
<td>PMF</td>
<td>peptide mass fingerprinting</td>
</tr>
<tr>
<td>pI</td>
<td>isoelectric point</td>
</tr>
<tr>
<td>pNIPAAm</td>
<td>poly-N-isopropylacrylamide</td>
</tr>
<tr>
<td>PTM</td>
<td>post-translational modifications</td>
</tr>
<tr>
<td>RSD</td>
<td>relative standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electrode microscopy</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>TOF</td>
<td>time of flight</td>
</tr>
<tr>
<td>Tris</td>
<td>tris (hydroxymethyl) aminomethane</td>
</tr>
<tr>
<td>tRNA</td>
<td>transfer Ribonucleic acid</td>
</tr>
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</table>
ABSTRACT

This dissertation describes the integration of micro-scale separations to matrix assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI TOF MS) for protein analysis. MALDI MS provides unsurpassed accurate mass measurements of intact bio-molecules, for example peptides and proteins, which in turn generate high molecular specificity enabling the identity, function and structure of these molecules to be characterized. However, in order to realize the full potential of MS in proteomic studies, integrated sample processing on automated and high throughput platforms is required to address the complexity, diversity and the dynamic range of proteomic analysis. The work described here contributes towards the development of automated and high throughput micro-total analysis systems (µ-TAS) for proteomics.

An overview of mass spectrometry instrumentation and techniques used in protein analysis is presented to highlight the significance of the work described. Microfluidics devices can serve as automated and high throughput platforms for integrating proteomics sample processing steps such as whole cell lyses, enrichment, solubilization, denaturation, protein separations, proteolytic digestion and chromatographic separations of peptides prior to MALDI TOF MS analysis. Therefore, coupling microfluidics devices to biological mass spectrometry is the first logical step towards developing fully integrated and automated systems for protein analysis. On-line and off-line approaches for analysis from microfluidic devices are discussed. The development of a specially tailored rotating ball inlet for automated on-line MALDI MS sample introduction from an electrophoresis-based separation platform is described. Electrophoresis-based micro-scale separations of peptides on fused silica capillary and polymer-based microfluidic devices were coupled to on-line
MALDI TOF MS using a rotating ball inlet. The rotating ball inlet allowed for individual technique optimization and automation thereby eliminating the need for fractionation and routine MALDI sample preparation. High throughput solid phase micro-reactors for efficient enzymatic cleavages and improved protein identification with MALDI MS in a microfluidic device were also developed for incorporation in an integrated protein analysis microfluidic system. Future work that outlines the framework and focus geared towards integrating the modules discussed in this dissertation into a functional micro-total analysis system for protein sample processing is discussed.
CHAPTER 1. INTRODUCTION

1.1 Proteins

Proteins are biological macromolecules that consist of one or more polypeptides. A polypeptide is a linear sequence (chain) of amino acids linked together by covalent peptide (amide) bonds. An amino acid is an amphoteric (zwitterionic) molecule that contains a basic amine functional group, an acidic carboxylic acid functional group and a side chain group designated $R$ as illustrated below. A short amino acid oligomer joined by an amide bond is called a peptide. When multiple peptide subunits are linked by peptide bonds, they form a polypeptide or a protein molecule. There are twenty known standard amino acids, which combine to form every single protein in the human body. Table 1.1 is a list the twenty known standard amino acids and their physicochemical properties.

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{C} \quad \text{OH} \\
\text{R} & \quad \text{NH} & \quad \text{O} \\
\text{C} & \quad \text{O} & \quad \text{OH}
\end{align*}
\]

Amino acid

The chemically diverse $R$ groups allows the synthesis of proteins with a wide range of physicochemical properties. The $R$ side group is specific to each amino acid and is used to classify amino acids as either weak acids, weak bases, hydrophilic or hydrophobic. For example glycine, the simplest amino acid molecule, has hydrogen as the side group, and is classified as hydrophobic. The ionization constant (pKa’s) of the amine and carboxylic groups in glycine amino acid molecule are 2.35 and 9.78, respectively. The isoelectric point (pI), value of 6.06 for this amino acid listed in Table 1.1 is therefore an average of the pKa values of the functional groups in the molecule.
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Abbrev.</th>
<th>Side chain (R)</th>
<th>Side chain property</th>
<th>pK(_1) (COOH)</th>
<th>pK(_2) ((^\ddagger)NH(_3))</th>
<th>pKr (R)</th>
<th>pI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>Ala, A</td>
<td>-CH(_3)</td>
<td>hydrophobic</td>
<td>2.35</td>
<td>9.87</td>
<td>6.01</td>
<td></td>
</tr>
<tr>
<td>Cysteine</td>
<td>Cys, C</td>
<td>-CH(_2)SH</td>
<td>hydrophobic</td>
<td>1.92</td>
<td>10.70</td>
<td>8.18</td>
<td>5.05</td>
</tr>
<tr>
<td>Aspartate</td>
<td>Asp, A</td>
<td>-CH(_2)COOH</td>
<td>acidic</td>
<td>1.99</td>
<td>9.90</td>
<td>3.90</td>
<td>2.85</td>
</tr>
<tr>
<td>Glutamate</td>
<td>Glu, G</td>
<td>-CH(_2)CH(_2)COOH</td>
<td>acidic</td>
<td>2.10</td>
<td>9.47</td>
<td>4.07</td>
<td>3.15</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>Phe, P</td>
<td>-CH(_2)C(_6)H(_5)</td>
<td>hydrophobic</td>
<td>2.20</td>
<td>9.31</td>
<td></td>
<td>5.49</td>
</tr>
<tr>
<td>Glycine</td>
<td>Gly, G</td>
<td>-H</td>
<td>hydrophobic</td>
<td>2.35</td>
<td>9.78</td>
<td></td>
<td>6.06</td>
</tr>
<tr>
<td>Histidine</td>
<td>His, H</td>
<td>-CH(_2)C(_3)H(_3)N(_2)</td>
<td>Basic</td>
<td>1.80</td>
<td>9.33</td>
<td>6.04</td>
<td>7.60</td>
</tr>
<tr>
<td>Isoluecine</td>
<td>Ile, I</td>
<td>-CH(CH(_3))CH(_2)CH(_3)</td>
<td>hydrophobic</td>
<td>2.32</td>
<td>9.76</td>
<td></td>
<td>6.05</td>
</tr>
<tr>
<td>Lysine</td>
<td>Lys, K</td>
<td>-(CH(_2))(_4)NH(_2)</td>
<td>Basic</td>
<td>2.16</td>
<td>9.06</td>
<td>10.54</td>
<td>9.60</td>
</tr>
<tr>
<td>Leucine</td>
<td>Lue, L</td>
<td>-CH(_3)CH(CH(_3))(_2)</td>
<td>hydrophobic</td>
<td>2.33</td>
<td>9.74</td>
<td></td>
<td>6.01</td>
</tr>
<tr>
<td>Methionine</td>
<td>Met, M</td>
<td>-CH(_2)CH(_2)SCH(_3)</td>
<td>hydrophobic</td>
<td>2.13</td>
<td>9.28</td>
<td></td>
<td>5.74</td>
</tr>
<tr>
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<td>-CH(_2)CONH(_2)</td>
<td>hydrophobic</td>
<td>2.14</td>
<td>8.72</td>
<td></td>
<td>5.41</td>
</tr>
<tr>
<td>Proline</td>
<td>Pro, P</td>
<td>-CH(_2)CH(_2)-</td>
<td>hydrophobic</td>
<td>1.95</td>
<td>10.64</td>
<td></td>
<td>6.30</td>
</tr>
<tr>
<td>Glutamine</td>
<td>Gln, Q</td>
<td>-CH(_2)CH(_2)CONH(_2)</td>
<td>hydrophilic</td>
<td>2.17</td>
<td>9.13</td>
<td></td>
<td>5.65</td>
</tr>
<tr>
<td>Arginine</td>
<td>Arg, R</td>
<td>-(CH(_2))(_2)NH-(C(NH)NH(_2))</td>
<td>Basic</td>
<td>1.82</td>
<td>8.99</td>
<td>12.48</td>
<td>10.76</td>
</tr>
<tr>
<td>Serine</td>
<td>Ser, S</td>
<td>-CH(_2)OH</td>
<td>hydrophilic</td>
<td>2.19</td>
<td>9.21</td>
<td></td>
<td>5.68</td>
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<tr>
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<td>Thr, T</td>
<td>-CH(OH)CH(_3)</td>
<td>hydrophilic</td>
<td>2.09</td>
<td>9.10</td>
<td></td>
<td>5.60</td>
</tr>
<tr>
<td>Valine</td>
<td>Val, V</td>
<td>-CH(CH(_3))(_2)</td>
<td>hydrophobic</td>
<td>2.39</td>
<td>9.74</td>
<td></td>
<td>6.00</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>Trp, W</td>
<td>-CH(_2)C(_6)H(_5)N</td>
<td>hydrophobic</td>
<td>2.46</td>
<td>9.41</td>
<td></td>
<td>5.89</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>Try, Y</td>
<td>-CH(_2)-C(_6)H(_4)OH</td>
<td>hydrophobic</td>
<td>2.20</td>
<td>9.21</td>
<td>10.46</td>
<td>5.64</td>
</tr>
</tbody>
</table>
1.2 **Protein Structure**

The amino acid sequence forms the *primary structure* of a protein. The peptide bond is oriented in a *trans*-configuration such that the carbonyl oxygen and the amide hydrogen of the adjacent amino acids point away from each other.

Although the peptide bond is rigid, the other bonds are flexible and allow the polypeptide backbone to fold in space. Therefore, proteins are not linear molecules as suggested by the amino acid sequence, but rather folds into secondary, tertiary and quaternary structures. Folding of a linear chain is stabilized by intramolecular hydrogen bonding within the peptide backbone resulting in local protein *secondary structures*, such as the alpha (α) helix and beta (β) sheet as shown in Figure 1.1. The alpha helix is characterized by hydrogen bonds between the co-axial main chain carbonyl (C=O) of each residue and the amide (N-H) residues along the chain. The beta sheet is characterized by hydrogen bonds crossing between chains. For long chains, the folding results into a three-dimensional domain structure that is unique to each protein. The overall three-dimensional structure of a single protein molecule resulting from stabilized secondary structures is called a *tertiary structure*. A tertiary structure is stabilized by non-bonding interactions, such as the formation of a hydrophobic core, hydrogen bonds and covalent disulfide bonds between
thiol group sulfur atoms in cysteine residues. Proteins further interact with other protein subunits to form a larger protein complex, called *quaternary structures*. In the context of functional rearrangements, tertiary or quaternary structures are referred to as "conformations," and can undergo conformational changes (transitions) induced by the binding of a substrate molecule to an enzyme's or antibody's active site, a physical region of the protein that participates in chemical catalysis or molecular recognition.

![Diagram of protein structures](image)

**Figure 1.1** Illustration of primary (amino acid sequence), secondary (α-helix, β-sheet), tertiary and quaternary (functional) structures of proteins.

### 1.3 Protein Classification

Proteins can be classified into three main types based on their tertiary structures; globular proteins, fibrous proteins, and membrane proteins. Most globular proteins are spherical in shape with their apolar motif oriented towards the inner core while the polar
moieties are oriented towards the outside. For this reason they are known to be soluble in aqueous media or less soluble forming a colloidal solution in aqueous media. Examples of globular proteins are enzymes that catalyze reactions (i.e. esterases), messengers that regulate biological processes (i.e. hormones) and other proteins like hemoglobin and immunoglobulins. Fibrous proteins are long filamentous molecules that serve as structural or storage proteins and are used to construct connective tissues, tendons, bone matrix and muscle fiber. They are insoluble in aqueous media and aggregate due to hydrophobic side groups that stick out of the molecule. Examples of fibrous proteins are keratins, collagens and elastins. Lastly, membrane proteins are covalently attached to, or electrostatically associated with the membrane of a cell or an organelle. They often serve as receptors or provide channels for polar or charged molecules to pass through the cell membrane. In terms of physical properties, proteins can also be divided into acidic, basic, hydrophobic and hydrophilic proteins depending on their amino acid composition.

1.4 **Protein Synthesis**

Proteins are bio-synthesized from genetic codes, a set of three-nucleotide sequences called codons present in the genome, that specify particular amino acid information encoded in the *messenger* ribonucleic acid (*mRNA*) of a given cell as illustrated in Figure 1.2.6.7 RNA and DNA molecules are made up of four different types of nucleotides linked together by phosphodiester bonds. However, they differ chemically in that nucleotides in RNA contain the sugar ribose rather than deoxyribose as in DNA. Like DNA, RNA contains the bases, adenine (A), guanine (G) and cytosine (C), however RNA has the base uracil (U) instead of thymine (T) as in DNA. In the first step of protein bio-synthesis, transcription, the double helix deoxyribonucleic acid (DNA) strands in a gene that codes for a protein unzips and the
strands unwind from each other to expose the code on each DNA strand.\textsuperscript{9} There are about six possible reading frames on double stranded DNA, three forward and three reverse. A reading frame contains a set of three adjacent and non-overlapping nucleotide codons in DNA or RNA. A reading frame that contains both a start and a stop codon is called an open reading frame (ORF). A protein enzyme called RNA polymerase, reads the sequence of nucleotides in the codon of one of the DNA strand that serves as the template and synthesizes a single strand \textit{m}RNA molecule from the 5′-to-3′ direction. The sequence of the RNA chain (transcript) produced by transcription is determined by the complementary base paring between the growing RNA nucleotides and the DNA template. The RNA chain is thereafter displaced and the DNA double-helix reforms.

The single stranded \textit{m}RNA once released from the DNA template migrates from the nucleus to the ribosomes in the cytoplasm and is translated into a transfer RNA (\textit{t}RNA). In the second step, translation, ribosome translates the codons from the \textit{m}RNA that specify the order in which new amino acids are added by the initiator, \textit{t}RNA, to a growing protein chain for elongation.\textsuperscript{9} The amino acid assembly is linked together by peptide bonds eventually forming a protein that is later released by the \textit{t}RNA.\textsuperscript{10} Different proteins can be generated by alternative use of start and stop codons. Cells also have the ability to change the protein expression levels depending on the state of the cell. Regulatory regions of DNA near the sites where transcription starts may act as a switch and respond to a signal from the cell to control gene expression while other regulatory regions serves as a microprocessor and respond to a variety of signals from the cell to control gene expression. For example the gene regulatory proteins can turn specific sets of genes on or off, hence, regulating protein expression levels in the cell.
Figure 1.2 Protein synthesis steps in a cell; (a) transcription at genetic level, (b) post-transcription at the mRNA level (c) translation at the tRNA level (d) post-translation at the protein level.

Synthesized proteins can be modified further in various ways (> 400) during or after translation.\textsuperscript{11} Post-translational modifications can occur either through attaching functional groups to specific amino acid chains on the protein, attaching other proteins or peptides, changing the chemical nature of amino acids or involving structural changes. Whereas some post-translational modifications such as glycosylation are permanent, others like phosphorylation are transitional and are used for cell cycle regulation. The genome (complete set of genes in an organism) is static while the transcriptome (complete set of RNA in a given cell) and the proteome (complete set of proteins in a given cell, tissue,
organelle or body fluid) are dynamic due to the regulation of transcription, RNA processing, protein synthesis and protein modifications. For example, the human genome has ~30,000 genes that can generate over $10^6$ proteins when post-translations modifications are taken into account.\textsuperscript{11}

1.5 **Protein Function**

Proteins are involved in most cellular processes as enzymes that catalyze biochemical reactions within the cell, such as metabolism, catabolism, DNA replications, DNA repair and RNA synthesis.\textsuperscript{12-14} Enzymes, that are proteins, also act on other proteins involved in signal transduction and in regulation of the cell cycle. Proteins act as receptors and cell adhesion molecules to transport ligands through the cell and through the body and to recognize extra cellular stimuli and signaling molecules, for example hemoglobin and antibodies. Other proteins play structural or mechanical roles, such as in the cytoskeleton, which allows the cell to maintain its shape and size. The mechanisms for controlling these functions often depend on controlling protein activity. Activity is regulated by post-translational modifications either through attaching functional groups to specific amino acid residues on the protein, attaching other proteins or peptides, changing the chemical nature of amino acids or inducing structural changes. Examples of post-translational modifications include addition of functional groups as in acetylation, alkylation, phosphorylation and glycosylation to specific amino acid residues, addition of other proteins/peptides as in ubiquitination, changing of amino acids as in deamidation and other structural changes due to disulfide bridges and proteolysis. Most of the protein’s post-translational modifications could serve to increase or decrease in activity, lifetime, or both and hence extend the range of protein functions.\textsuperscript{11} For example, acetylation of the N-terminus increases the lifespan of
proteins in the cell while phosphorylation is used to activate or deactivate enzymes and to modulate the specificity of receptors.

1.6 Why Analyze Proteins?

Following the elucidation of the genome sequences of many organisms, there has been a shift in focus from genomics, the study of global properties and functions of genes, to proteomics, which is the large-scale study of identity, structures, functions, interactions and expression levels of proteins that form complete sets of proteins (proteome) of a given organisms, organelle, tissue or cell line. Genomic information is of great use to proteomic studies because it provides a “blueprint” of the probable complementary proteome. Since proteomics relies on the existence of genomic information, it plays the role of identifying and defining functional genomic codes from a given DNA sequence along with annotating the genome. As a result, proteomic studies have focused on a large-scale characterization of the entire protein complement of a cell line, tissue or organism, protein-protein and protein-gene interactions, modifications, function, and define where these proteins are located in cellular and sub-cellular compartments. In this regard, the goal of proteomics is to obtain a global and integrated view of how biological systems function. This information, when combined with gene expression and metabolic profiling, forms the basis for understanding the fundamental mechanisms of life, a field known as systems biology.

Proteomics is currently studied in a number of complementary ways categorized either under descriptive, structural, interaction, functional, or expression proteomics as shown in Figure 1.3. Descriptive or qualitative proteomics focuses on the identification of new expressed proteins to validate hypothetical proteins predicted from gene sequences, or to add new proteins to existing protein databases or to serve as biomarkers for disease
states. Proteins are readily accessible in biological samples such as serum, cerebrospinal fluid and urine when shedding from tissues and can be used as diagnostic, staging and prognosis disease biomarkers. Further, proteins are the most therapeutically relevant molecules in systems biology, lending themselves as therapeutic agents or for use in therapeutic drug monitoring studies. On the other hand, structural proteomics entails mapping out the overall structure of proteins and their complexes in an effort to characterize protein-protein interactions. These interactions are useful in drug discovery since they help in studying protein-drug interactions and activity. Structural proteomics also exposes post-translational modifications in proteins that alter their activity.

Expression proteomics involves the quantitative study of protein expression levels between samples that differ by some property e.g. cancer. Studies have shown that there is no measurable correlation between expression levels for a given gene transcript, mRNA quantities and the corresponding proteins quantities. One reason being that the majority of protein diversity is generated post-transcription. The comparison of protein expression levels within samples that differ by some parameter gives rise to differentially expressed protein profiles that are used to discover proteins that can serve as biomarkers. Functional proteomics involves monitoring fluxes, spatial and temporal properties of molecular networks in living cells in an effort to provide information about pathway signaling, aging and disease mechanisms or protein-drug interactions. Another reason for analyzing proteins rather than the genetic code is that protein activity is dependent on post-translational modifications, which cannot be predicted from the corresponding DNA sequence. In terms of location, some proteins move from one cellular compartment to another, for example from the cytosol to the nucleus, as a form of regulation and in such
cases, it has been shown that the cellular distribution of a given protein is more informative than the protein expression level. By identifying the molecular species involved in such biological networks, proteins can be classified by function. Induced changes in the protein primary structure and RNA interference also allows for large-scale functional evaluations in these molecules.

**Figure 1.3** Overview of proteomics, which can be categorized into functional proteomics (defining protein functions and their role in systems biology), structural proteomics (elucidating structures of proteins, complexes and interactions), expression proteomics (measurement of protein expression levels and their relation to physiological state of the cell) and qualitative proteomics (identifying expressed proteins).

1.7 **Challenges of Analyzing Proteins**

A number of technologies have been developed to analyze proteins; however it is clear that no one technology platform is suitable for all applications.\(^\text{35}\) Despite the strengths and weaknesses of existing technologies, one significant drawback is their lack of integration and automation. Another limitation of protein analysis is the lack of an
amplification method equivalent to the polymerase chain reaction (PCR) for preparation of less abundant proteins that are difficult to detect. The issue of sensitivity becomes paramount considering the dynamic range of protein abundance in biological samples.\textsuperscript{36} Its has been estimated that this dynamic range is in the order of $10^5$ for tissues and $10^9$ for body fluids such as serum.\textsuperscript{37} Low abundant proteins are present in nanomolar concentrations ($\sim 10$-50 ng/ml or less) in body fluids.\textsuperscript{37, 38} Therefore, they are difficult to detect in the presence of high abundant ones ($\sim$50-80 mg/ml) due to limited dynamic range of commonly used technologies. The diversity of proteins between different cell lines even within the same tissue or organism is still another challenge. Whereas human cells may express up to 20,000 proteins at one time, the approximately 30,000 genes in the human genome may code for more than 800,000 proteins.\textsuperscript{15, 39} Further, these proteins can be modified after translation in over 400 ways.\textsuperscript{40} These tasks have already brought about a number of intimidating challenges that continue to prove that a comprehensive study of the proteome is sometimes a humbling exercise that is far from completion.\textsuperscript{36, 41} However, a significant progress has already been realized in the development of techniques for analyzing proteins.\textsuperscript{42-44} Another issue with some common technologies is the high rate of false positives and false negatives, for example, the yeast two-hybrid system, which is used to detect protein interactions.\textsuperscript{45, 46}

1.8 Sample Processing for Proteomics

Typical sample preparation for proteins entails a series of laborious and low throughput steps ranging from protein extraction methods from tissues such as laser microdissection, whole cell lyses, protein isolation and enrichment from body fluids, solubilization, reduction, alkylation and denaturation before gel electrophoresis separation, staining and spot excision, proteolytic digestion of visualized protein spots, chromatographic
separations of generated peptide fragments followed by either off-line fractionation or on-line analysis or both, identification of the peptide fragments and proteins using mass spectrometry. A schematic representation of the routine sequence of analytical steps used for proteomic sample processing is shown in Figure 1.4.

**Figure 1.4** Overview of proteomic sample processing steps and techniques used in the extraction, pre-concentration, separation, identification and discovery of proteins from tissues, cells and body fluids.

There are two popular strategies used in analyzing proteins by mass spectrometry, the “bottom-up” and “shotgun” approaches. In the “bottom-up” approach, protein mixtures are first separated, then the separated proteins are digested before the identification process by peptide mass fingerprinting or sequence tag is begun as shown in Figure 1.4.
above. Since the digestion step occurs after protein separation, the peptide fragments generated are specific to a particular protein and in most cases allow identification without Tandem MS. The “bottom-up” approach is time consuming and limited by the resolution of two dimensional gel electrophoresis. On the other hand, the “shotgun” approach begins with proteolytic digestion of protein mixtures followed by a variety of chromatographic separation approaches as shown in Figure 1.5 below.

**Figure 1.5** General strategies used in proteomic studies; shotgun approach begins with the digestion of all the extracted proteins followed by separation and fractionation before analysis with a mass spectrometer, the bottom-up approach begins with the separation of all extracted proteins, followed by staining, digestion and then identification with mass spectrometry.

For the shotgun approach, digestion of complex proteins mixtures result in a large number of peptides that cannot be resolved by any analytical technique and also complicates the protein identification process. A combination of multi-dimensional liquid
chromatography and tandem MS is usually performed to improve the protein identification efficiency.\textsuperscript{52} When shotgun and bottom-up approaches are combined, one can sacrifice the high resolution requirements in two dimensional gel electrophoresis for the bottom-up approach by extensive fractionation at the peptide level with shotgun protocols. Another less commonly used approach is “top-down” which typically refers to measuring the mass of a protein in a mass spectrometer prior to gas phase fragmentation usually by electron capture dissociation.\textsuperscript{53-55}

1.8.1 Extraction of Proteins

Extraction of protein from tissues, cells and body fluids requires efficient release of biomolecules since it is the first step in a multi-step sample processing scheme. The quantity and nature of released proteins affect the success of downstream processing. A general sample extraction entails removal of salts, nucleic acids, polysaccharides, lipids and insoluble material. Current technologies for protein extraction employ either mechanical methods like mortar and pestle grinding, or semi-automated methods, such as bead beating, rotor-stator homogenizations, enzymatic digestion, chemical dissolution and sonication.\textsuperscript{56-58} These methods suffer from potential contaminations, extended processing time, shearing molecules-of-interest that result in distortion of native states of protein complexes, lack of automation, selective to a subset of proteins or narrow range of sample types and poor reproducibility.\textsuperscript{59, 60} They also destroy the protein compartmentalization (localization) information and release hydrolases (phosphatases, glycosidases and proteases) into the homogeneous lysate leading to potential alteration of the native protein composition and rendering differential expression difficult.\textsuperscript{37} Some proteins, for example, the plasma membrane proteins are difficult to isolate due to their propensity to exist in various
structures and also due to their low abundance in relation to mitochondria, endoplasmic reticulum and other membranes in the cell.61 Removal of protein disulfide bonds followed by reduction and alkylation of the resulting free sulfide groups is necessary to improve solubility.47

New, reliable and improved protein extraction methods are therefore needed to advance and accelerate proteomic discoveries. First, novel sample processing platforms are needed to address fast solubilization of all cellular proteins and naturally occurring protein complexes. The platforms also need to alleviate unwanted protein aggregations, promote efficient extraction, release and isolation of all protein components (especially 30% of membrane proteins which constitute more than 50% of current drug targets) from cellular debris without altering their native form or states in an automated and high throughput sample processing system.24, 62, 63

1.8.1.1 Pressure Cycling Technology Sample Preparation System (PCT SPS)

The state-of-the-art technology for protein extraction from solid tissues, body fluids, liquid cultured cells, including a variety of hard-to-lyse materials, employs an automated pressure cycling technology sample preparation system (PCT SPS).64 The system utilizes a pressure instrument (barocycler) to generate alternating cycles of ambient and 35 kpsi (235 MPa) of hydrostatic pressure. Samples are placed inside the tubes and then the tubes are placed inside the barometer for cycles of pressure to be applied. The alternating cycles of pressure are used to control biomolecular interactions and hence release proteins from cells and tissues in temperature controlled single use PULSE (pressure utilized to lyse sample for extraction) tubes shown in Figure 1.6.64, 65
On one end of the tube is a movable polypropylene ram that is put in place after the sample is loaded into the tube, on the other end of the tube is a fluid retention chamber sealed with a threaded cap for introducing processing buffers and withdrawing extracted samples from the tube. In the middle of the tube there is a perforated lysis disk. The temperature is controlled using an external circulating water bath to maintain sample processing between 4 and 37\(^0\) C. At high pressure, the movable polypropylene ram (disk) and the perforated lysis disk placed inside the tube squeezes and macerates the solid tissues, increasing the surface area, which is exposed to the extraction buffer. When the pressure is released, the partially homogenized sample is pulled back through the lysis disk by the receding ram. The combination of physical passage through the lysis disk, rapid pressure changes, temperature, solvent exchange and other bio-physical mechanisms, breaks up the cellular structures thereby releasing the nucleic acids, small molecules and proteins into the collection chamber. Proteins are thereafter isolated from the homogenized sample.

![Figure 1.6](image)

**Figure 1.6** Pressure used to lyse sample and extract (PULSE) tube consisting of (a) a ram, (b) a perforated lysis disk, (c) a collection chamber and (d) a threaded cap.

1.8.1.2 **Laser Capture Microdissection**

Laser capture microdissection employs a finely focused laser beam to isolate individual cells from a heterogeneous tissue sample section as illustrated in Figure 1.7.\textsuperscript{66, 67} It is basically an inverted microscope fitted with a low-power near-infrared laser. Tissue
sections are placed on standard glass slides, and a transparent, 100-mm-thick, ethylene–vinyl acetate film placed over the dry tissue section. A focused laser beam with adjustable diameter (7 to 30 µm) provides enough energy to transiently melt a thermoplastic film in a precise location, binding it specifically to the targeted cells. The plastic film absorbs most of the thermal energy and hence little or no detectable damage of tissue or biomolecules occurs. The film with the adhering cells is thereafter lifted off the thin tissue section, leaving all unwanted cells and tissue behind in contact with the glass slide. Under the microscope, one views the thin tissue section through the glass slide on which it is mounted and chooses microscopic clusters of cells to be targeted for extraction and further molecular analysis. Targeted cells are delivered directly into a microcentrifuge tube, cap or a thermoplastic film for purification and molecular processing. Proteins are thereafter isolated from these cells using appropriate protocols. The major limitation of this technique is that it isolates minute amounts of sample, which limits analysis to the collection of numerous cells for protein analysis. The isolation of large numbers of cells from many tissue sections takes a long time.

**Figure 1.7** Laser capture microdissection apparatus used for selective isolation and extraction of individual cells from a tissue section. (http://www.nano.geo.uni-muenchen.de/nanobio/laser.html).
1.8.2 **Protein Pre-concentration**

After extraction, sample pre-concentration is required to address protein components present in low amounts. Traditional concentration methods such as solvent evaporation, molecular weight cut-off filters and dialysis are time consuming, have high sample loss, and lack selectivity, hence results in contaminants and other proteins being concentrated along with the targeted proteins. Pre-concentration platforms should address selective depletion of high abundant proteins. For example, accessible body fluids like blood plasma, is postulated to have 30,000 proteins. The albumins, immunoglobulin G, alpha-1-antitrypsin, immunoglobulin A, transferrin and heptaglobin are the most abundant proteins (60-80 mg/ml) while the biologically interesting and clinically relevant low abundant proteins present in > 10 ng/ml accounts for ~ 15 % of unknown proteins as shown in Figure 1.8. Cutting edge techniques in the field utilize bio-specific molecular recognition elements, such as immuno-affinity ligands, zwitterionic detergents, ion-exchange, size exclusion and separation techniques to deplete the high abundant proteins.

![Figure 1.8](image_url) **Figure 1.8** Pie chart illustration of human serum proteins; albumin are the most abundant proteins in serum present in ~ 60-80 mg/ml while the unknown proteins account for 15% of the serum proteins and are present in less than 10 ng/ml.
Affinity ligands such as antibodies, antigens, aptamers, enzymes, hormones and avidin have been used to remove interference by immuno-depletion of high abundant proteins in order to improve the detection of low abundant proteins. IgG immuno-affinity-based systems with specific antibodies that target abundant proteins have been demonstrated to provide effective removal of targeted abundant proteins, with minimal carryover, high longevity, and minimal nonspecific binding. Immobilized metal ion affinity chromatography (IMAC) has also been used for protein pre-concentration. IMAC employs a column material to bind divalent metal ions such as copper, nickel, zinc, cobalt, iron, and calcium. For example, these metal ions specifically interact with exposed amino acid residues like histidines, tryptophans, cysteines, and tyrosines (common phosphorylation sites) to form coordination complexes between the metals and amino acid residues on the protein. This results in affinity enrichment of phosphorylated proteins and serves to eliminate interferences thereby increasing the signal intensity for protein identification.

Centrifugal ultrafiltration has also been used to pre-concentrate low molecular weight proteins by using denaturing conditions that disrupt protein-protein interactions. The technique can be tuned by varying the spin time, sample amount and protein recovery method; however, it lacks specificity. Protein pre-concentration has also been demonstrated from solution by adsorption of a protein or protein mixture onto a hydrophobic resin immobilized within a microcolumn. Sample loading is accomplished by flowing the protein solution through the microcolumn, where the protein adsorbs onto the hydrophobic resin surface. Another approach employs microbeads to concentrate proteins from an aqueous phase by adsorption onto reverse-phase polymer beads. The beads are then washed to remove contaminants before the bound proteins are released from the beads by digestion.
1.8.3 **Protein Separation**

Protein separation allows simplification of complex mixtures and improves the detection of low abundant proteins. A number of separation technologies have been on the forefront of proteomics studies in efforts to elucidate the identity of proteins. Commonly used protein separation methodologies, their strengths, limitations and the need for new enabling technologies are discussed in the following section.

1.8.3.1 **One and Two Dimensional Gel Electrophoresis**

For a long time, one-dimensional sodium dodecyl sulphate poly-acrylamide gel electrophoresis (1D SDS PAGE) has been used as the method of choice to resolve protein mixtures.\(^88\) The SDS surfactant denatures proteins by binding along the polypeptide backbone and hence imparting a negative charge along its length. A reducing agent is added in order to reduce the disulfide bridges allowing the protein to adopt a random-coil configuration necessary for separation by size in the cross-linked polyacrylamide sieving gel matrix cast on a slab upon application of an electric field.\(^88\) Protein separations in the cross-linked gel matrix pore structures is based on molecular weight and the technique has a dynamic range of approximately 10 to 300 kDa. The viscous gel serves as an anticonvective medium eliminating conductive transport, reducing diffusion and hence increases the resolving power and peak capacity.

One dimensional gel electrophoresis is limited in resolving power with maximum peak capacity of ~ 400.\(^89, \, 90\) The low peak capacity results from the fact that the peaks are restricted to a definite interstitial retention volume and maximum solvent volume of the gel. For more complex protein mixtures, two-dimensional sodium dodecyl sulphate poly-acrylamide gel electrophoresis (2D-SDS PAGE) is usually the method of choice for the
“bottom-up” approaches. In two-dimensional gel electrophoresis, proteins are initially separated based on their isoelectric point (pI) using isoelectric focusing (IEF) and thereafter separated in the second dimension using SDS PAGE based on their molecular weight. In IEF, protein mixtures are electrophoretically driven through a pH gradient, and since proteins are zwitterionic, they migrate to a final position where the net charge is zero. The pH at this point is usually the same as the pI of the protein. The theoretical limit of IEF is governed by the resolution of the pH gradient. The resolution in IEF can be increased by using immobilized pH gradients or specialized pH gradients that can zoom in on a narrow pI range to resolve more complex mixtures. The increased resolving power and hence peak capacity (> 1000) in 2D gel electrophoresis has been used to resolve post-translational modified proteins and different forms of proteins.

Despite these strengths, a number of limitations to 2D gel electrophoresis still exist. It remains a laborious and is a slow process taking over 24 h and is capable of a single run per gel. Although, the dynamic range is wide, it is limited by the number and type of proteins that can be resolved. Loading of hydrophobic proteins is a challenge. For example, plasma membrane proteins analysis require cleaving of protein disulfide bonds followed by reduction and alkylation of the resulting free sulfide groups to improve solubility. In addition, most abundant proteins usually dominate the gel thereby making visualization and detection of low-copy proteins difficult. The technique is widely used for qualitative and expression profiling where differentially expressed proteins between two samples that differ by some parameter are exposed by the appearance or disappearance of spots on the gel. The intensity of the spots on the gel provides quantitative information about protein expression levels while a mass spectrometer is used to identify
or even quantity the proteins in the gel spots. A schematic of current equipment used in 2D gel electrophoresis and an example of typical data used for differential display is shown in Figure 1.9.

Figure 1.9 A representation of current 2D gel electrophoresis equipment which employs IEF in the first dimension and SDS PAGE in the second dimension. A typical data output is also shown as used to display differentially expressed proteins in diseased versus normal tissues.

- **Staining**

Protein spots resolved in a gel need to be visualized for ease of detection, excision and identification. Visualization of spots in gel can be done in situ or following “western” electroblot transfer of separated proteins onto polymeric membrane support materials. Most of the staining is done after electrophoresis, however, there have been attempts to stain proteins before loading them onto the gel. Protein spots are visualized using staining methods such as silver stains, commassie blue, fluorescent dyes or radioactive labels. In situ staining is required for most proteins using organic dyes for example coomassie blue, which is a non-polar sulfonated aromatic dye that stains basic amino acid residues in acid
conditions. It is less sensitive compared to silver stains, which is the most sensitive non-radioactive protein visualization stain. However, silver stains are not universal, they stain some proteins poorly if not at all, for example calcium binding proteins. Fluorescent dyes, for example fluorescamine, have also been used to stain proteins before or after gel separation. Radioactive isotopes such as $^{14}$C, $^{35}$S, $^{32}$P, $^{3}$H and $^{125}$I have also been used to label proteins. Radiolabeling is the most sensitive protein visualization and detection method. Staining proteins is time consuming and depends on the type of stain, the visualization and image processing steps adds processing time to sample preparation and analysis.$^{104}$

- **Digestion**

For identification purposes, visualized protein spots are excised from the gel and processed using proteolytic digestion or chemical cleavage.$^{105}$ Chemical reagents used for digestion, for example cyanogen bromide, cleave peptide bonds at specific amino acid residues while others like hydrazine cleaves all of the peptide bonds yielding amino-acyl hydrazides of all the amino acids present in a protein. Most of the protein digestion is carried out using in-solution or in-gel digestion protocols that take between 6 to 24 hours.$^{105}$ The process usually involves protein denaturing, reducing the disulfide bonds, mixing with an alkylating agent to prevent disulfide bonds from reforming and finally incubation of the protein mixture with a lytic reagent. Enzymatic proteolytic cleavage employs proteases that hydrolyse the peptide bond. Proteases are divided into two broad categories: proteinases or endopeptidases, which attack inside the protein to produce large peptide fragments and the peptidases or exopeptidases, which attack ends of proteins to produce smaller peptide fragments or amino acids.$^{106, 107}$ The proteinases can further be subdivided into six groups
based on the mechanism of action at the active site. These sub-divisions are serine, cysteine (thiol), aspartic (acid), metallo and glutamic acid proteases named according to the amino acid participating in catalysis. On the other hand, peptidases are classified based on the action pattern: for example, aminopeptidase cleaves amino acids from the amino end, carboxypeptidase cleaves amino acids from the carboxyl end, dipeptidyl peptidase cleaves two amino acids, dipeptidase splits a dipeptide and tripeptidase cleaves an amino acid from a tripeptide.

Examples of serine proteinases such as trypsin, cleaves the peptide bond at the c-terminal next to lysine and arginine residues if the residues are not next to praline. Another serine protease chymotrypsin, cleaves the peptide bond at the c-terminal next to tryptophan and tyrosine if these residues are not next to proline. Other cysteine (thiol) proteinases include bromelain and papain. Aspartic (acid proteinases) for example pepsin cleave at the N-terminus next to leucine, tryptophan and tyrosine if the residues are not next to praline. Examples of metallo-proteinase include collagenase, keratinase and collagenase. Examples of aminopeptidase include alanyl and lysine aminopeptidase, di and tripeptidase like lysosomal peptidases. Carboxypeptidase e.g. prolyl carboxypeptidase A does not cleave when C-terminus residue is arginine, lysine or proline or if proline resides next to terminus residue.

1.8.3.2 Liquid Chromatography

Liquid chromatography (LC) is a powerful technique for separating proteins and peptides. Sample loading in LC is controlled using fixed volume sample loops that are typically in the micro-liter range. Sample is pumped through the column at 1000-5000 psi with a flow rate dependent on the column internal diameter (i.d.). Generally, LC is practiced
in column formats of greater than 10 mm i.d. and at flow rates of 1000 μl/min for prep-scale purpose. For analytical separations, columns of less than 5 mm i.d. are employed with varying flow rates. The columns consist of a stationary solid phase such as 5 μm size beads that interacts with solutes in a mobile liquid phase. Recent developments have shown that capillary LC, which employs smaller i.d. capillaries of ~ 50 μm or less provide a linear response over a wide range of sample concentrations and improved sensitivity due to constrained sample size when interfaced to a mass spectrometer.

LC is practiced in different formats namely; ion exchange, size exclusion, normal and reverse-phase liquid chromatography. In ion exchange chromatography, proteins are reversibly adsorbed on to solid phase resins that contain cationic exchangers such as -SO₃⁻, -OPO₃⁻ and -COO⁻ anionic functional groups or anionic exchanger that contain the cationic tertiary (i.e. -C₂H₄NH(C₂H₅)₂) and quaternary (i.e. -CH₂N+(CH₃)₃) ammonium groups to separate proteins based on their charge properties. Proteins possess a net charge in solution, dependent upon the pH, structure and pI. In solutions with pH below their pI, proteins have a net positive charge and will bind to cationic exchangers, whereas in solutions of pH above their pI they have a net negative charge and will bind to anionic exchangers. The procedure involves gradient elution using a mobile phase consisting of buffers of gradually increasing ionic strengths to displace bound proteins from the stationary phase or changing the buffer pH so that the net charge on the proteins is altered.¹⁰⁹

In a different format, size exclusion chromatography also referred to as gel permeation or gel filtration chromatography, proteins are separated based on their size.¹¹⁰ The columns employ a gel medium such as silica, cross-linked polystyrene, polyacrylamide, dextran or inert porous agarose beads that trap small proteins in the pores resulting in longer
retention times compared to large proteins. The pore size is dependent on the gel medium, for example silica-based medium has a pore size in the range of 125 to 1000 Å and molecular weight exclusion limit of 2 kDa to 50 kDa for pore sizes of 125 Å, 5 kDa to 500 kDa for pore sizes of 500 Å and so forth. Cross-linked polystyrene-divinylbenzene has pore sizes in the range of $10^2$ to $10^6$ Å while agarose pore sizes in the range of 500 to 2500 Å. For a protein domain with an apparent hydrodynamic radius (radius of gyration) of 14 Å when folded and 36 Å when unfolded, the folded form will elute much later due to its smaller size.

Normal phase LC employs polar stationary phase such as cyano (-C$_2$H$_6$CN), diol (-C$_3$H$_6$OCH$_2$CHOHCH$_2$OH), amino (-C$_3$H$_6$NH$_2$) dimethylamino (-C$_3$H$_6$N(CH$_3$)$_2$) and non-polar mobile liquid phases to separate proteins based on their polarity. Reverse-phase chromatography, the most commonly used LC format, employs stationary phases that are non-polar like hydrocarbons, waxy liquids or bonded hydrocarbons such as C$_{18}$, C$_8$, C$_4$. One common stationary phase is silica, which has been treated with RMe$_2$SiCl, where R is a straight chain alkyl group such as C$_{18}$H$_{37}$ or C$_8$H$_{17}$. The mobile phase is comprised of solvents that are polar-aqueous mixtures like methanol-water or acetonitrile-water. Elution is accomplished using either a single (isocratic) or a programmed (gradient) mobile phase composition. Solvophobic interactions of the solutes in the mobile phase and the stationary phase results in differences in adsorption, selectivity and partitioning. As a result, solute retention occurs and components in the liquid phase are separated as they elute out of the column.

Generally, for large proteins (i.e. with molecular weight above 20 kDa), a digestion step prior to separation with LC is required. A typical digestion of a protein mixture present
in a given sample generates a more complicated peptide mixture that no single separation
technique can resolve. For example, the proteome of many cell lines may contain more than
10,000 proteins and a tryptic digest of one protein may result in an average of 30-50
peptides, this translates to ~3 x 10⁵ to 5 x 10⁵ or more peptides. Usually multi-dimensional
LC, typically strong cation exchange followed and reverse-phase LC followed by multiple
fractionation steps, are carried out to address sample complexity of this magnitude.¹¹² A
typical multidimensional LC can resolve up to 2 x 10⁴ peptides in the best-case scenario,
which is still one order of magnitude less than required to analyze all peptides present in the
proteome of a cell line.¹¹³, ¹¹⁴ Further, the sample processing, like in the best case scenario
described above, takes three days of work.¹¹⁴ Nonetheless, liquid chromatography
is the workhorse of “shotgun” proteomics where proteins are digested and the resulting
peptides subjected to multiple LC steps.⁵⁰, ¹¹⁵ When this LC approach is combined with 2D
gel electrophoresis, one can sacrifice the high resolution in 2D gel electrophoresis at the
protein level by extensive fractionation after LC separations at the peptide level.

1.9 Microfluidic Devices and Systems for Proteomic Sample Processing

A microfluidic device consists of a network of fluidic channels that are a few tens of
microns wide and fabricated in planar substrates such as glass, silicon or polymers. The
fabrication of microfluidic devices is discussed in Chapter 3. Microfluidic devices are being
explored as alternative platforms for protein analysis. On reason being that the transitioning
of analytical techniques to chip-based formats promises potential revolutionary impact on
bioanalytical analyses.¹¹⁶-¹²¹ The advantages associated with microfluidic platforms include,
small sample volumes,¹²², ¹²³ less reagent consumption, disposability, portability, fast
analysis, parallel analysis, reduced cost\textsuperscript{124-126} and integrated sample processing that eliminates sample losses and contamination.\textsuperscript{116,127-129}

1.9.1 \textbf{Microfluidic Devices for Discrete Protein Sample Processing Steps}

Samples are usually flowed through a network of channels in a microfluidic device for discrete processing steps like extraction, pre-concentration, separations, sorting, capture, lyses, digestion, desalting and clean-up.

1.9.1.1 \textbf{Extraction}

Microfluidic devices have been demonstrated for cell lyses and protein extraction.\textsuperscript{130,131} In one of these examples, a diffusion-based microfluidic device for continuous lysis of bacterial cells and fractionation of protein components was reported. A cell suspension and a chemical lytic reagent were hydrodynamically introduced into the device, the lytic reagent permeates into the cell membrane allowing intracellular components to leave the cell and diffuse into the same fluidic channel depending on their size and diffusion coefficients.\textsuperscript{131} In a different approach, phosphocholine immobilized on agarose beads was used for solid phase extraction in a microfluidic device to extract C-reactive protein directly from unadulterated human serum.\textsuperscript{132} The beads were packed into the fluidic channel and solution containing C-reactive protein introduced using a syringe pump. The protein was subsequently released by changing the elution buffer.

1.9.1.2 \textbf{Pre-concentration}

Microfluidic devices have been used to pre-concentrate protein samples from solution.\textsuperscript{133-138} One sample pre-concentration technique employs sample stacking with buffers plugs of different conductivity.\textsuperscript{137-140} Dialysis has also been used to pre-concentrate proteins in a microfluidic device.\textsuperscript{133,136,141,142} In one example, protein concentration in a
A microfluidic device was achieved using a nano-capillary array placed in a microfluidic channel for concentrating large molecules in front of the array, and porous silica membranes placed between adjacent microchannels that allowed buffer ions to pass but excluded larger molecules. Nanoporous membranes in a microfluidic channel were also used to achieve a molecular weight cutoff of protein molecules larger than 5700 Da. Ion exclusion enrichment effects as a result of electrical double layer in PDMS/glass devices has also been reported to pre-concentrate proteins. Another example employs a thermally responsive polymer poly (N-isopropylacrylamide) (pNIPAm) film. This polymer has a liquid critical solution temperature (LCST) and exhibits reversible wettability that can be exploited to capture and release proteins from solution. The polymer film was coated in a fluidic channel and a heater was used for rapid and controlled heating and cooling in small volumes. Above the LCST, the film switches to a protein-adsorbing state that is more hydrophobic, capturing proteins from solution; below the LCST, it switches to an antifouling hydrophilic state, hence releasing the captured proteins.

Affinity-depletion methods have also been employed in microfluidic devices for protein pre-concentration. In one example, affinity capture of lysozyme and albumin from human cerebrospinal fluid was achieved in a microfluidic channel using UV photopolymerization of glycidyl methacrylate and trimethylolpropane trimethacrylate that was also derivatized with Cibacron-blue-3G-A, a triazine dye, known to have group specificity for albumin and lysozyme. In a similar example, a microfluidic channel with immobilized metal affinity chromatography beads was used to enrich targeted phosphopeptides and allowed identification of trace proteins from human prostatic cancer cell extracts. Integrated selective enrichment target fabricated on silicon have been
used for characterization of prostate-specific antigen from human seminal fluid co-isolated by affinity chromatography.

1.9.1.3 Separation

LC has been demonstrated in microfluidic devices for peptide and protein separations. In one example an array of methacrylate monolithic columns was prepared in a microfluidic channel by UV-initiated polymerization to separate a peptide mixture from trypsin digested proteins. In a separate approach a microfluidic device was used to separate a digest obtained from the MCF7 breast cancer cell line. The cytosolic protein extract was processed off-chip using a shotgun protocol, tryptic digestion and prefractionation using strong cation exchange chromatography (SCX). The selected sample subfractions were then separated in a microfluidic LC device using reverse-phase chromatography. In a different approach, reverse-phase LC using a C-18 side chain polymer monolith prepared in a microfluidic device was used to separate proteins and peptides with resolution comparable to a conventional capillary HPLC column. Multi-dimensional LC in microfluidic devices are still being investigated as a method for high peak capacity for analyzing complex peptide mixtures in shotgun proteomics.

Electrophoretic separation of proteins and peptides has also been demonstrated in microfluidic devices and reviewed by Lion et. al. Since the work described in this dissertation employed electrophoresis, this technique will be discussed in detail. Capillary electrophoresis concepts were first introduced in the early 80’s by Mikkers, Jorgenson and co-workers. Compared to LC, CE is fast and achieves high separation efficiency with nano-liter injection volumes. There are various modes of CE, namely capillary zone electrophoresis (CZE), micellar electrokinetic capillary chromatography (MEKC),
capillary gel electrophoresis (CGE), capillary isoelectric focusing (CIEF), and capillary electrochromatography (CEC) which can be employed in protein separations. CZE is the simplest form of CE where separations occur based on charge-to-size ratios. MEKC employs micelles above their critical micelle concentration to act as mobile stationary phase and modify sample mobility through hydrophobic interactions. The surfactants also serve to minimize sample adsorption onto the capillary wall. CEC combines the principles of electrokinetic pumping with high performance liquid chromatography. In CGE, separations are based on size as they migrate through a gel matrix. The gel provides both static and dynamic coating of the capillary wall suppressing the electro-osmotic flow to near-zero. CIEF employs a pH gradient between the cathode and anode and hence samples focus at their isoelectric point (pI) where net charge on the molecules is zero.

For the purpose of this dissertation, CZE will be discussed since it was the technique used in this research. Capillary or microchip electrophoresis employs a voltage applied across a capillary or channel to manipulate sample movements by exploiting their ability to migrate in solution under a certain field strength. CZE is the simplest form of CE where charged species are separated in a buffered solution based on charge-to-size ratio. Charged molecules in solution inside a capillary/channel column exhibit differences in their electrophoretic mobility \( \mu \) due to their differences in size, charge \( q \) and frictional coefficients \( f \), when a voltage, \( V \), is applied.

\[
\mu_{ep} = \frac{q}{f} = \frac{V}{E} \quad (1.1)
\]

The migration velocity \( v \) of analytes in solution is a function of their electrophoretic mobility \( \mu \) and the electric field strength \( E \).
\[ \nu = \mu E \quad (1.2) \]

The electrophoretic mobility is an intrinsic property of the molecule and for separation and hence resolution \( R \) between two species to occur, analytes must exhibit a difference in mobility as shown in equation 1.3 below where \( N \) is the separation efficiency.

\[ R = \frac{1}{4} \frac{\Delta \mu_{app}}{\mu_{app,avg}} N^{1/2} \quad (1.3) \]

The migration time \( t_m \) along the capillary is determined by the equation;

\[ t_m = \frac{L_d L_i}{\mu_{app} V} = \frac{L}{\mu_{app} E} \quad (1.4) \]

where \( L_d \) is the capillary/channel length to the detector and \( L_i \) is the total capillary/channel length. From equation 1.2, the migration velocity is affected by changes in the electric field strength. However, increasing \( E \) results in an increase in current and therefore joule heating. Typically, the current in CZE is directly proportional to the electric field strength, the square of the capillary radius \( r \) and conductivity \( \kappa \) of the buffer used.

\[ I \sim E r^2 \kappa \quad (1.5) \]

From equation 1.5, it is clear that lower conductivity buffers and narrow inner diameter capillaries/channels are required in order to increase the analyte migration velocity without excessive joule heating. Normally, capillaries/channels of 25 to 100 \( \mu \)m internal diameter are used in CZE to allow application of high field strengths while maintaining low current. While for microchips, the channel properties will depend on the material used for its fabrication, capillaries employed in CZE are mostly made from fused silica which consists
of silanol functional groups on the inner wall surface. The silanol groups ionize in the presence of appropriate buffer pH, typically above their pKa. At pH greater than 5, the capillary wall bears a negative charge and results in an increase in surface charge density. Upon application of a voltage, current flows through the capillary and an electric potential arises due to positively charged buffer ions aligned near the capillary wall. This potential is referred to as the zeta potential ($\zeta$). The zeta potential causes a resistance to bulk flow of solution, a phenomena called electroosmotic force (EOF) as shown in equation 1.6, where, $\varepsilon$ is the permittivity (dielectric constant) of the buffer solution, $\zeta$ is the zeta potential and $\eta$ is the buffer viscosity.

$$\mu_{\text{eof}} = \frac{\varepsilon \zeta}{4\pi\eta}$$

(1.6)

The velocity of this bulk solution flow is computed from the equation below.

$$v_{\text{EO}} = \frac{\varepsilon \zeta E}{\eta}$$

(1.7)

The magnitude of the EOF is also dependent of the ionic strength of the buffer through its effects on the zeta potential. In bare fused capillaries, the direction of the EOF is usually from the anode to the cathode since the wall is negatively charged as shown in Figure 1.10. At the same time, positively charged species move towards the cathode while negatively charged species migrate towards the anode. Usually if the magnitude of the EOF is greater than the electrophoretic mobility of the negatively charged species, the apparent mobility of negatively charged species is towards the cathode. However, in cases where the magnitude of the electrophoretic mobility of the negatively charged ions is greater than that of EOF, these ions are never detected by detectors placed towards the cathode side. Neutral molecules are swept by the magnitude and velocity vector of the EOF towards the cathode.
For charged analytes, the apparent electrophoretic mobility \( (\mu_{\text{app}}) \) is therefore determined by the magnitude of their individual electrophoretic mobility \( (\mu) \) and velocity of the electroosmotic force \( (v_{\text{EO}}) \) as expressed in equation 1.8.

\[
\mu_{\text{app}} = \mu_{\text{ep}} \pm \mu_{\text{eof}} \tag{1.8}
\]

Figure 1.10 A schematic illustrating the migration of ions inside a capillary upon application of an electric field. Positive and negative ions move towards the negative and positive electrodes respectively.

Full characterization of peak profiles typical of a CZE separation requires a description of the concentration distribution of a solute as a function of position and time within the capillary. The average position of a zone or band is determined by the net migration time, while the concentration distribution or profile is governed by diffusion and other dispersive parameters. The average position or migration time \( t \) is expressed as a
function of the total length migrated by the zone $L$, apparent mobility $\mu_{\text{app}}$, and the applied electric field $E$.

$$t = \frac{L}{\mu_{\text{app}} E}$$  \hspace{1cm} (1.9) \textsuperscript{165, 166}

The efficiency of Gaussian-shaped CZE peaks is measured by theoretical plates $N$ or the height of a theoretical plate $H$. Normally $H$ is defined as

$$H = \frac{L}{N} = \frac{\sigma^2}{L}$$  \hspace{1cm} (1.10) \textsuperscript{165}

where $\sigma^2_{\text{total}}$ accounts for all the dispersive parameters. As a sample zone/band migrates along the capillary, it undergoes band broadening (zone dispersion) mainly due to longitudinal diffusion, $\sigma^2_{\text{diff}}$.\textsuperscript{168} This dispersion is inevitable in CZE due to the concentration gradient along the direction of the electric field. However, other factors can contribute to the total peak dispersion such as joule heating, $\sigma^2_{\text{joule}}$.\textsuperscript{169} This affects the electrophoretic mobility due to the parabolic temperature profile and hence parabolic velocity profile across the capillary. The length of the finite injection volume $\sigma^2_{\text{inj}}$, as well as finite detection volume $\sigma^2_{\text{det}}$ affects zone dispersion. Long injection plugs or long detector cells contribute more to zone dispersion. Other factors include solute-wall adsorption, $\sigma^2_{\text{ads}}$,\textsuperscript{170} concentration overload, $\sigma^2_{\text{conc}}$ in cases where the analyte ion concentration is higher than that of buffer ions,\textsuperscript{171} and also coiling or turns\textsuperscript{172, 173} introduced to the separation capillary and extra column effects, $\sigma^2_{\text{ext.col}}$.\textsuperscript{168, 174, 175} These factors are assumed to be independent and the total dispersion is therefore expressed as a summation of the individual second moments of the concentration profile, the variance ($\sigma^2_{\text{total}}$).

$$\sigma^2_{\text{total}} = \sigma^2_{\text{diff}} + \sigma^2_{\text{joule}} + \sigma^2_{\text{inj}} + \sigma^2_{\text{ext.col}} + \sigma^2_{\text{coil}} + \sigma^2_{\text{ads}} + \sigma^2_{\text{conc}}$$  \hspace{1cm} (1.11) \textsuperscript{168}
Recently, microfluidic devices have been used to demonstrate two-dimensional separation of proteins and peptides.\textsuperscript{176-180} In one example, MEKC was employed in the first dimension followed by CZE for peptide separations. This device produced a peak capacity of 4200 (110 in the first dimension and 38 in the second dimension) and was used to identify a peptide from a tryptic digest of ovalbumin and to distinguish between tryptic digests of human and bovine hemoglobin.\textsuperscript{176} Two-dimensional separation of proteins has also been reported. In one example, IEF was coupled to CZE separations.\textsuperscript{178} In a different example, a combination of IEF and SDS PAGE was demonstrated to achieve a peak capacity of 1700.\textsuperscript{177} Another example reported a peak capacity of 1000 using sodium dodecyl sulfate microcapillary gel electrophoresis (SDS µ-CGE) in the first dimension and MEKC in the second dimension.\textsuperscript{179}

1.9.2 Microfluidic Systems for Integrated Protein Sample Processing

Microfluidic systems or (micro-total analysis systems, µ-TAS) are integrated sample processing platforms assembled by integrating several modular microfluidic devices as analysis components in a fashion similar to how integrated circuits for electronics are fabricated.\textsuperscript{118-120} The planar substrates used in fabricating microfluidic devices are being exploited for integration, miniaturization, multiplexing, high throughput and automation of sample processing steps used in protein analysis to accelerate biomarker and drug discovery.\textsuperscript{118-120, 181} Whereas integration of processing steps circumvents sample losses, contaminations and false positives or negatives arising from transfer steps,\textsuperscript{116, 128, 182} miniaturization affords the assembly of modular devices that offer potentially portable and disposable diagnostic platforms that can be used for point-of-care testing.\textsuperscript{117-120} On the other hand, high throughput assays can be used for large scale screening that can be automated to
reduce the turnaround time for biomarker discovery, clinical diagnostics and also to improve the accuracy of results. Further, the ability to perform multiplex assays on μTAS devices reduces the cost of clinical diagnostics, research and development and allows assay controls to be run with every sample processing step.

There have been no attempts reported to date that address the integration of entire sample processing steps such as whole cell lyses, protein extraction or isolation, enrichment, gel separation, proteolytic digestion and chromatographic separations onto a single microfluidic device. Hence, there exist no systems that can process protein samples in a single automated platform. However, a few efforts have been directed towards the integration of selected processing steps. Examples of integrated systems reported in the literature include devices for proteolytic digestion of protein mixtures followed by subsequent electrophoretic separation of the generated peptide fragments. In one example, a system was described where a model protein, oxidized insulin B-chain, was enzymatically digested in a heated channel and the resulting peptides electrophoretically separated. The peptides were thereafter labeled with a fluorophore in the same device for fluorescence detection. This system is shown in Figure 1.1.187

Another report of an integrated system addressed the integration of solid-phase extraction of proteins followed by proteolytic digestions, or digestion of proteins followed by phosphopeptide enrichment. An example of a microfluidic system used for enzymatic digestion of a protein followed by enrichment of generated phosphopeptides from the digest with IMAC is shown in Figure 1.12. The first portion of the channel was the digestion zone while the downstream portion of the channel was used for IMAC capture of the phosphopeptides. There have also been systems focused on solid-phase extraction and
concentration of peptides followed by a downstream electrophoretic or chromatographic separation of the enriched peptides. An integrated microfluidic system has also been described for protein pre-concentration using a porous silica membrane in a fluidic junction that allowed buffer ions to pass but excluded proteins, which were later injected into the separation channel for electrophoretic separation. A schematic of this system is shown in Figure 1.13.

Figure 1.11 A microfluidic system used for on-chip proteolytic digestion of proteins, separations and post column labeling of peptide fragments, (Reproduced from J. Chromatography B 745 (2000) 243 –249).
Figure 1.12. A microfluidic system used to integrate trypsin digestion of proteins and selective affinity capture of the generated phosphopeptides, (Reproduced from Analytica Chimica Acta 564 (2006) 116–122).

Figure 1.13 A microfluidic system used for protein pre-concentration with porous silica membrane and electrophoretic separation of the pre-concentrated proteins, (Reproduced from Anal. Chem. 2005, 77, 57-63. Copyright, 2005 American Chemical Society).
Recently a device has been reported that integrates separation of proteins with downstream proteolytic cleavage of the separated proteins. Using this system, myoglobin was isolated from bovine serum albumin using integrated valves and then selected for enzymatic digestion in a rotary micromixer. The peptide fragments were later recovered and used for protein identification. A sketch of the system is shown in Figure 1.14.

![Figure 1.14 A sketch of an integrated microfluidic system comprised of injection/separation module, protein trapping module, a circular micromixer and an enzyme activation module. The system actuation and pumping is achieved using electro-osmotic driven flow and was used to separate proteins followed by digestion of the isolated proteins. (Reproduced from The Analyst, 2006, 131 (10), 1122 – 1128. Copyright 2006, Royal Society of Chemistry).](image)

1.10 Protein Identification

Mass spectrometry (MS) has become one of the most widely used analytical tools for protein identification. MS is an analytical tool used for measuring the mass-to-charge ratio of both individual atoms and molecules or fragments from intact molecules in order to deduce structure. MS is a separation technique that separates ions in the gaseous phase. MS enables protein characterization and de novo amino acid sequencing by mass measurements.
that provide identity, sequence and structural information without inference to protein databases. This information can also be used to determine the type and location of protein modifications. The identity of a protein can also be determined using peptide mass mapping by performing searches on available protein databases. The peptides are generated from proteolytic digestion of proteins using “shotgun” or “bottom-up” protocols. Another protein identification approach employs sub-sequence of a transcribed protein-coding or non-protein-coding nucleotide sequence by searching expressed sequence tag databases. Proteins can also be identified using “top-down” approaches by subjecting intact protein ions and large protein fragments directly to tandem mass spectrometry in a Fourier transform mass spectrometer.

Protein quantification can be accomplished using a number of MS approaches that allow direct correlation of the relative amounts of analyte present in a given sample to the MS ion intensity signal and by using stable isotope tagging. However, there exists an enormous difference in physical properties and protein abundances or concentration in most proteomes in the order of $10^6$ to $10^9$. For example in serum, albumin, which is the most abundant plasma protein occurs at a concentration of approximately 50 mg/ml. On the other hand, cytokeratins commonly used tumor markers, although abundant in epithelial cells and carcinomas are nevertheless in low abundance (~ 10 to 50 ng/ml) in patient blood. Although MS is capable of routine femtomolar limits of detection, the nanomolar concentration typical of tumor makers in serum is a challenge in the presence of abundant proteins. The difference in protein concentration is usually higher than the dynamic range of ~ $10^4$ for most MS.

In addition, ionization of low abundant peptides is often suppressed and their spectra are masked by high abundant species. Therefore, sequential
analytical processing steps are the key to simplification of sample complexity in order to improve sensitivity and limits of detection for MS analysis.

Integration of microfluidic devices to biological mass spectrometry is a logical step toward developing automated platforms for high throughput processing of biologically complex samples available in limited amounts or in relatively low concentrations. Chemical and biochemical analyses often probe qualitative, structural and quantitative information and many efforts still continue to progress towards realizing the full potential of these devices by integration with existing analytical detectors. A long-term challenge lies in scaling-down most of the traditional analytical techniques into miniaturized platforms without compromising their superb performance. This dissertation focuses on the coupling of integrated sample processing systems to biological mass spectrometry. In particular, the proteolytic cleavage of proteins in micro-reactors followed by the separation of the resultant peptides with on-line MS detection will be discussed in detail.

### 1.10.1 Basics of Mass Spectrometry

A typical mass spectrometer (MS) consists of a sample inlet, an ionization chamber and source, a mass analyzer and a detector. In the ionization chamber, analyte molecules are converted into gaseous ions using various ionization methods, the ions are then accelerated into a field free region called a mass analyzer. A mass analyzer, which in most cases is under high vacuum, separates the ions in the gaseous phase based on their mass-to-charge \(m/z\) ratio. The separated ions are then detected using a suitable sensing element and the current arising from these ions is digitized to form a mass spectrum. Mass spectrometers are credited for their unsurpassed accurate mass measurements that provide molecular specificity and identification capabilities. Figure 1.15 shows a block diagram of the
individual components; sample inlet, ionization chamber, mass analyzer, detector data acquisition and a vacuum system that constitute a MS.

Figure 1.15 A block diagram of a mass spectrometer consisting of a sample inlet, ionization chamber, mass analyzer, vacuum pump, detector and data acquisition modules.

1.10.2 History of Mass Spectrometry

In 1897, Joseph J. Thomson gave a lecture at the Royal Institute about observations on the existence and properties of canal rays (positive ions).\textsuperscript{218-220} Earlier, Eugene Goldstein had demonstrated that the presence of gases in cathode ray tubes gave rise to rays that behaved differently from cathode rays. In 1898, Wilhelm Wein deflected these rays in the opposite direction of cathode rays using electric and magnetic fields and concluded that they were positive. Thomson improved on Wein’s data and their work formed the foundation of mass spectrometry in subsequent measurements of masses of ions in gases at low pressures.\textsuperscript{221} However, it was not until two decades later that an application for Thomson’s experiments was developed by Francis W. Aston and Arthur Jeffrey Dempster to
discover new isotopes, their relative abundances and exact masses. Since then, MS has undergone extensive innovations driven by a wide range of applications. Most MS applications were driven by the petroleum industry and uranium isotope separation in the 1940’s. The improvements in high resolution MS and exact mass measurements extended the applications to organic chemistry in the 1950’s through the 70’s. All along, MS application to biological fields was limited by lack of suitable soft ionization techniques for large biological molecules. The discoveries of plasma desorption, fast-atom bombardment (FAB) electrospay ionization (ESI) and matrix assisted laser desorption/ ionization (MALDI) extended the upper mass range of MS allowing analysis of large biopolymers with MW over 100 kDa. These developments coupled to other innovative developments in mass analyzer technology have elevated MS to an indispensable technique in biomedical research, and brought about a new field known today as biological mass spectrometry. Today, MS is the primary technique used in the identification of proteins.

1.10.3 Types of Mass Spectrometers

Mass spectrometers differ mostly depending on the type of ionization technique or the type of mass analyzer employed. The mass analyzer and ionization technique in any given mass spectrometer is governed by the intended application. In this dissertation, only the time-of-flight (TOF) mass analyzer will be discussed in detail. Further for ionization techniques, only MALDI and ESI, the commonly used and dominant ionization techniques employed for a variety of biological molecules ranging from medium to fairly large polar molecules, will be discussed. A detailed discussion on different types of mass analyzers and other ionization techniques can be found in a standard mass spectrometry textbook.
1.10.3.1 Ionization Techniques

Ionization techniques can be classified as either soft or hard ionization depending on the type of ions generated. Hard ionization techniques are generally characterized by extensive fragmentation while soft ionization techniques yield mostly intact ions. Examples of hard ionization techniques include electron ionization,\textsuperscript{234} chemical ionization,\textsuperscript{235-237} atmospheric pressure chemical ionization, thermospray,\textsuperscript{238-240} field desorption\textsuperscript{241} and laser desorption ionization.\textsuperscript{242, 243} Example of soft ionization techniques includes; plasma desorption,\textsuperscript{244} electrospray ionization which uses a voltage bias at the tip of a capillary probe to exert an electrostatic force on liquid flowing through the capillary as shown in Figure 1.16.\textsuperscript{245} The liquid therefore emerges from the exit of the capillary as a “Taylor cone” jet of fine droplets which undergo further disintegration and evaporation to form multiply charged ion species.\textsuperscript{231} Atmospheric pressure chemical ionization is similar to ESI, however it employs a corona-discharge needle at the end of a metal capillary to create a plasma that allows proton transfer reactions and a lesser extent of fragmentation to occur.\textsuperscript{246}

![Figure 1.16](image-url) **Figure 1.16** The ionization process in ESI; voltage application at the tip of a capillary forms charged droplets, which undergo evaporation and division as they shrink in size due to charge repulsion resulting in multiply charged ions that are accelerated to a mass analyzer.
Another type of soft ionization technique is MALDI, which employs a focused pulsed laser beam to deposit energy into an excess of matrix host molecules for desorption and ionization of the analyte guest molecules in the gas phase as depicted in Figure 1.17.\textsuperscript{247} Most commonly used MALDI matrices are acidic molecules that are used in excess of the analyte molecules to absorb the laser energy. Initial studies with laser desorption employed direct irradiation of sample with a laser beam without the use of a matrix, an approach commonly referred to as laser desorption ionization (LDI).\textsuperscript{242, 243} In LDI, the extent of energy transfer that leads to thermal degradation is difficult to control. Further, analyte compounds exhibit selective absorption of radiation at different laser wavelengths. These limitations led to the development of MALDI in 1985. One effort led by Karas and Hillenkamp\textsuperscript{232} used tryptophan as a matrix with a 266 nm laser. Two years later, Tanaka and coworkers\textsuperscript{248} mixed their sample with a slurry of 10 nm finely divided cobalt particles in glycerol with a 337 nm laser and was able to form ions from intact proteins. The mixing of sample with an appropriate matrix and use of appropriate laser wavelength was critical to the success of MALDI. Typically, guest analyte molecules are mixed with an excess of host matrix molecules, which absorbs irradiation energy at the laser wavelength and transfers the energy to the sample in a controlled manner. The ionization is considered to be soft because no extensive fragmentation of analyte molecules is observed. Singly charged species are typical of the MALDI process and results in the formation of both positive and negative ions. In some cases, double or triply charged protonated ions are formed in low abundance. The mechanism of MALDI is not well understood, one popular theory postulates that the desorption of sample and matrix into a gas-phase plume may result in ionization through gas-phase proton transfer reactions.\textsuperscript{249}
1.10.3.2 Mass Analyzers

For most mass spectrometers, the mass analyzer is the heart of a MS where ions are separated based on the ratio of the mass to charge \((m/z)\). For TOF, a nominally monoenergetic beam of ions enters the mass analyzer and ion transmission based on velocity occurs as mass resolved ions arrive at the detector. The Figures of merit for a typical mass analyzer comprises the mass range, resolution, scan speed and detection sensitivity.

![Figure 1.17](image)

**Figure 1.17** A schematic of the MALDI process with a linear time-of-flight mass analyzer. Focused UV or IR laser is used for desorption and ionization of sample mixed with a matrix resulting in the formation of singly charged ion.

*Time-of-flight* (TOF) mass analyzers employ differences in velocity to separate ions in the gas phase\(^{250,251}\). Ions drift in a field free region inside a flight tube after acceleration to a constant kinetic energy, \(E_{\text{kinetic}}\) given by the equation:
\[ E_{\text{kinetic}} = \frac{1}{2} m v^2 \]  

(1.12)  

The initial kinetic energy of ions is dependent on their acceleration voltage \( V \) and the ion charge \( z \).

\[ E_{\text{kinetic}} = z V = \frac{1}{2} m v^2 \]  

(1.13)  

Rearranging equation 1.10 shows that the ion velocities are an inverse function of the square root of their \( m/z \) values:

\[ v = \sqrt{\frac{2zV}{m}} \]  

(1.14)  

The actual time-of-flight is a summation of the time ions spend in the ion source, flight tube and any post-acceleration at the detector. However, for most configurations, the flight tube distance and therefore an ion’s flight tube time is very large compared to the time spent in the ion source. The approximate time-of-flight for an ion (in the 100 \( \mu \)s time frame for typical conditions) in a flight tube of length \( L \), is estimated by the equation below;

\[ t = \frac{L}{v} = L \sqrt{\frac{m}{2zV}} \]  

(1.15)  

The mass resolving power between any two ions of mass \( m \) with a discernable mass difference of \( \Delta m \) is given the equation;

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

(1.16)  

Traversing ion velocities are an inverse function of the square root of their \( m/z \) values. Therefore, light ions travel faster and have shorter flight times to reach the detector.
compared to the heavy ones. The time-based spectrum is converted to a mass spectrum by measuring the flight times of two different ions (a, b) of known masses.

\[ m = at^{1/2} + b \]  

(1.17)

Pulsed ionization or pulsed acceleration potentials in case of a continuous ion beam, allows ions to enter the flight tube at the same time as discrete ion packets, fulfilling the primary requirement of TOF analyzers. The high ion transmission efficiency and fast data acquisition rates for TOF mass analyzers allow complete mass spectra to be recorded in microsecond time intervals. Ion detection after separation by a mass analyzer is accomplished using electron multipliers such as microchannel plate or photomultipliers that require ion-to-photon conversion after ions first strike a scintillation material to emit photons.

1.11 A New Microfluidic System for Processing Protein Samples

We are developing a novel microfluidic system for integrated sample processing of proteins that can be interfaced to a variety of different biological mass spectrometers (MS), such as MALDI or ESI. The system is comprised of a protein extraction and pre-concentration unit in series with a fluidic network for performing two-dimensional separations of extracted proteins. After the two-dimensional protein separation unit is another unit consisting of a series of solid phase micro-reactors arrayed orthogonally to the second dimension fluidic channel for proteolytic cleavage of individual components selected from the components eluting in the second dimension separation channel. The reactors are connected to a downstream separation unit for sorting peptide fragments that finally feed the separated peptide fragments to a mass spectrometer for protein identification. A schematic diagram for the integrated sample processing device is shown in Figure 1.18.
The system input is whole cells that are lysed and the protein components isolated from the cellular matrix using nanopillars decorated with thermally-responsive polymers. Extracted proteins are pre-concentrated using a thermally responsive polymer such as poly(N-isopropylacrylamide) (pNIPAAm). This polymer exhibits reversible wettability and protein adsorption properties that can be exploited to capture and release proteins. Above the liquid critical solution temperature (LCST), the polymer is hydrophobic and below the LCST, the polymer is extremely hydrophilic, therefore acting as an on-off switch to pre-concentrate extracted protein in the channel.

Figure 1.18 A schematic diagram illustrating an integrated sample processing system for protein analysis. The system is comprised of a protein pre-concentration module, two-dimensional protein separation module, solid-phase micro-reactors for digestion of separated proteins, and peptide separation module connected to a MS.
Extracted proteins are then injected into the two-dimensional separation unit for separation. In this unit, SDS PAGE and MEKC are used to separate protein mixtures. Two conductivity detectors are used to monitor separations in this unit. Protein bands eluting from the second dimension are injected to individual solid phase micro-reactor for digestion. The solid phase micro-reactors consist of trypsin immobilized on high surface area nanopillars for rapid digestion. Peptide fragments generated from these nano-reactors are further separated downstream using microchip electro-chromatography or free solution electrophoresis. The peptide fragments are recovered and detected using MALDI MS and thereafter the proteins are identified using peptide mass fingerprinting.

This dissertation will cover the proteolytic microfluidic reactors module, peptide fragment separation module and the interfacing module to on-line MALDI MS using a rotating ball inlet described above. Chapter 2 describes a rotating ball inlet for interfacing on-line MALDI MS to microfluidic devices. In Chapter 3, a detailed discussion of the peptide separation module is outlined. Chapter 4 covers the proteolytic digestion of proteins in microfluidic reactors and Chapter 5 describes future work towards integrating the modules to a fully functional proteomics microfluidic system.

1.12 References


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CHAPTER 2. ROTATING BALL INLET FOR ON-LINE MALDI TOF MS

Most MALDI TOF MS instruments are designed for off-line sample introduction, which requires ion source vacuum break to allow the transfer of samples into the ionization chamber for analysis. This step often can result in sample losses, increased sample processing time and can create operational difficulties due to manual intervention into the processing pipeline.¹⁻³ Modification of a MALDI TOF MS ion source is required to allow on-line transfer of liquid samples from a microfluidic device into the essentially high vacuum and solid sample MALDI MS technique.⁴⁻⁹ On-line MALDI MS analyses are desired to increase sample throughput, for automation of sample preparation and to provide the ability to acquire real-time data. On-line MALDI MS also allows easy switching from one sample to another without having to break the ion source vacuum and thereby reducing instrument downtime.¹⁰⁻¹² Discussed here are approaches for analyzing samples from a microfluidics device using MALDI MS.

2.1 MALDI MS Analysis From a Microfluidic Device

The developments of matrix assisted laser desorption and ionization mass spectrometry (MALDI MS) for on-line and off-line analysis from microfluidic devices stems from a number of facts. The low sample volumes typical of analyses in a microfluidic device are readily compatible with low sample consumption in MALDI MS.¹³, ¹⁴ Further, sample preparation often involves a series of steps to address sample complexity and microfluidic devices are attractive platforms for integrated sample processing prior to MALDI MS analysis. The fast response and data acquisition rates with MALDI TOF MS readout allows for integration with rapid analysis in microfluidic devices. MALDI MS provides high resolution and precise mass measurements that generate high molecular specificity and
identification capabilities. These Figures of merit coupled with the sensitivity and high mass range makes MALDI MS a versatile detector and increases the scope of microfluidic assays.

2.1.1 **Off-line Analysis**

Off-line analysis from a microfluidic device is rather straightforward. Common approaches entail either fraction collection from a microfluidic device and spotting them onto a MALDI sample plate or placing the entire device inside a MALDI MS ionization chamber for analysis. In the first approach, bottlenecks are often encountered in the deposition technique when sampling low volumes. However, automation of the deposition step can increase the throughput of this off-line approach. In one example, a piezo-electric microdispenser was used to automate the sample deposition into micro-vials before MALDI MS analysis. The second approach entails sample processing on an open substrate or in enclosed fluidic network then removing the cover plate of a microfluidic device before analysis with MALDI MS. Usually, no sample transfer steps are involved with this approach hence limiting sample losses. Limitations with this off-line approach are encountered when the size of the device cannot be fitted into the ionization chamber to be addressed with MALDI MS readout. In such cases, the device is split into smaller addressable sizes, or if the device is of required size, slight modifications are made on the sample target to allow MALDI MS readout. When the cover of a microfluidic device has to be peeled off after sample processing, caution is required to preserve the integrity of the sample.

2.1.1.1 **Off-Line MALDI Interfaces**

Off-line MALDI analysis from microfluidic devices may be automated using various interfaces. In one approach, a impulse-driven momentum transfer droplet deposition or a
heated droplet interface employed a transfer tube manipulated by a robotic arm which was mounted on an x-y-z translation stage to transfer samples from a liquid chromatography separation platform to a MALDI target.\textsuperscript{23} In a similar development, a deposition system based on non-contact pulsed electric field\textsuperscript{24} was demonstrated to transfer the effluents from multiple microfluidic channels directly onto MALDI targets.\textsuperscript{25} A continuous contact deposition using a capillary dragged on a matrix-precoated membrane target was also developed to combine capillary electrophoresis to MALDI MS.\textsuperscript{26} Recently, a system based on discrete nanoliter droplet plugs as microreactors for performing solution reactions in microfluidic with off-line MALDI MS detection was reported.\textsuperscript{27}

Off-line analysis can also be accomplished by placing an entire device inside the ionization chamber.\textsuperscript{19, 21, 28} Gustafsson \textit{et al.}\textsuperscript{19} described a microfluidic device in a compact disk format that employed centrifugal force to control movement of liquids within the device. Sample applied into the microfluidic device was washed and eluted through a 10nL reversed-phase column followed by downstream co-crystallization with MALDI matrix in the desorption area located at the outlet port. In a somewhat similar off-line approach, electrowetting-on dielectric (EWOD) was demonstrated for MALDI sample preparation and purification in a microfluidic device.\textsuperscript{18, 29} Liquid droplets of sample and matrix were digitally moved to specific locations using EWOD array of electrodes patterned on a substrate material for electrical contacts.

In another example, sample processing was carried inside an enclosed microfluidic device; the cover of the device was then peeled off, no matrix was required before loading the entire device inside the vacuum for interrogation with an IR laser.\textsuperscript{21} Briefly, a PMMA/PDMS hybrid device was used to perform SDS-polyacrylamide gel electrophoresis.
in an enclosed fluidic channel. The PDMS cover used to enclose the fluidic channel was carefully removed in order to access the gel-line. No matrix addition step was required since compounds present in the cross-linked gel served as matrix for IR-laser desorption ionization. The laser was either scanned across the channel while the device was stationary under high vacuum or the device was moved across the path of a focused laser line inside the ionization chamber.

2.1.2 On-line MALDI Interfaces

On-line analyses are highly desired for their high sample throughput, automation and ability to acquire real-time data while experiments progress. A number of attempts for on-line sample introduction into a MALDI MS have been reported in literature and are briefly mentioned below.

2.1.2.1 Continuous Flow (CF) MALDI

Continuous flow MALDI employs a capillary to deliver samples premixed with a matrix to a metal porous frit embedded in a sample stage.\textsuperscript{30-32} The CF porous probe allows liquid samples to be introduced into a MALDI ionization source at controlled flow rates. The CF probe approach has had limited success due to water freezing and clogging of liquid matrices commonly used in UV-MALDI which block the pores of the metal frit at the capillary end leading into vacuum.\textsuperscript{8} Developments in IR-MALDI indicate that strong material ablation associated with IR lasers may alleviate freezing problems if protic solvents serve as matrix.\textsuperscript{31} Adapting the CF probe for microfluidic is not practical because it requires connecting a capillary to the microfluidic device, placing a metal frit on top of a fluidic channel or fabrication the frit at the end of a fluidic channel. This approach results in wasteful sample consumption, material mismatch, extra-column and large dead volume effects from capillary interconnect.
2.1.2.2 **Vacuum Draw Interface**

In this approach, the MS vacuum is used to induce a self activated pressure-driven pumping mechanism as a driving force to initiate liquid flow inside a microfluidic device.\(^{33}\) Briefly, a device carefully filled with reagents in the reservoirs is mounted onto a standard MALDI sample target. The MS vacuum thereafter used to induce a pressure driven flow of the reagent towards an exit reservoir where products formed in the device are detected. The device was recently modified to include a sub-micron hole on the device cover to desorb effluents as the flow along the fluidic channel.\(^{34}\)

2.1.2.3 **Aerosol MALDI**

Aerosol MALDI involves generation of aerosol particles at atmospheric pressure using pneumatic, ultrasonic, thermal or electrostatic potential means. The particles are then introduced into a mass analyzer through a differentially pumped capillary inlet.\(^{35}\) A matrix is usually added to the aerosol particles before entering the mass spectrometer or sample is premixed with a proper matrix before spraying.\(^{36}\) Aerosols have a rapid solvent evaporation, which minimizes the incompatibility of liquid introduction into the high vacuum of a mass spectrometer; however it is uneconomical with sample. A pulsed UV laser is used to form ions from the aerosol particles. The aerosol interface is limited in mass range and resolution due to particle transmission efficiency.\(^{8}\)

2.1.2.4 **Vacuum Deposition**

On-line MALDI has also been demonstrated with a vacuum deposition approach. A fused silica capillary is used to deliver liquid samples and matrix to a rotating wheel placed inside the ion source of a mass spectrometer.\(^{10}\) The capillary is in contact with the wheel to minimize clogging at the exit. By rotating the wheel, a narrow sample trace deposit is
transported into the desorption region of the mass spectrometer. A major drawback of the approach is limited operation time that requires retrieving the wheel from vacuum after one complete rotation. In a recent development, the wheel was substituted with a disposable Mylar tape to increase the operation time and for sample archiving.11, 12

2.1.2.5 Atmospheric Pressure (AP) MALDI

AP MALDI alleviates the vacuum requirements for operating the MS ion source. Ionization is performed with a laser at atmospheric pressure and the ions generated are guided into the vacuum of the mass spectrometer electrostatically or using a carrier gas.37-39 Normally, a quadrupole ion guide is required between the AP MALDI source and the vacuum to collimate the ions into the mass analyzer. The AP MALDI source when combined with the appropriate mass analyzer has the potential to couple high throughput analyses in microfluidic devices.

2.1.2.6 Rotating Ball Inlet

As mentioned previously, on-line analysis from a microfluidic device requires a specially tailored interface for coupling the device to allow sample transfer from the fluidic channels into the ionization chamber of the MS. One approach already reported in literature employs a rotating ball inlet for on-line sample transfers from microfluidic devices into an ionization chamber of a MALDI MS.40 Although the rotating ball inlet was originally developed for continuous sampling of volatile species,41, 42 further modifications have allowed for coupling of micro-scale separation to on-line MALDI MS.43-45 Briefly, the interface consists of a 0.75” stainless steel ball fitted with a drive shaft and was mounted on an ISO-100 flange. The drive shaft was attached to a motor on the other end to facilitate rotation of the ball. This ion source configuration allowed the rotating ball inlet to be
mounted entirely at atmospheric pressure with a small portion of ball exposed through a gasket seal to the ion source vacuum. The rotating ball served as a mechanical transport mechanism that introduced samples deposited on its surface past the gasket into the ionization chamber. To eliminate sample cross-talk, a cleaning system made from a solvent saturated felt pad was mounted on-line for surface regeneration. A clean surface was always presented for subsequent deposits after the previous deposition region passed out of the ionization chamber, thereby making this inlet self-replenishing. A schematic representation of the interface coupled to a microfluidic device for on-line analyses is shown in Figure 2.1.

![Figure 2.1 Schematic representation of a rotating ball inlet; (A) stainless steel ball (B) drive shaft (C) gasket (D) ISO-100 flange (E) ion extraction grid (F) matrix syringe pump (G) Cleaning solvent pump (H) microfluidic chip (I) separation channel (J) reservoirs (K) cleaning system (L) waste solvent drain.](image)

### 2.1.3 Off-line Versus On-line Analysis

Generally, off-line and on-line analysis approaches from microfluidic devices are complementary. Despite the strengths and weaknesses associated with either approach, a comparison can be made with regard to performance, ease of implementation and the robustness of the approach. Off-line approaches are performed without major modifications
of either the microfluidic device or the mass spectrometer and the systems performance is not significantly affected. However, in some approaches, during readout time, the laser or device is moved and depending on the modifications done on the ion source; the mass spectrometer performance can be affected. Mass resolution is often compromised in cases where the ions focusing field is affected by device movements and position within the ion source. Careful consideration of these issues during design and modification of the ion source can tremendously reduce the effects on mass resolution. Some off-line approaches generally suffer from sample losses associated with sample transfer steps and limited sample throughput. However, sample losses arising from sample transfer steps can be avoided by the direct analysis of samples within the microfluidic device. On the other hand, on-line approaches like rotating ball inlet require major adjustments on a typical MALDI MS ion source that can affect mass resolution and sensitivity. On-line analysis reduce instrument down time and have a high sample throughput compared to off-line approach. Modifications required for a typical MALDI MS ion source for on-line analysis are demanding and difficult to optimize but avails the instrument to various front-end sample processing techniques.

2.2 Experimental Section

2.2.1 Ball Inlet

To integrate microfluidic platforms to on-line MALDI TOF MS, an interface based on a rotating ball inlet was developed with a configuration that allowed on-line transfer of sample and matrix from atmospheric pressure into the high vacuum of a MS ionization chamber. The interface was made from type 316 stainless steel ball of 19 mm (0.75 in.) in diameter that were obtained from (Small parts, Miami Lakes, FL). A 6 mm hole (0.25 in.)
was drilled through the center of the ball and a stainless steel drive shaft of same diameter was press-fit into the hole. The ball assembly was mounted on a modified stainless steel ISO-100 flange, which had a 15 mm circular hole machined to a thickness of 3 mm on-center. A circular Teflon gasket with a 9 mm central hole was specially designed and machined to fit between the ball and an ISO-100 flange face. Vacuum grease was applied between the gasket and the flange before the gasket was sandwiched between the ball and the flange to form a vacuum seal. The ball was held in place by two stainless steel blocks with sintered bronze bearings press fit to allow free rotation of the shaft. The stainless steel bearing blocks were held against the ISO-100 flange by four adjustment screws. The majority of the ball was at atmospheric pressure and exposing only a small portion of the ball to the vacuum. Centering the ball on the shaft was critical, since slight wobble in the shaft caused a vacuum breach as the ball rotated. The ball-gasket seal was sufficiently vacuum-tight to maintain a pressure of $10^{-5}$ Torr or lower when methanol solvent was applied to the air-side of the ball.

The ISO-100 flange with the ball assembly were mounted on an 8 in.-diameter 25 mm-thick Delrin (acetyl resin) insulator flange that allowed the entire ball assembly to be raised to the mass spectrometer acceleration voltage while the remainder of the mass spectrometer remains at ground potential. During operation, the rotating ball and ISO-100 flange assembly were held at 10 kV, and an acceleration grid 13 mm from the flange surface was held at ground potential. Rotation of the ball was accomplished using a multi-speed transmission motor scavenged from a withdrawal and infusion syringe pump (Model 901, Harvard Apparatus, Holliston, MA). Further rotational control was achieved by connecting the motor to a variable AC transformer for fine speed adjustment on each particular gear. A
A digital image of a rotating ball interface with the assembly mounted on an ISO flange is shown in Figure 2.2.

**Figure 2.2** A digital image of the entire rotating ball inlet assembly mounted onto an ISO-100 flange, the drive shaft and the ball inlet are marked with yellow arrows, the gasket seal is seen as a white mark behind the ball position. The drive shaft and the ball are rotated clockwise facilitating sample transports into the ionization chamber of the MALDI MS.

### 2.2.2 MALDI TOF Mass Spectrometer

The ion source chamber was modified so that the desorption and ionization laser could be directed at the center of the rotating ball flange through a quartz window at a 45° angle with respect to the flange. A video camera and macroscopic lens was directed through a second port on the opposite side of the chamber and was used to view the ball in operation. A 355-nm Nd:YAG laser (Minilite, Continuum, Santa Clara, CA) was used for the MALDI experiment. The laser repetition rate was set at 10 Hz, and the laser energy was attenuated with a polarizer to ~ 10-15 µJ for desorption. The laser was focused to a spot size of 100 x 300 µm measured using laser burn paper. The ion source was evacuated using a 1500 L/s 6-in. diffusion pump (M-6, Varian, Lexington, MA), and the flight tube was evacuated using a
345 L/s turbomolecular pump (Turbovac 360, Leybold, Export, PA). A 25-mm aperture separated the flight tube from the ion source. During operation, the ion source operated at a pressure of $3 \times 10^{-6}$ Torr, and the flight tube, at a pressure of $2 \times 10^{-7}$ Torr. A was used. A 18 mm bipolar ion-to-photon conversion detector and a standard microchannel plate detector (Burle, Sturbridge, MA) were used interchangeable in a home-built 1 m linear time-of-flight mass spectrometer in this work and was similar to that described previously. A schematic diagram of the home built MALDI TOF MS is shown in Figure 2.3.

**Figure 2.3** A schematic diagram of the home built MALDI TOF MS; the ion source has a ball inlet interface for sample introduction. The ball is rotated by a motor placed outside the ion source. There are two side ports on each side of the ionization chamber; one port is used to focus the laser into the ball inlet while the other port is used to view the ball in order to align the sample to the laser. The instrument has a 1 meter linear time-of-flight mass analyzer and a detector placed at the end of the flight tube. The laser triggers the oscilloscope to digitize the signal from the detector for downloading into a computer.
2.2.3 **On-line Sample Transport Mechanism**

The rotating ball serves as a mechanical transport mechanism that introduces sample deposited on its surface to the ionization chamber.\(^41\, 42\) To test the sample transport mechanism, a Teflon rod mounted on a ring stand was used to hold capillaries in contact with the ball. Two holes were drilled through the rod for mounting two dual-lumen tubing sleeves. One sleeve held a sample capillary, and the other held the matrix capillary. Sample deposition occurred first, followed by matrix deposition on the same spot while the ball rotated as shown in Figure 2.4.

![Figure 2.4](image.png)

**Figure 2.4** An illustration of the rotating ball inlet sample transport mechanism; sample deposited on the surface of the ball is rotated past a polymer gasket and interrogated with a laser aligned with the sample trace. Once the trace passes out of the desorption region, its cleaned with a solvent saturated felt placed in contact with the ball outside vacuum before subsequent deposits are made.

The ball was cleaned on-line using a solvent-saturated felt pad placed in contact with the ball surface by wiping off the matrix and sample left after the desorption process to regenerate the surface for subsequent sample deposits. The cleaning solvent was delivered to the felt pad using a syringe pump (model 2374, Harvard). The sample deposition is similar
to the bottom layer method. A solution of 50 mM $\alpha$-cyano-4-hydroxycinamic acid dissolved in methanol served as the matrix and was delivered onto the rotating ball by a syringe pump (Model 55-2222, Harvard) at a flow rate of 0.5 µL/min. The matrix solvent evaporated upon deposition onto the ball leaving a uniform 300 µm-width trace of matrix and analyte. The sample trace had to pass the polymer gasket seal in order to enter the ionization chamber.

### 2.2.4 Materials

The MALDI matrices, $\alpha$-cyano-4-hydroxycinamic acid ($\alpha$-CHCA, C-2020), sinapinic acid (SA, 85429), 2, 5-di-hydroxy benzoic acid (DHB; G-5254), caffeic acid (60018, Sigma), nicotinic acid (72311), succinic acid (14078), 2-(4-hydroxyphenylazo) benzoic acid, (HABA; 54793), and peptides, bradykinin (B-3259), [Met-OH11] substance P (S-2136), neurotensin (N-6383), [Lsy8] vasopressin (V-6879), peptide separation buffer (P-2188) and insulin (I-5500) were obtained from Sigma Aldrich (St. Louis, MO) and used as received without further purification. Matrix solutions were prepared at 20 mg/ml in a mixture of acetonitrile and water in a volumetric ratio of 1:1. A 1 mg/ml of insulin solution was prepared in the same solvent system as the analyte.

### 2.2.5 MALDI Surface Profilometry

One major challenge was to transport samples past the gasket with minimal losses while still maintaining the vacuum seal. To evaluate sample transport, measurements were taken to estimate the sample trace thickness, which may vary from one matrix to another. A study was carried out to characterize MALDI matrix/sample morphology as a function of sample deposition method with commonly used matrices. Samples were prepared using the droplet deposition method such as the dried droplet, seed layer, bottom layer, sandwich and
upper layer deposition. When using the dried droplet method, the matrix and analyte are deposited on the target in a single solution. The application of a “seed” layer of matrix prior to the addition of the analyte has been shown to improve crystallization when the matrix and analyte solution is subsequently added.\textsuperscript{47-51} Another variation on the dried droplet method is the sandwich method, where the analyte is deposited on the seed layer, followed by the separate addition of matrix.\textsuperscript{52,53} In the bottom layer method, the analyte is first deposited on the target, followed by the matrix. Spray deposition has also been reported using pneumatic nebulization\textsuperscript{54-56} and electrospray\textsuperscript{57,58} for droplet creation. A schematic of these droplet deposition methods is shown in Figure 2.5.

![Diagram of droplet deposition methods]

**Figure 2.5** A schematic representation of the droplet deposition methods; dried droplet deposits matrix and sample at same time, seed layer deposits matrix first then a sample/matrix mixture follows, sandwich has sample between two layers of matrix, bottom and upper layer deposits sample and matrix first respectively.

Surface profilometry is a versatile technique that can be used to measure the thickness of thin films of solid materials on a micrometer scale. A surface profilometer maps the surface topography of the material by dragging a sharp micron-sized probe across the sample surface. It uses a mass cantilever system to keep the tip force constant while scanning. The dimension of the tip dictates the vertical and horizontal resolution, and is
typically capable of a lateral resolution of 100 nm and nanometer vertical resolution. The morphologies of matrix compounds α-cyano-4-hydroxycinamic acid, sinapinic acid, 2, 5-dihydroxy benzoic acid, caffeic acid, succinic acid, 2-(4-hydroxyphenylazo) benzoic acid, and nicotinic acid were studied. Scanning electron microscopy (SEM) and a Tencor surface profiler were used to observe and characterize the sample morphologies.

The samples were deposited using dried droplet, seed layer, bottom layer, sandwich and upper layer methods on a standard MALDI sample target. Although the rotating ball inlet cannot use the sandwich method, the method was included in this study for comparison of sample preparation thicknesses only. With the dried droplet method, 2 µl of matrix and 1 µl of sample were premixed and deposited on a MALDI target using a pipette and solvent, which was left to evaporate. For the seed layer preparation, 1 µl of matrix was deposited on the target using a pipette and left to dry, then 2 µl of a 1:1 mixture of the analyte and matrix solutions were deposited on the same spot and left to dry. Bottom layer samples were prepared by depositing 1 µl of analyte on the target using a pipette and letting it dry before 2 µl of matrix was deposited on the same spot. In the sandwich method, 1 µl of matrix was deposited on target and left to dry, then 1 µl of analyte was deposited on the same spot, followed by the addition of 1 µl of matrix. For the upper layer approach, 2 µl of matrix was deposited on the target; upon drying 1 µl of analyte was deposited and left to dry.

A contact surface profilometer (P-11, Tencor, Santa Clara, CA) was used to measure the thickness of sample deposits prepared using different sample deposition techniques. Samples deposited on MALDI target are profiled at atmospheric pressure. The MALDI target was loaded onto the motorized profiler stage and manipulated with software to place it in position with the profiler tip. The profiler is equipped with a video camera that allows the
user to position the tip on a desired location and to visualize the sample while scanning takes place. A Cambridge Stereoscan 260 scanning electrode microscope was used to acquire SEM images of the sample deposits. A MALDI-TOF MS instrument (Omniflex, Bruker, Billerica, MA) was used to analyze samples deposited using the above mention techniques. The profiler tip was adjusted by eye such that the tip passed through the central of each sample spot.

2.2.6 **Flow Injection Analysis (FIA)**

Sample transport past the gasket was first tested using FIA. In FIA, a sample plug is introduced into a mobile phase using a fixed volume injector. The pressure driven flow of the mobile phase transports the plug along a column toward a detector placed on one end. A rotary valve micro sample injector (Model ABR0592, Valco, Houston, TX) was used to carry out repeated injections. FIA was accomplished by contacting the rotating ball with a single transfer capillary connected to the outlet of the rotary valve injector. The sample was introduced manually using a 2 µL sample loop of the injector. The injector valve was then switched to inject position, allowing a syringe pump (Model 55-2222, Harvard Apparatus) attached to the inlet of the injector to pump matrix at a flow rate of 2 µL /min through the transfer capillary and carry the sample plug from the sample loop of the injector onto the rotating ball.

2.2.7 **Capillary Zone Electrophoresis (CZE)**

The rotating ball inlet was then tested with capillary electrophoresis (CE) separations. Fused-silica capillaries 20 cm long with 50 µm i.d. (Polymicron, Phoenix, AZ) were used for CE separations. A mixture of three peptides was run on an HP 3DCE system with a photodiode array detector (Model GX1600AX, Hewlett Packard, Walbron, Germany)
to obtain optimal separation conditions. The peptide mixture consisted of 60 µM of vasopressin, substance P, and neurotensin. The optimized separation conditions were then used to separate the mixture on the CE system interfaced to the MALDI-MS. The home-built CE system consisted of sample and buffer vials mounted on an insulating stand, a CE power supply (CZE 1000R, Spellman, Hauppauge, NY) and another power supply for the ball interface (Gamma High Voltage Research, Ormond Beach, FL). The two power supplies were connected to share the same ground. The ball acted as the CE cathode and was held at 10-kV positive potential necessary for ion acceleration (see Figure 2.6). The capillary anode was biased at 18-22 kV relative to ground and 8-12 kV across the 20-cm capillary. Field strengths between 400 and 600 V/cm were used for all separations, and electrokinetic injections were performed at 400 V/cm for 10 s. A 50 mM phosphate electrolyte at pH 2.5 was used as the separation buffer.

Figure 2.6 A schematic representation of the rotating ball inlet interface for CE-MS indicating (A) ball, (B) driveshaft, (C) Teflon gasket, (D) ISO-100 flange, (E) extraction lens, (F) matrix capillary, (G) buffer capillary, (H) anode and running buffer, (I) electrophoresis capillary, (J) cleaning solvent capillary, (K) felt pad and holder, and (L) solvent drain. (Reproduced with permission from Anal. Chem. 2004, 76, 5968-5973. Copyright, 2004 American Chemical Society)
In CE mode, one of the dual lumen sleeves held the separation and make-up buffer capillaries, while the other sleeve held the matrix capillary. The CE system was operated manually by switching the separation capillary from the buffer vial to sample vial during injections. The separation capillary was rinsed and conditioned between runs for 2 min with methanol and then buffer. A 50 mM phosphate supporting electrolyte was supplied through another capillary via a syringe pump (Model 55-2222, Harvard Apparatus) at a flow rate of 0.5 µL/min to the separation capillary tip, which was in contact with the ball to keep the electrical connection. At pH 2.5, the capillary supports a low EOF and also suppresses analyte adsorption to the capillary wall.\textsuperscript{59} The peptides used in this study had pI values above the buffer pH and, thus, were expected to be positively charged.

2.2.8 Data Acquisition

A 500 MHz digital oscilloscope (Model LT372, LeCroy, Chestnut Ridge, NY) was used to record mass spectra. Communication with the oscilloscope was accomplished using a personal computer (Apple, Cupertino, CA) via general purpose interface bus (GPIB). The oscilloscope was externally triggered by the laser output signal and remotely controlled by in-house LabView software (National Instruments, Austin, TX) for downloading averaged mass spectra. The mass spectra were averaged over 10 laser shots with 50 000 data points and a flight time resolution of 1 ns per data point. The mass spectra were downloaded and stored over 1s integration times as the experimental data collection progressed in real time. Since the laser duty cycle was 10 Hz and each mass spectrum was an average of 10 laser shots integrated over 1s, the file download duty cycle of ~1s per mass spectrum using a GPIB interface allowed 50 % of the mass spectra acquired to be recorded. Following data acquisition, stored mass spectra were processed with in-house LabView programs. Two-
dimensional CE-MS plots were generated using NIH Image and Image J software (National Institutes of Health, Bethesda, MD).

2.3 Results and Discussion

2.3.1 Surface Profilometry

Surface characterization using a profilometer is a simple and fast technique for measuring film thickness and requires no special sample preparation prior to scanning. Samples deposited at ambient pressure on a MALDI target were left to dry upon which the MALDI target was loaded onto the profiler programmable motorized stage and placed in a desired location under the profiler tip with the help of software control and a video camera.

Surface profiles generated from different matrices deposited using the dried droplet methods are shown in Figure 2.7. The profiles demonstrated that the sample thickness varied greatly with the matrix by more than an order of magnitude for matrices prepared from solutions of the same volume and concentration. The CHCA, HABA and SA deposits are relatively thin, all being under 100 µm in thickness. Succinic acid and DHB were relatively thicker at approximately 0.5 mm average thickness. The sample morphology was obviously heterogeneous as a result of different sizes of the matrix crystals as indicated by varying thicknesses for each matrix profile. The profiles for the dried droplet deposit variations (seed layer, bottom layer, sandwich and upper layer) were qualitatively similar to the dried droplet results. The roughness of the sample morphologies varied as a result of different sizes of the matrix crystal matrix. An estimate of the roughness for each matrix could be computed from the ratio $R_a / \lambda_a$, where $R_a$ is the average deviation of peak height in microns of random points in a typical surface profiler trace and $\lambda_a$ is the average distance between peaks of the surface features in a typical surface profiler trace.
Figure 2.7 Surface profiles of dried droplet sample deposits obtained from α-CHCA, DHB, Succinic, HABA and SA MALDI matrices. The scan length and height units are in μm and were obtained by scanning a surface profiler tip horizontally along and over the matrix deposits with a 2 mg-force applied on the stylus and steps (scan speed) of 20 μm/sec.
A bar graph representation of the measured sample film thicknesses for the dried droplet deposition methods is shown in Figure 2.8. The data resulted from ten replicate measurements and the error bars in Figure 2.8 correspond to one standard deviation. The average thickness was obtained from surface profiles by averaging the deviations in height at random points. The multi-layer deposition methods (sandwich, bottom, upper and seed layer) were consistently less thick compared to the single dried droplet deposit. This was likely due to the second solution deposit dissolving the crystals that were formed by the first solution deposit. DHB and SA were consistently the thickest samples within a particular deposit method while HABA and CHCA were the thinnest.

Figure 2.8 Droplet thickness variations in samples deposited using the dried droplet, sandwich, bottom, upper and seed layer methods. Dried droplet was consistently thick compared to the other techniques that require multiple layers deposition approach as shown in the bar graph
The corresponding data for the bar graph shown in Figure 8 is presented in the Table 2.1 below.

**Table 2.1** Surface profile average thickness by all matrix and droplet deposit methods

<table>
<thead>
<tr>
<th>Deposition method</th>
<th>α-CHCA</th>
<th>Sinapinic</th>
<th>DHB</th>
<th>Caffeic</th>
<th>Succinic</th>
<th>HABA</th>
<th>Nicotinic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dried droplet</td>
<td>40±16μm</td>
<td>135±22μm</td>
<td>600±20μm</td>
<td>80±18μm</td>
<td>500±136μm</td>
<td>36±7μm</td>
<td>85±20μm</td>
</tr>
<tr>
<td>Seed layer</td>
<td>18 ±2μm</td>
<td>85 ±18μm</td>
<td>350±35μm</td>
<td>70±13μm</td>
<td>180 ±35μm</td>
<td>10±2μm</td>
<td>40±17μm</td>
</tr>
<tr>
<td>Bottom layer</td>
<td>22 ±5μm</td>
<td>90 ±27μm</td>
<td>225±33μm</td>
<td>50±10μm</td>
<td>120 ±23μm</td>
<td>5 ±1μm</td>
<td>60 ±7μm</td>
</tr>
<tr>
<td>Sandwich</td>
<td>24 ±6μm</td>
<td>80 ±10μm</td>
<td>500±45μm</td>
<td>50±14μm</td>
<td>250±35μm</td>
<td>5±1μm</td>
<td>65±15μm</td>
</tr>
<tr>
<td>Upper layer</td>
<td>30 ±5μm</td>
<td>70 ±7μm</td>
<td>250±2μm</td>
<td>60±12μm</td>
<td>135±33μm</td>
<td>8±1μm</td>
<td>27±2 μm</td>
</tr>
</tbody>
</table>

From these measurements, α-CHCA a standard solid matrix commonly used for low molecular weight peptides and proteins gave thin matrix/sample deposits compared to DHB. Although HABA deposits were comparable in thickness to α-CHCA, HABA was not selected because it was not a suitable matrix for the samples used. The bottom layer approach is more suitable for the rotating ball inlet when the matrix and sample have to be introduced separately. Again for the bottom layer approach, α-CHCA is a preferred matrix than HABA for the analysis of low molecular weight peptides and proteins. Measurements were then taken to estimate the surface roughness of the ball and the thickness of α-CHCA matrix/sample deposits prepared by drag contact droplet deposition from a capillary as the ball was rotating. The ball assembly was removed from the flange after the sample was deposited on-line. Different portions of the matrix/sample trace on the ball surface were measured by scanning a length of 2 mm at 20 μm/s with a 2 mg-force applied on the stylus. Ten replicate measurements were made to ascertain the ball roughness and the matrix/sample deposit thickness before and after the trace passes past the gasket seal.
Surface profile measurements on the rotating ball are shown in Figure 2.9. The profile of the ball surface after coating with a solution of 50 mM α-cyano-4-hydroxycinamic acid dissolved in methanol at a flow rate of 2 µL/min is shown in Figure 2.9a. The scan was perpendicular to the direction of the track; the thickness of the sample and matrix deposit before it passes under the gasket was 15 µm. After the track passes under the gasket, the thickness is reduced to 10 µm, as indicated in Figure 2.9b. At the edges of the track, the deposit is slightly thicker at 12 µm. These results indicate that approximately one-third of the sample deposit is scraped off the ball surface as it passes into the vacuum system. The uncoated ball surface is shown in Figure 2.9c. The unpolished ball surface has indentations ~2 nm deep and 500 nm in diameter with an aspect ratio of 0.004, obtained by dividing the depth with diameter.

![Figure 2.9](image-url) 

**Figure 2.9** Surface profiles obtained on the surface of the rotating ball: (a) ball surface coated with sample, (b) ball surface after the sample deposit has passed under the polymer gasket, and (c) the uncoated ball surface. (Reproduced with permission from Anal. Chem. 2004, 76, 5968-5973. Copyright, 2004 American Chemical Society)
2.3.2 Flow Injection Analysis (FIA)

The performance of the interface was compared between a static and on-line mode of operation of the stainless steel ball using a flow injection analysis (FIA) set-up. Figure 1.10 shows mass spectra of bovine insulin in static and continuous flow mode with FIA. The mass spectra are an average of 20-shot spectra obtained with the acceleration voltage set at 10 kV. In both cases, the samples were deposited on the rotating ball at atmospheric pressure. A 2µl sample of 20 µM solution of insulin was injected with FIA and deposited on the ball at a flow rate of 2 µL/min using a matrix solution that was continuously infused with a syringe pump. In static mode (Figure 10a), the ball rotation was stopped, and after breaking vacuum, the ball was manually rotated to align the sample track with the laser and restore vacuum before acquiring the mass spectrum. For on-line operation, the ball was rotated continuously at 0.3 rpm, and the sample track passed under the Teflon gasket before entering the mass spectrometer. At this rate of ball rotation, the solvent evaporated from the ball surface before the sample reached the gasket. Although acquired from rotational and static mode of ball operation, the resulting mass spectra were nearly identical in signal intensity and mass resolution. The mass spectrum in Figure 2.10b has a greater contribution from low-mass signal, possibly due to salts or other contaminants picked up from the gasket surface. The other mass spectrum in Figure 2.10a which was deposited on the ball and placed into the vacuum without passing under the gasket seal has less contribution from low mass signals. In both cases, the [M + 2H]⁺ and the [2M + H]⁺ adduct peaks were observed but the [M + H]⁺ was still the most intense peak in the mass spectra. The mass spectral data indicated that, although much of the sample deposited was removed from the ball surface, with on-line operations, there was still sufficient material left to generate mass spectra.
Figure 2.10 Mass spectra acquired from a 2µl sample of 20 µM insulin sample mixed with 20 mM α-cyano-4-hydroxycinamic acid matrix in (a) static ball mode by pipetting onto the stationary ball, and placing the sample portion on the ball into the vacuum without passing it under the gasket and (b) continuous flow mode on the rotating ball using flow injection analysis. (Reproduced with permission from Anal. Chem. 2004, 76, 5968-5973. Copyright, 2004 American Chemical Society)

2.3.3 Capillary Zone Electrophoresis (CZE)

To achieve successful operation for CE with the rotating ball as the cathode and transfer interface to MALDI-MS, two parameters were critical in preserving the separation efficiency: the linear speed of the ball and the flow rate of the makeup buffer. First, the makeup buffer flow rate was critical in keeping the capillary tip wet for stable currents; however, caution was taken to minimize zonal dispersion. The makeup buffer was delivered by a syringe pump at a flow rate of 8 nL/s, which is typical of bulk solution flow in CE experiments. Second, the linear speed of the ball was selected to minimize the sample
zonal dispersion arising from the finite detection volume. It was initially anticipated that matching the linear speed of the ball with the velocity of the species migrating through the separation capillary would preserve the separation efficiency; however, this was found not to be the case.

A plot of separation efficiency (plate number) as a function of ball rotation rate is shown in Figure 2.11. This plot was obtained from single ion electropherograms of the peptide bradykinin obtained at different linear velocities of the ball. The plate numbers were obtained from the relationship \( N = \frac{L^2}{\sigma_{\text{total}}^2} \), where \( L \) is the length of the separation capillary. For this calculation, it was assumed that the major contribution to the total peak dispersion (\( \sigma_{\text{total}}^2 \)), for UV detection was longitudinal diffusion and therefore \( \sigma_{\text{total}}^2 \sim \sigma_{\text{Diffusion}}^2 \).

![Figure 2.11](image)

**Figure 2.11** A plot of theoretical plate numbers (separation efficiency) as a function linear speed of the rotating ball. The plate numbers were calculated an individual peak at varying linear speeds of the ball inlet. At low linear velocity better separation efficiency was observed compared to high linear velocity. (Reproduced with permission from *Anal. Chem.* 2004, 76, 5968-5973. Copyright, 2004 American Chemical Society)
Because the separations were first optimized using UV detection, the value for $\sigma^2_{\text{Diffusion}}$ was obtained from the UV trace in Figure 2.14. Operating the ball at high linear velocity did not allow enough time for the sample, make-up buffer and matrix deposits to dry by solvent evaporation before passing the gasket vacuum seal. As results, smearing/lateral spreading at the ball-gasket contact point for wet samples significantly affected the resolution and hence the separation efficiency at the detection point. This is because at higher rotation rates, contributions to the total peak dispersion resulted not only from longitudinal diffusion but also from the finite detection volume as well: $\sigma^2_{\text{total}} \sim \sigma^2_{\text{Diffusion}} + \sigma^2_{\text{detector}}$, where $\sigma$ is the peak dispersion.\textsuperscript{62, 63} Further, the number of data points acquired for characterizing peak profiles as they passed through the detection point at high linear velocity of the ball was limited by the laser duty cycle. However at very low linear speed of the ball, it was predicted that the resolution and hence separation efficiency would be limited since separated components would elute to the same position resulting. Very low linear speeds were not investigated because the analysis would take a long time and significantly reduce the intended sample throughput associated with this on-line approach. A linear speed of 0.04 rpm was chosen as the optimal speed of the ball for CE separations in order to preserve the CE separation efficiency ($10^4$ plates per meter) and to operate at acceptable sample throughput. At the optimized speed, the separation efficiency at the detection point was improved possibly due minimal lateral spreading or smearing at the gasket-ball contact point for dry samples that fixed the separated components on the ball past the gasket seal into the ionization chamber.

The relatively low flow rates observed in the CE mode, combined with the optimized linear speed of the ball resulted to a narrow (200 μm wide) sample track. Individual sample
solutions containing 20 μM of the peptides bradykinin, substance P, and neurotensin were
electrokinetically injected into the capillary to gauge their elution behavior in the capillary.
The injected sample volume is estimated to be 5 nL, corresponding to a 100-fmol quantity
for each of the three peptides. Mass spectra of the three peptides acquired after migrating on
capillary and upon deposition on the ball are shown in Figure 2.12. There is a significant
contribution to the mass spectra from impurity peaks in the low \( m/z \) region, but the mass
spectra are free of detectable interference peaks above \( m/z \) 700. The mass resolution of these
peptide peaks was calculated to be \( \sim 250 \) by fitting a Gaussian distribution at half height on
the peak profile using in-house algorithms. This mass resolution is low and may be
improved by using a reflectron in the flight tube or delayed extraction in the ion source.

![Figure 2.12](image)

**Figure 2.12** Mass spectra obtained on-line on the surface of the rotating ball after individual
electrokinetic injections of 100-fmol samples of (a) bradykinin, (b) substance P, and (c)
neurotensin. (Reproduced with permission from *Anal. Chem.* 2004, 76, 5968-5973. Copyright, 2004 American Chemical Society)
A separation of the three peptides, vasopressin, substance P, and neurotensin, was carried out, and a two-dimensional contour plot was generated on a grayscale map, as shown in Figure 2.13. The migration times were determined by first injecting the individual peptides and selective ion monitoring at their corresponding m/z. The x-axis corresponds to electrophoresis time, the y-axis corresponds to m/z, and the third dimension of signal intensity is represented by the grayscale at a particular m/z and migration time. Substance P was detected at 14.80 min, vasopressin at 15.75 min, and neurotensin at 16.80 min. Low m/z interfering peaks from matrix and running buffer are seen as the dark stripes across the bottom of Figure 2.13. Data acquisition was initiated at 5 min. The drift in m/z of the interference peaks was a symptom of drift in the high voltage power supply connected to the interface flange.

**Figure 2.13** A two-dimensional representation of a CE-MALDI separation of the peptides substance P, vasopressin, and neurotensin. The grayscale intensity represents the ion signal at a particular m/z and elution time while the x-axis corresponds to electrophoresis time and the y-axis corresponds to m/z. (Reproduced with permission from *Anal. Chem.*2004, 76, 5968-5973. Copyright, 2004 American Chemical Society)
A comparison between a UV detector electropherogram and the mass spectral electropherogram is shown in Figure 2.14. This was done to assess peak profiles as eluted from the separation capillary onto the interface for detection. A photodiode array electropherogram (obtained from a different run under nominally identical conditions) is shown. The single ion electropherograms obtained by integrating the ion intensities along the \( x \)-axis for the data in Figure 2.13 are overlaid above the corresponding photodiode array electropherogram peaks. The integration limits were set to \( \pm 0.5 \) µs about the peak of interest. The calculated plate numbers for UV trace and the single ion electropherogram were \( \sim 10^4 \), evidence that the separation efficiency was preserved with optimized linear speed of the rotating ball interface.

![Graph showing selected ion electropherograms of substance P, vasopressin, and neurotensin](image)

**Figure 2.14** Selected ion electropherograms of substance P, vasopressin, and neurotensin obtained from Figure 2.13, plotted above a photodiode array detector electropherogram obtained from a different run under nominally identical conditions monitored at 214 nm. (Reproduced with permission from *Anal. Chem.* 2004, 76, 5968-5973. Copyright, 2004 American Chemical Society)
Another important parameter for the optimization of the detector performance is the laser repetition rate, which sets the sampling rate. The repetition rate should be fast enough to sufficiently characterize peak profiles eluting from the separation capillary. From the peak widths detected on the separation capillary and with the interface operated at the optimized linear speed of 0.04 mm/s, an average electrophoretic peak width of 20 s resulted. With a laser duty cycle of 10 Hz, ~100 data points were collected for each peak with a download duty cycle of ~1 s. The time-of-flight (TOF) mass analyzer has an intrinsic advantage of fast acquisition rates, which provides an extremely short time to produce a full mass spectrum. This allows a large number of mass spectra data per unit time to be acquired for efficient characterization of CE separation peak profiles.

2.4 Conclusion

We have demonstrated a new rotating ball interface for online MALDI, in which the majority of the ball surface is at atmospheric pressure and easily accessible for sample deposition. Samples can be deposited on the ball surface, where they dry and are delivered into high vacuum of a MS past a polymer gasket seal. The ball surface is interrogated by MALDI and then cleaned on-line after it passes out of the ionization chamber for subsequent sample deposits. A CE separation of three peptides, substance P, vasopressin, and neurotensin, was demonstrated. Optimization of the makeup buffer flow rate and the ball rotation rate was critical for maintaining separation efficiency. Continued development of the rotating ball interface is focused on coupling microfluidic chip separations to MALDI using this interface. Improvements in mass resolution for the TOF instrument can be obtained by either implementing delayed ion extraction or adding a reflectron in the mass analyzer or both.
2.5 References


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CHAPTER 3. DIRECT COUPLING OF POLYMER-BASED MICROCHIP ELECTROPHORESIS TO ON-LINE MALDI TOF MS USING A ROTATING BALL INLET

3.1 Introduction

3.1.1 Microfabrication Techniques for Polymer-Based Microfluidics Devices

Microfluidic devices have been fabricated in various materials such as glass, silicon and polymer.1-4 The choice of substrate material is dependent on physical properties such as optical clarity and glass transition temperature, chemical properties such as solvent compatibility and surface chemistry, electrical properties such as dielectric strength, thermal conductivity and expansion coefficients, availability and ease of machining.5 The material’s physical and chemical properties play a significant role in microfabrication, sample handling and processing within a microfluidic device. In departure from originally used material such as glass and silicon, there has been a recent shift to polymer-based microfluidic devices.6 Polymers exhibit a wide range of surface properties such as wettability, adsorption and zeta potential,7, 8 depending on chemical functionality. Such physico-chemical properties affects sample processing, experimental conditions and quality of data in diverse ways.5 Often, different surface modification chemistries are required on polymer substrates to tailor the surface to the sample processing needs.

Polymers are easy to machine and the microfabrication process is cost effective for mass production. Microstructures can be fabricated directly onto polymers using laser ablation, micromilling or wire imprinting techniques. An alternative approach involves microfabrication of a molding master with desired microstructures using high precision micromilling or photolithographic techniques,6 9-11 then using polymer replication technologies such as hot embossing or injection molding to transfer microstructures and
micro-part onto polymer substrates.\textsuperscript{10, 12} Once stamped out, the devices are assembled by thermally annealing a cover plate to enclose the micro-structures patterned onto the polymer substrate.

One example of a photolithographic technique used for the microfabrication of a molding master is LIGA, a German acronym for lithography, electroplating and molding.\textsuperscript{2} Initially, a desired microfluidic network is designed using computer-aided design (CAD) software. The design is transferred to an optical mask which consists of a chromium coated quartz plate with a positive photoresist layer on top. An X-ray mask is developed from the optical mask after exposure to UV radiation. The chromium coating on the optical mask blocks UV radiation while exposing a resist-coated kapton film coated with a gold plating layer which serves as an X-ray absorber. The developed X-ray mask is then aligned over a layer of PMMA bonded onto a stainless steel plating base for exposure to X-rays. PMMA in this case serves as an X-ray photoresist. The exposed PMMA layer disintegrates and can be removed with an appropriate solvent leaving behind the unexposed PMMA parts, the resultant voids are filled by electroplating Ni. The Ni electroform molding master is then mechanically polished with the intact PMMA parts serving a support material for the raised Ni microstructures. The master can then be used for replication of the microstructures either using hot embossing or injection molding. LIGA is the preferred method when fabricating sub-micron sized features of microstructures that require extremely high-aspect ratio in a microfluidic device. A schematic diagram of procedures involved in the micro-fabrication of mold inserts using the LIGA (lithography, electroplating, and molding) technique and replication in polymer substrates using a hot embossing technique is illustrated in Figure 3.1.
**Figure 3.1** A schematic illustrating the steps involved in the microfabrication of mold inserts using LIGA technique and replication of the microstructures using molding technique: (a) X-ray or UV exposure of synchrotron radiation to a photoresist with an X-ray mask, (b) development of exposed area and electroplating the voids, (c) mechanical separation of the electroform metal structure from the starting base plate then molding polymer substrate using the mold insert.

A high precision micromilling machine can also be used to fabricate a molding master by micromilling the desired microstructures onto a brass plate. The milling machine is capable of achieving positional and repetition accuracy of ±1 µm by use of a laser measuring system and an optical microscope. Varying sizes of solid carbide milling bits operating at different feed rates are employed for milling and rapid prototyping of desired microstructures on brass plates. The sizes of features that can be fabricated using micromilling technique are limited by the size of the milling bit. The burrs created at the upper surface of the microstructures after milling are removed by mechanical polishing before the master is used for replication of microstructures with the hot embossing
technique. A digital picture of a Kern high precision micromilling machine used for the microfabrication of mold inserts is shown in Figure 3.2.

![Image of Kern high precision micromilling machine and milling bits](image)

**Figure 3.2** A Kern high precision micromilling machine and milling bits used in the microfabrication of mold insert on brass plates.

Replication of polymer micro-parts can be accomplished using hot embossing.\(^1\)\(^,\)\(^9\) Hot-embossing is an easy and cost-effective method for reproduction of large numbers of identical polymer devices with similar microstructures. The molding master is mounted onto a temperature controlled fixture and the planer polymer substrate sandwiched between the molding master and an evacuated embossing chamber at controlled temperature and pressure. The assembly is heated and pressed for a specified time and upon cooling, the molding master is mechanically separated from the polymer substrate, which already has the desired featured embossed onto the surface. Hundreds of devices can be made from one master, allowing for rapid prototyping and cost-effective mass production of polymer microfluidic devices. The final step of assembling a microfluidic device involves cleaning the embossed microstructures and thermal bonding of a cover plate at a controlled temperature to enclose the embossed microstructures and to allow for fluidic transport within the microfluidic channel network.
3.1.2 **Integration to Biological Mass Spectrometry**

Most of the early work in coupling microfluidics devices to biological mass spectrometry focused on electrospray ionization (ESI). On-chip CE ESI-MS has some drawbacks associated with the non-compatibility of ESI with aqueous buffers and the need to decouple separation voltage from ESI voltage. Further, the generation of ESI directly from the chip usually results in a large droplet constituting a highly mixed volume, limiting the utility of upstream separation. It is also worth noting that the directional control of the spray can be difficult for a high density array of spray tips, potentially leading to crosstalk. In some cases, the sprays can be difficult to start and stop because they are heavily dependent on the tip geometry and the liquid phase. Microfluidic chip interfaces with ESI also suffer from problems when sprays are started or stopped and this limits the speed of moving from one sample to another and hence less desirable for high throughput applications. However, the obvious preference of ESI interfaces between microchips and mass spectrometry is due to the fact that ESI is a continuous liquid introduction technique and therefore naturally easier to implement in microfluidics.

Nonetheless, ESI and MALDI are complementary techniques that provide soft ionization of biomolecules in liquid and solid samples respectively. A few works have been reported on on-line and off-line coupling of micro-column separations to matrix assisted laser desorption ionization (MALDI). Unlike ESI, MALDI is essentially a solid sample technique that employs an excess of solid matrix crystals as host for guest sample molecules. Recent studies have also shown that MALDI can work with liquid matrices when infrared lasers are employed for desorption and ionization. MALDI has the strengths of high tolerance to impurities such as buffer additives, a wide mass range, easily interpreted
mass spectra and the potential for high throughput sampling availed for by rapid switching, such as a fast x-y translation stage or a galvanometer-driven mirror. Limitations of off-line MALDI have resulted in efforts towards the developments of rugged and robust interfaces for on-line MALDI \(^{39-43}\) that can delivery sample continuously into vacuum in real time and thereby exploit the potential for high throughput sampling.

Recently, an on-line continuous pressure driven self-activating flow on-chip microfluidics device connected to a MALDI-TOF mass spectrometer was reported.\(^ {30}\) The integration was achieved by placing the micro-device on a standard MALDI sample plate, which had a rectangular hole milled to fit the entire device. The reagents were loaded on the device off-line before mounting it into the mass spectrometer ion source. Chemical syntheses as well as biochemical reactions were carried out under vacuum inside the MALDI-MS ionization chamber and the products analyzed by MALDI-TOF MS. The device was later modified to include a sub-micron hole for monitoring the kinetics of chemical reactions in real time using MALDI MS.\(^ {29}\)

Previous works on integrating microfluidics to MALDI MS have reported either off-line electrophoresis,\(^ {36}\) or off-line manipulation of samples in the fluidic channel using a centrifugal force,\(^ {34}\) electrowetting-on-dielectric,\(^ {33,\,35}\) or on-line continuous pressure driven self-activating flow.\(^ {29,\,30}\) We report here the integration of microchip electrophoresis to on-line MALDI MS. The coupling of MALDI MS to microchip electrophoresis employs a direct contact deposition approach to transfer samples eluting from a microfluidic device onto a rotating ball inlet.\(^ {44}\) Samples deposited at atmospheric pressure are transported into the high vacuum of the mass spectrometer without breaking the ion source vacuum. The interface has the advantage of decoupling the ionization process from the separation step.
This is important in optimizing the separations and ionization process independently without compromising the sample processing step and overall system performance. The interface performance was demonstrated by the separation of peptides and a proteolytic digest of a model protein with subsequent MALDI MS detection of the generated peptide fragments.

3.2 Experimental Section

3.2.1 Reagents and Methods

Bradykinin (B-3259), [Met-OH\textsuperscript{11}] substance p (S-2136), bombesin (B-4272), cytochrome C (C-2506), peptide separation buffer (P-2188), α-cyano-4-hydroxycinamic acid (C-2020), ammonium bicarbonate (A-6141), trypsin digestion buffer and trypsin (T-6567) were all obtained from Sigma-Aldrich (St. Louis, MO). The separation buffer (phosphate, pH 2.5) stock solution was diluted to 10 mM in de-ionized water and sonicated for 15 minutes before use. The peptides bradykin, substance p and bombesin were prepared to a concentration of 10 µM in the phosphate buffer. A 50 mM solution of α-Cyano-4-hydroxycinamic acid (α-CHCA) prepared in methanol and 0.1 % trifluoroacetic acid (TFA) was used as the MALDI matrix.

*Tryptic digestion:* A 50 mM ammonium bicarbonate digestion buffer was prepared in de-ionized water. A 20 µM solution of cytochrome c was prepared in the ammonium bicarbonate digestion buffer whereas trypsin was prepared in trypsin reaction buffer obtained from Sigma-Aldrich (T-6567, St. Louis, MO). Cytochrome c (20 µM) and trypsin (0.1 mg/ml) solutions were then mixed in 50:1 (w: w) ratio and incubated for 18 h at 37\textdegree C. The reaction was stopped by the addition of 1% trifluoroacetic acid in the incubation mixture. The digest was thereafter concentrated using a speed vacuum at 40\textdegree C and resuspended in a 10 mM phosphate buffer.
3.2.2 Microchips

A molding tool was first micromilled on a 0.250” thick brass plate (alloy 353 engravers brass McMaster-Carr, Atlanta, GA). The brass plate was cut into a 12 cm diameter round plate and a specified pattern of microstructures was milled onto the surface of the brass plate with a high-precision micromilling machine (KERN MMP 2522, KERN Micro- und Feinwerktechnik GmbH & Co.KG; Germany). The milling machine is capable of achieving positional and repetition accuracy of ±1 µm by use of a laser measuring system (LaserControl NT, Blum-Novotest GmbH, Germany) and an optical microscope (Zoom 6000, Navitar, Inc. Rochester, NY). A 50 µm solid carbide milling bit (McMaster-Carr or Quality Tools, Hammond, LA) was employed for milling the microstructures. The micromilling was accomplished at a feed rate of 10-20 mm/min for a 50 µm bit and at 40,000 rpm. The burrs created at the surface of the microstructures after milling were removed by mechanical polishing. The polishing step was done manually, first using a 3 µm grain size polishing paper (Fibrmct Discs - PSA, Buehler, Lake Bluff, IL) and then with a polypropylene cloth (Engis, Wheeling, IL) using a 1 µm diamond suspension (Metadi Diamond Suspension, Buehler). Both the polishing paper and the cloth were attached to 12” x 12” x 0.25” glass plates obtained from McMaster-Carr. A SEM image of a section of the mold insert is shown in the right panel of Figure 3.3. The milled pattern had a 8 cm linear separation channel that was 50 µm wide and 100 µm deep with an offset T-injector with a fixed volume ($V_{inj} = 10$ nL).

The molding die was then used for replicating polymer micro-parts via hot embossing as the polymer replication technique on 5 mm thick, 12 cm diameter poly-methyl methacrylate (PMMA) disks. PMMA sheets were obtained from (MSC Industrial
Supply Company, Melville, NY). The sheets were mechanically cut to 12 cm disks and baked on the oven at 80 °C for 8 h to remove the residual moisture before embossing. Embossing was accomplished by first mounting the molding die in a PHI Precision Press hot embossing machine (Model TS-21-H-C (4A)-5, City of Industry, CA) together with planar PMMA disks of 5 mm thickness and 12 cm in diameter. Both the die and the PMMA disk were heated separately in a vacuum chamber to a temperature of 155 °C, just above the glass transition temperature of PMMA (T_g = 107 °C). The molding die was then brought into contact with the PMMA substrate and embossed at a pressure of 950 P.S.I for 150 s. The PMMA-molding die was rapidly cooled to just below the glass transition temperature before mechanically separating the die from the embossed substrate.

![Figure 3.3](image)

**Figure 3.3** A schematic diagram showing the top view of chip to ball direct contact deposition: (A) sample reservoir, (B) waste reservoir, (C) buffer reservoir, (D) fluidic channel exit tip, (E) ball. The panels on the right show two SEM images of (1) mold insert (2) embossed microchip tip. (Reproduced with permission from *Electrophoresis*, 2005, 26, 4703-4710, Copyright, 2005 Wiley-VCH)

After embossing, the PMMA device was machine cut as shown in Figure 3.3. The upper circular portion was 8 cm in diameter and the entire device was 10.5 cm long. The
fluidic channel exit was mechanically cut to a tapered V-shaped tip (see Figure 3.3). The final device was sonicated and cleaned with water and IPA and air-dried. The channels were enclosed by thermally annealing a 125 µm PMMA cover slip at 107 °C for 20 min on the embossed side of the substrate.

3.2.3 Microchip Electrophoresis.

An in-house built high voltage controller, which consisted of four independent power supplies sharing a common ground, was used to drive the electrophoretic process. Timing relays were incorporated into the high voltage power supply controller to allow four different outputs to sequentially carry out the electrokinetic injection, separation and activate pull back voltages required for minimizing sample leakage from the sample reservoir into the separation channel. A schematic of microchip electrophoresis configuration is shown in Figure 3.4.

Figure 3.4 An illustration of microchip electrophoresis set-up; sample injection is carried out using a fixed volume injection cross when a voltage is applied across the double T junction of the two side short channels labeled sample and waste where the rotating ball is placed. The sample plug at the junction is separated along the channel toward the port labeled waste. The potential at the reservoirs is controlled using a programmable power supply that employs high voltage relays for switching the potential.
The electrical connection to reservoirs A, B and C was via Pt electrodes inserted into the appropriate wells while that for the fluidic channel exit (tip D, see Figure 3.3) was through an ISO-100 flange. Microchips were integrated to on-line MALDI MS by adapting the rotating ball inlet to function as an electrophoresis electrode in addition to sample transfer and MALDI target function.\(^46, 47\) The voltage applied on the rotating ball served as a reference to operate microchip electrophoresis in a reversed or normal mode and also a reference for MALDI MS operation in either positive or negative mode. Caution! MALDI MS and electrophoresis systems use high voltage and must be operated carefully.

The chip was mounted on an electrically insulated holder and attached to a XYZ micro-translational stage for precise alignment of the channel exit with the matrix trace deposited on the surface of the rotating ball. Two external current meters were used for monitoring the current between the sample (A) and waste (B) reservoirs during injection and also between the buffer reservoir (C) and the ball (E) during separation. The chip was initially filled with buffer through reservoir C using a syringe until the buffer at the exit of the fluidic channel was in contact with the ball, which was rotated at 0.2 rpm. The buffer in reservoir A was removed with a Hamilton syringe and replaced with the sample. The sample was then immediately injected at a field strength of 150 V/cm for 15 s between reservoirs A and B while reservoir C and tip D were floating. The high voltage controller was thereafter switched to the appropriate voltage between reservoirs C and D providing a field strength of 200 V/cm, while floating reservoirs A and B. The pull-back voltages at reservoirs A and B were activated 10 s after injection to allow the plug enough time to migrate past the second arm of the offset T-injector. The electrolyte used to prepare the sample and carry out the electrophoresis was 10 mM phosphate buffer at pH 2.5. These experimental conditions were
used in all cases unless otherwise stated. The voltage at point D was always set to a lower positive potential relative to the other reservoirs producing a normal mode electrophoresis configuration.

3.3 Results and Discussion

In departure from traditional materials such as silicon or glass, we employed polymers as the substrates of choice for fabricating the separation platform. Polymers are attractive because of the ease of machining and a wide choice in fabrication technologies that can be employed to manufacture the prerequisite microstructures including replication using hot embossing. The replication technique used is capable of mass production of devices at high rates and minimum costs making these devices attractive for clinical diagnostic applications where one-time use is necessary to eliminate false positives and negatives arising from sample contaminations.

In this case study, PMMA was a suitable substrate for fabricating the device because it supports a lower electro-osmotic flow ($2.07 \times 10^{-4}$ cm$^2$/Vs) independent of the pH, compared to glass whose electro-osmotic flow varies with pH. PMMA is also a low cost and easy to machine substrate that can readily be molded using hot embossing. The replication quality of the embossed device was assessed by comparing the aspect ratio of the microstructures on the master to those embossed on the substrate using microscopy. Thermal annealing of the device at the glass transition of the polymer allowed assembly of the device without significantly compromising the integrity of the embossed microstructures. Visual inspection of the device under a microscope after pealing off a thermally annealed cover slip did not reveal any collapsed microstructures. An assembled chip was tested for leaks by manually flowing de-ionized water through the enclosed fluidic channel using a hand-held
syringe. The planar substrate geometries used to manufacture microfluidic devices have large area that allow for integrated sample processing and a modular systems approach to integrate platforms. However, it is worth noting here that the microfluidic planar footprint is technically different from tubular platforms used in capillary electrophoresis and require specialized approach when coupling to MALDI MS. This is true when capillary interconnects for sample transfer from the device to MS is not desired. Indeed, the direct contact approach eliminates the need for such and thereby circumvents the dead volume and extra-column broadening associated with capillary sample transfer interconnects.\(^{49}\)

In coupling microchip electrophoresis to on-line MALDI MS using the rotating ball interface geometry, some of the challenges addressed included providing an efficient sample transfer from the exit of the fluidic channel onto the ball surface, maintaining a stable electrical connection between the chip and the ball, online matrix addition, sample transfer into vacuum, laser alignment on the sample trace and the regeneration of a clean ball surface for subsequent sample deposits. The alignment of the microchip tip to the matrix trace at atmospheric pressure was accomplished using a xyz micro-translational stage. The laser spot was aligned with respect to the matrix trace using an adjustable mirror mount and a video camera. This alignment was critical to achieve optimal signal for the MALDI process; the sample was deposited beneath the matrix trace in order to be aligned with the laser for desorption. The microchip’s v-shaped 50 µm tip minimized the surface area of the chip in contact with the ball. This allowed the sample zones to be transferred from the fluidic channel by direct contact deposition onto the rotating ball surface with minimal lateral spreading. The direct contact deposition configuration maintained stable electrical
connection (and stable current) between the ball and the chip without the need for a make-up buffer.

Initially, 10 fmol of each test peptides were electrokinetically injected into the separation channel to determine the migration times. The conditions used were as described in the experimental section. The corrected migration times were established to be 1.15 min for bradykinin (m/z 1061), 1.42 min for substance p (m/z 1349) and 1.78 min for bombesin (m/z 1619). The RSD obtained from three measurements for the migration times was less than 2 %. Online mass spectra acquired for bradykinin, substance p and bombesin are shown in Figure 3.5. The mass spectra are plotted from bottom up with bradykinin at the bottom. The peaks below m/z 500 arise from salts, matrix, matrix fragments and matrix adducts. The low background in the low mass region of spectrum A, where most matrix and buffer salts adducts peaks show up could be due to competitive ionization or laser energy effect. No contamination arising from the dissolution of PMMA by the phosphate buffer was observed. The mass resolution averaged about 150; however, this value might be improved by adding delayed extraction in the ion source and/or a reflectron in the flight tube.

A mixture of the three peptides containing 10 fmol of each test peptide was separated in the 8 cm channel. Experimental conditions used here were as earlier described in the experimental section. A separation contour plot of three peptides, bradykinin, substance p and bombesin, generated using the 2D graphing software is shown in Figure 3.6. Also a single ion electropherogram generated by integration the ion intensities in the 2D contour plot is shown in Figure 3.6. The gray scale represents normalized signal intensity; the x-axis represents m/z while the y-axis represents separation and read-out time. In the low m/z region, several peaks from the salts in the separation buffer and matrix adducts are observed.
Figure 3.5 Averaged mass spectra from 5 laser shots acquired on-line from electrokinetic injections 10 femtomoles each of (a) bombesin, (b) substance p and (c) bradykinin. (Reproduced with permission from *Electrophoresis*, 2005, 26, 4703-4710, Copyright, 2005 Wiley-VCH)

Figure 3.6 The upper panel represents a two dimensional separation contour plot of a separation of (a) bradykinin, (b) substance p and (c) bombesin on a PMMA chip. The x and y-axis represents m/z and separation times respectively while the gray shade shows normalized ion intensities. The panel below is a representative electropherogram generated by integrating ion intensities from the 2D separation contour plot. (Reproduced with permission from *Electrophoresis*, 2005, 26, 4703-4710, Copyright, 2005 Wiley-VCH)
Electropherograms were constructed by integrating the signal intensities for the peaks of interest in the contour plot within a specified m/z window using Image J software. A typical electropherogram is shown in Figure 3.6 beneath the 2D contour plot and represents a separation of the three peptides with the corrected migration time scale on the x-axis and normalized intensity on the y-axis. Assuming Gaussian peak profiles, the separation efficiencies generated in the 8 cm channel were calculated to be approximately 10³ plates, which translated to 10⁴ plates per meter. A comparison of these results was made to those previously observed using capillary electrophoresis to evaluate the microfluidic advantages. First, the chip was designed to achieve a higher sample loading leading to improved sensitivity. Compared to the capillary, samples with a 10 fold lower concentration were routinely injected in the device and detected. Average linear migration velocity in the chip was about three times faster than that in the capillary. Apparent mobility is a function of both the analyte electrophoretic mobility and EOF. The high migration velocity in the chip is due to the higher EOF of native PMMA (2.07 x 10⁻⁴ cm²/Vs), compared to that observed for fused silica (< 0.5 x 10⁻⁴ cm²/Vs) at low pH.

The linear speed of the ball when coupled to the chip was operated five times faster (0.2 rpm) than that in capillary electrophoresis. It was possible to operated the ball at higher linear speed with the chip because, for one, no make-up buffer was required when interfacing microchip electrophoresis as was the case in capillary electrophoresis. This was because of the presence of a stronger EOF in PMMA than that of glass at low pH. Therefore sample and matrix deposits dried fast before passing the gasket vacuum seal due to less aqueous solutions on the ball surface. Secondly, the hydrophobic nature of PMMA minimized the possibility of droplet formation at the chip’s tapered tip to ball contact point.
that could have caused some sample lateral spreading. Further, separations in the microchip
developed in a shorter time compared to capillary electrophoresis and allowed the ball to be
rotated faster. CE separations took considerably longer time leading to evaporation of the
buffer at the capillary tip, and thereby necessitated the need for a make-up buffer since the
EOF was suppressed at low pH. The above operating parameters resulted in fast analysis
time using the microchip compared to the capillary format and generated comparable
separation efficiencies despite the shorter separation channel.

We next analyzed a tryptic digest of cytochrome c by microchip CE interfaced to
MALDI MS using the rotating ball inlet. A tryptic digest of cytochrome c that was
performed off-chip using in-solution digestion protocol was first analyzed off-line in static
mode by manually spotting 1 μL of a premixed tryptic digest and matrix on the ball and then
the spot was rotated into vacuum. Typical mass spectra acquired off-line without a
separation step from the digest are shown in Figure 3.7. The first panel shows the mass
spectrum acquired from a cytochrome c digest while the second panel shows the mass
spectrum obtained from a cytochrome c digest that had been spiked with bradykinin. As can
be seen in the first panel, only a few fragments were detected in the unspiked digest without
a separation step. Moreover, in the second panel where the digest had been spiked with a
high concentration of bradykinin, the fragments from the digest were suppressed and only an
intense bradykinin signal was seen. From these results, the presence of an intense signal
from an abundant fragment often appeared to suppress ionization of the other fragments
present in a digest mixture that were in lower abundance. The cytochrome c digest was next
injected and separated in the micro-device to further test the performance of the coupling.
Figure 3.7 The mass spectra acquired off line in static mode are shown below the contour plot and represents (a) cytochrome c digest and (b) cytochrome c digest spiked with bradykinin. (Reproduced with permission from *Electrophoresis*, 2005, 26, 4703-4710, Copyright, 2005 Wiley-VCH)

Figure 3.8 A two dimensional contour plot showing the separation of cytochrome C digest. The x-axis represents m/z, y-axis represents electrophoresis time, and gray shade represents ion intensities. Bradykinin (m/z 1061) was continuously infused for mass calibration. (Reproduced with permission from *Electrophoresis*, 2005, 26, 4703-4710, Copyright, 2005 Wiley-VCH)
Based on the off-set T-injector volume, the injected amount was estimated to be less than 20 fmol. The separation conditions used were the same as described in the materials and methods section. A separation contour plot of the protein digest is shown in Figure 3.8. The numbered spots in the contour plot indicate peptide fragments detected from the digest. The intense peak at \( m/z \) 1061 in the contour plot as shown in Figure 3.8 corresponds to intact bradykinin, which was added in the buffer reservoir C such that it was continuously infused during the separation of the digest and used for calibration. There are about ten intense fragments appearing above \( m/z \) 700 in the contour plot from the digest that are detected online, even with continuous infusion of a higher concentration of bradykinin.

There are two intense peaks arising from the auto-proteolysis of trypsin at \( m/z \) 1154 and \( m/z \) 2211. These peaks were not labeled and therefore not included in the tentative identifications listed in Table 3.1. As shown here, a separation step was required to improve the sensitivity of low abundant species when analyzing the digest with MALDI TOF MS. The detected peptide fragments were given tentative assignments by matching their peptide masses to those in the MS-digest database expected for a horse heart cytochrome c tryptic digest. The digest was also analyzed with a MALDI-TOF MS instrument (Ominiflex, Bruker Daltonics Inc., Billerica, MA) to identify the intense fragment peaks. Tentative peptide assignments given (using the one letter symbol) to the labeled fragments in the contour plot are listed in Table 3.1. The sequence coverage for the digested cytochrome c was estimated to be 62 % based on the sequence information obtained from the tentative identification assigned to the fragments. Potential sample loss during off-chip digestion and peptide recovery, non-specific binding of peptide fragments on PMMA or undetected fragments due to ion suppression effects could account for some of 38 % loss of sequence.
coverage information. We have investigated other polymer substrates with desired characteristic to complement PMMA surface.\(^5\) Indeed the PMMA surface has a water contact angle of 73\(^\circ\), indicating a more hydrophobic surface compared to commonly used glass devices (35\(^\circ\)). Unlike optical detection schemes, when a mass spectrometer is the detector of choice, optical clarity of the polymer substrate is not a requirement thereby availing many plastics for potential use as the fluidic system. We are also incorporating digestion chambers onto the device to improve the digestion and protein identification efficiencies and also reduce any potential sample losses associated with processing and transfer steps.

Table 3.1 A listing of tentative assignments of peptide fragments by PMF.

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<tr>
<th>Pep. ID</th>
<th>Predicted Mass</th>
<th>observed Mass</th>
<th>Da. Error ±</th>
<th>Start</th>
<th>End</th>
<th>Missed Cleavages</th>
<th>Sequence</th>
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</tr>
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</tr>
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</table>
3.4 Concluding Remarks

We have described the coupling of a PMMA microfluidic device to online MALDI TOF MS using a rotating ball interface. The approach was simple in design and did not employ a sample transfer capillary connection from the fluidic channel to the mass spectrometer. This eliminated any broadening of the electrophoresis peaks associated with extra column effects, material mismatch and automates MALDI sample preparation. The coupling was robust and showed high separation efficiencies and improved sensitivity with routine analysis of samples in the lower femtomole range. Although MALDI MS is credited for a high tolerance of impurities, the sensitivity of low abundant species is often compromised in complex samples due to ion suppression effects. Coupling microchip electrophoresis to MALDI MS is one way of simplifying complex mixtures before analysis, and thereby improving the sensitivity of constituents present in low abundance. To improve the mass resolution, mass accuracy and mass range, we are modifying the home built instrument to incorporate a delayed extraction in the ion source and a reflectron in the flight tube, respectively.

3.5 References


(6) de Mello, A. Lab Chip 2002, 2, 31N-36N.


CHAPTER 4. A SOLID-PHASE PROTEOLYTIC MICRO-REACTOR FOR EFFICIENT DIGESTION AND IMPROVED PROTEIN IDENTIFICATION WITH MALDI MS

4.1 Introduction

Sample processing for proteomic applications requires high throughput and automated technologies to accelerate target identification and biomarker discovery.¹ Most sample preparation protocols, for example protein digestion, are time-consuming and labor intensive taking on average 6-24 h and are prone to sample losses and manual handling that may introduce contaminants, such as human keratins.²⁻⁴ Digestion of proteins to peptides is an important step when mass spectrometry is used for the identification of proteins. Peptides have lower molecular weights compared to proteins and their masses can be measured with higher accuracy using mass spectrometers. Protein digestion can be accomplished using chemical or proteolytic cleavage methods as discussed in Chapter 1. The choice of cleavage method is dependent on the type and objective of the study; for example, chemical cleavage using hydrazine cleaves all of the peptide bonds yielding amino-acyl hydrazides of all the amino acids present in a protein and is mostly used to determine the amino acid composition of proteins. On the other hand, proteolytic cleavage is specific to particular amino acid residues depending on the mechanism of action of the protease employed and is used to study structural modifications of proteins or identification of proteins using their peptide maps.

For proteomic applications, there are two popular approaches used for elucidation protein composition namely; “shotgun” or “bottom-up” were proteolysis is routinely performed before protein identification using mass spectrometry. In the bottom-up approach, a mixture of proteins is first separated using two-dimensional gel electrophoresis and the
visualized proteins band are excised from the gel for digestion using in-gel-based protocols. In the shotgun approach, a mixture of proteins is first digested using solution (homogeneous) protocols followed by multidimensional separation before analysis with tandem mass spectrometry to identify the original proteins. In both approaches, protein identification with mass spectrometry is mostly performed using peptide mass fingerprinting (PMF), where peptide masses generated from the proteins are subjected to a protein database search for identification.

The protein identification efficiency using PMF is often limited by the extent of proteolytic digestion. For example in the shotgun approach, protein identifications are based on the assumption that the digestion of all proteins in the mixture is driven to completion. However, it is known that digestion of a complex protein mixture is limited by the physical properties of the proteins, poor solvation, denaturation, inadequate local enzyme concentrations, autodigestion, insufficient reaction times, and long incubation times, degradation of proteins below the detectable molecular weight range. During long incubation times, some peptide fragments remain intact but they may also undergo chemical modifications such as deamidation, oxidation and increased trypsin autolysis products, thereby complicating peptide mass assignments during PMF. Further, some of the generated fragments within the incubation mixture compete for and/or inhibit protease activity. Modifications to standard digestion protocols for example reaction time, local enzyme concentration, inclusion of reduction, alkylation and on-column digestion rather than incubation tubes have been demonstrated to generate large improvements in the identification of proteins present in a sample, hence pointing to the fact that digestion is a major limiting factor in protein identification efficiency.
On the other hand, bottom-up approaches involve digestion of excised protein bands or spots after a gel-based electrophoresis separation or direct in-gel digestion without spot excision. Spot visualization in the gel after separation requires staining procedures, which are dependent on the physicochemical properties of the protein, such as molecular weight, isoelectric point and solubility of the protein, and can limit the sensitivity for visualization via staining.\textsuperscript{16} Compatibility and success of MS detection are also dependent on the staining procedure. For example, coomassie blue stains are compatible with MS, however, they are less sensitive than silver stains therefore restricting identification to only the most abundant proteins in the gel. On the other hand, silver-stained proteins require destaining before digestion to improve the sensitivity and quality of mass spectral data.\textsuperscript{17, 18} In general, regardless of the stain used, the efficiency and accuracy of protein identification depends on the number of missed cleavages during digestion, protein abundance and properties of the protein.\textsuperscript{18} In cases where protein spots are digested directly in the gel, digestion is limited by the accessibility of the gel-entrapped proteins by the proteolytic enzyme. Peptide fragments generated may diffuse into the gel media, which results in low peptide recovery. Usually, careful peptide extraction from the gel is required in order to improve peptide recovery and hence protein identification. These factors affect digestion and typically results in less than 50 % sequence coverage when performing in-gel digestion. Such low coverage affect for example the analysis of integral membrane proteins with hydrophobic domains and post-translational modifications and because modified residues may be excluded from the analysis, this may limit the protein identification efficiency.\textsuperscript{19, 20} However, in-gel digestion of proteins followed by peptide recovery is sometimes favored over extraction of proteins from the gel followed by digestion. This is because protein
recovery from gels using electro-elution, chemical extraction or passive diffusion methods is
time-consuming and results in low protein yields, especially for low abundant proteins. Alternative approaches employ electroblotting to transfer gel free proteins onto membrane supports.

In order to improve the efficiency of protein digestions and hence the reproducibility of proteomic studies, there has been a shift in focus from solution-based or in-gel protein digestion to immobilized microfluidic enzymatic reactors (IMERS). Immobilization of proteolytic enzymes onto solid supports alleviates limitations encountered in solution-based or in-gel protein digestion and results in less autodigestion of the enzyme, greater stability, longer catalytic activity, reusability, and higher digestion efficiency. Reactors in micro-column flow-through formats can be easily adapted into an integrated sample preparation platform for automated sample processing. Enzymatic reactors have been prepared inside microfluidic devices by immobilization of proteases onto polymer monoliths, beads, and membranes, or encapsulation in sol-gels. However, immobilization of protease enzymes within, for example, a polymer monolith or encapsulation in sol-gels can unfortunately limit or hinder substrate accessibility to the enzyme active sites, thereby affecting the efficiency of the digestion. On the other hand, the use of beads in microfluidic devices has been limited by difficulties encountered in packing the beads into the desired locations within the chip as well as maintaining their entrapment when operated in flow conditions. Use of magnetic beads to simplify handling within a microfluidic device has been shown to overcome some of the aforementioned problems associated with beads. Wang et.al reported a microfluidic-based reactor that was packed with immobilized trypsin gel beads. Protein samples were flowed through the reactor using a syringe pump at
rates between 0.5 and 1 µl/min, which afforded a digestion time of 3-6 min. In another study, Ekstrom et.al demonstrated a reactor with trypsin immobilized onto a porous silicon wafer for rapid protein digestion within 1 to 3 min.\(^{45}\) A membrane reactor consisting of trypsin adsorbed onto poly-vinylidene fluoride (PVDF) membranes was used in a poly-dimethylsiloxane (PDMS) microfluidic device.\(^{37}\) Samples were flowed through the membrane-based reactor using a syringe pump at rates of 0.3, 0.2 and 0.1 µl/min with digestion times of 3, 5 and 10 min, respectively. Jin et.al described an electroosmotic flow (EOF) driven reactor consisting of immobilized trypsin gel beads packed into a microfluidic channel for digestion of proteins with a reaction time of 12 min.\(^{46}\)

A compelling advantage of micro-reactors is that they can provide a large surface-to-volume ratio as a direct result of downscaling the micro-reactor bed volume.\(^{47}\) Decreasing the linear dimension of the reactor reduces the surface area quadratically while the volume decreases by the cube. The increased surface area-to-volume ratio results in reduced diffusion paths and thus, increases encounters between the substrate and enzyme.\(^{48}\) This phenomenon decreases kinetic barriers imposed by mass transport of substrate to the surface resulting in shorter processing times for proteolytic digestion.\(^{47}\) For example, high aspect ratio microstructures can provide increased surface area-to-volume ratios within a miniaturized reactor with defined lateral dimensions and these structures can be embossed inside the fluidic channel \textit{in situ} using micro-replication technologies.\(^{26,49-51}\) Patterning high surface area support structures within the micro-reactor bed eliminates the difficulty associated with packing beads and also allows the microstructures to be fixed in a desired location within a device with an open architecture that provides unrestricted substrate access to the immobilized enzyme.
In this Chapter, we report on the fabrication, assembly and operational parameters of a high surface area-to-volume ratio solid-phase micro-reactor consisting of a 3.5 cm x 230 μm x 100 μm microfluidic channel with trypsin immobilized onto an array of free-standing 30 μm x 30 μm diamond-shaped micro-post support structures with 20 μm edge-to-edge interspacing embossed into a fluidic channel. The microchip was embossed in poly-methyl methacrylate (PMMA) substrate from a metal master prepared using LiGA. The proteolytic enzyme, trypsin, was covalently strapped to the surface of the PMMA microstructure supports using a UV-directed surface modification protocol. The performance characteristics of the solid-phase reactor were assessed using model proteins. The sequence coverage for model proteins was optimized as a function of linear flow velocity, micro-reactor geometry, physical and material properties of proteins and substrate respectively.

4.2 Experimental

4.2.1 Reagents and Materials

Proteomics grade trypsin (T-6567), cytochrome c (C-2506), myoglobin (M-1882), β-casein (C-6905), BSA (A-0281), α-cyano-4-hydroxycinnamic acid (C-2020) and ammonium bicarbonate (A-6141) were all obtained from Sigma Aldrich (St. Louis, MO) and used as received without further purification. Sodium phosphate buffered saline pH 7.0 (PBS, Product No. 28732), MES (2-[morpholino] ethanesulfonic acid) buffer (Product No.28390), sulfo-NHS (N-hydroxysuccinimide) (Product No.24510), 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC, Product No.22980) were obtained from Pierce Biotechnology (Rockford, IL) and used as received without further purifications. Polymethyl methacrylate (PMMA) sheets were obtained from MSC industrial supply Co. (Melville, NY).
4.2.2 Micro-reactor Fabrication

A mold master was prepared using the LiGA technique, which was subsequently used to replicate polymer micro-parts via hot embossing as described in Chapter 3. The embossed microfluidic devices had 16 micro-reactor chambers each consisting of a 3.5 cm long x 230 μm wide x 100 μm deep microfluidic channel and an array of free standing 30 μm x 30 μm x 100 μm tall diamond-shaped microposts with a 20 μm edge-to-edge interspacing embossed within the polymer fluidic channel bed. Figure 4.1 shows these micro-post structures used in the micro-reactor to achieve the desired high surface area-to-volume ratio. There where approximately 2,500 posts in each bed which increased the surface area-to-volume ratio of the chamber up to three times compared to that of a similar dimensional open chamber without micro-posts. Two reservoirs of approximately 1 mm in diameter were manually drilled at the start and end of each reactor, one to allow for liquid introduction to the reactor chambers and another as the exit reservoir for collection.

4.2.3 UV Activation of PMMA and Trypsin Immobilization

After embossing, the microstructures were sonicated in water for 15 min then rinsed with copious amounts of distilled water and air dried. The microstructures and cover plate were then exposed to UV light at 254 nm for 20 min. A final rinse of the microstructures and the cover plate was done with distilled water followed by air drying. The reactor chambers were then enclosed by thermally annealing a cover plate at a controlled temperature of 102 °C. The enzyme attachment protocol adopted was based on PMMA modification protocols developed in our laboratories and is shown schematically in Figure 4.2. A buffer solution comprised of 0.1 M MES (2-[morpholino]ethanesulfonic acid) and 0.5 M NaCl at pH 6.0 was used to prepare 5 mM of sulfo-NHS (N-hydroxysuccinimide) and 5 mM of 1-
ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) coupling reagent. This solution was introduced into the micro-reactor chambers using a syringe and incubated inside the reactor for 15 min. It was thereafter replaced with a 100 mM phosphate buffer solution at pH 7.0 containing 100 µg of trypsin. After refilling the chamber, the reservoirs were sealed with scotch tape to prevent evaporation and the solution was left to stand inside the chambers for 2-3 h. The solution containing excess trypsin was removed from the reactors; the chambers were then rinsed with buffer and air dried. After air drying, the reactor chambers with immobilized trypsin were ready for use. A fused silica capillary was then glued to each of the chamber entrance reservoir ports to allow protein solution to be introduced into the chambers using a syringe pump.

4.2.4 Protein Digestions

Solutions containing 1 mg/ml of protein samples (cytochrome C, myoglobin, β-casein, and BSA) were prepared separately in 20 mM ammonium bicarbonate buffer at pH 7.0 and introduced into the micro-reactor chambers using a syringe pump (Harvard Apparatus, model 55-2222). For each protein sample, the solution was flowed through the micro-reactors at flow rates ranging from 2 to 10 µl/min. The residence time taken by the fluid to travel inside the micro-reactor at a given volumetric flow rate was experimentally determined by observing under a bright field microscopy. At 2 µl/min, the solution residence time inside the micro-reactor was estimated to be 3.8 s while at 10 µl/min, the residence time was 13.8 s. The solution at the exit reservoir was sampled by pipetting 0.5 µl aliquots in a timely fashion using a syringe and deposited onto a MALDI sample target as shown in Figure 4.1. The MALDI matrix (α-cyano-4-hydroxycinamic acid, α-CHCA) was
then added to the sample deposit followed by MALDI TOF MS analysis. Peptide masses obtained were used to identify the proteins using MS-digest database searches.

**Figure 4.1** Solid-phase micro-reactor chambers consisting of fluidic channels with free standing micro-post patterned on the floor of the channel bed. The chamber measured 35 mm x 230 µm x 100 µm and had an array of 30 µm x 30 µm posts with 20 µm interspacing. On one end was a fused capillary fit to introduce sample into the chamber and on the other end was an open reservoir for sampling aliquots for deposition onto a MALDI sample target.

**Figure 4.2** A three step protocol for immobilizing trypsin onto PMMA microstructures; (i) UV exposure on native PMMA microstructures to introduce surface carboxylic groups; (ii) activation of surface carboxylic groups with NHS/EDC; (iii) covalent coupling of amine groups of trypsin onto the microstructures.
4.3 Results and Discussion

Surface immobilization and test of activity. To evaluate the ability of trypsin when immobilized onto PMMA microstructures to maintain its catalytic activity, we first tested the immobilization protocol using a thin polymer sheet and exposed the immobilized trypsin to the model proteins. A sheet of PMMA was activated with UV light at 254 nm for 15 min, rinsed with copious amounts of distilled water and thereafter air dried. One µl of a buffer solution containing 0.1 M MES, 0.5 M NaCl at pH 6.0, 5 mM sulfo-NHS (N-hydroxysuccinimide) and 5 mM 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) was deposited on the polymer sheet and left to stand for 15 min. It was then replaced with another 1 µl of a 100 mM phosphate buffer solution at pH 7.0 containing 100 µg of trypsin then covered to prevent evaporation and left to stand on the polymer sheet for about 2-3 h. A schematic representation of the trypsin immobilization procedure is shown in Figure 4.2. First, the methacrylate moieties on the native PMMA surface were converted to carboxylic functional groups by exposure to UV light. The carboxylic groups were then activated by sulfo-NHS (N-hydroxysuccinimide) and 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC), which served as the coupling reagent. The primary amine groups present in trypsin were then coupled directly to the carboxylic groups using EDC. After the coupling reaction was completed, the polymer sheet was thoroughly rinsed with distilled water and dried under a stream of air.

After a matrix was deposed on the polymer sheet, the sheet was attached onto a standard MALDI target using double-sided adhesive tape and the deposit was analyzed with MALDI TOF MS. A mass spectrum acquired directly from the polymer sheet showed the expected trypsin peak at ~ m/z 24,000 (see Figure 4.3). As deduced from this experiment,
trypsin was successfully covalently immobilized onto the PMMA surface as shown in the mass spectrum.

![Trypsin Immobilization Mass Spectra](image)

**Figure 4.3** Mass spectra acquired on a PMMA surface a) before trypsin immobilization b) after trypsin immobilization.

The activity of the immobilized trypsin was then evaluated by first depositing 1 µl of a solution containing 1mg/ml of β-casein onto the trypsin immobilized polymer sheet. The digestion was stopped after ~3 s by addition of trifluoroacetic acid followed by immediate addition of a MALDI matrix. In the flow through format, a solution containing 1mg/ml of β-casein was introduced into the micro-reactor at a flow rate of 10 µl/min. The mass spectrum in Figure 4.4a shows a ~3 s tryptic digestion of β-casein acquired on the polymer sheet and Figure 4.4b shows a mass spectrum of a β-casein digested in the flow through micro-reactor format. As can be seen in both cases, peptide fragments were generated and the mass of the peaks observed agreed with the expected fragments from a tryptic digest of β-casein, an
indication that the immobilized trypsin retained its enzymatic activity. However, digestion in on the polymer sheet showed larger fragments an indication of incomplete digestion while the digestion in the flow through reactor showed most of the fragments within the expected m/z region for a complete digestion typical of β-casein.

![Mass spectra data acquired from: (a) static digestion of β-casein on a polymer sheet immobilized with trypsin; (B) flow through digestion of β-casein in a micro-reactor with immobilized trypsin.](image)

**Figure 4.4** Mass spectra data acquired from: (a) static digestion of β-casein on a polymer sheet immobilized with trypsin; (B) flow through digestion of β-casein in a micro-reactor with immobilized trypsin.

*Flow through trypsin micro-reactors.* In the micro-reactor format, test proteins were digested by flowing them through the micro-reactor chamber containing the micro-posts with immobilized trypsin at varying flow rates and hence, controlled residence times. The
peptide fragments generated from the reactor were collected as fractions at the exit reservoir and analyzed with a MALDI TOF MS by depositing the effluent of the micro-reactor onto a MALDI target plate. The peptide fragments generated were identified using a MS-digest database search and were used to compute the sequence coverage of the proteins. The sequence coverage was calculated from the ratio of the number of identified amino acids to the total number of amino acids present in the protein and reported as percent sequence coverage. High sequence coverage indicated improved digestion and protein identification efficiencies. The geometry of the proteolytic chamber was first investigated and digestion efficiencies were compared. Three chambers with different geometries were tested: (i) a 35 mm long $\times$ 230 µm wide $\times$ 100 µm deep chamber that had an array of 30 µm $\times$ 30 µm micro-posts with 20 µm interspacing; (ii) a chamber with same lateral dimensions as the micro-reactor described in (i) above but without the micro-post architecture (open channel format); and (iii) a 35 mm long $\times$ 50 µm wide $\times$ 100 µm deep open channel format chamber without micro-posts. Figure 4.5 shows a comparison of sequence coverage obtained from these three chambers of different geometries as a function of flow rate.

As seen in Figure 4.5, there was 60 to 100% enhancement in sequence coverage using the chamber with the micro-post architecture as compared to the open channel architecture of similar dimensions for the model protein, cytochrome c. Mass transport is directly proportional to the diffusion coefficients and inversely proportional to the distance the protein travels to encounter the immobilized enzyme. In the posted channel the mass transport was increased due to the shortened distance that the protein in solution traveled to encounter the immobilized trypsin. This resulted to more frequent encounters between the solution-phase protein and the surface-immobilized enzyme. Further, the microstructures
inside the reactor chamber provide increased surface area, which resulted in a higher trypsin load compared to the open channel. The factors resulted in higher digestion efficiency and better sequence coverage in the micro-posted chamber. The digestion efficiency is measured by the substrate modulus, which is a ratio of reaction velocity to transport velocity. This modulus is inversely proportional to rate of mass transport and approaches zero at high transport velocity when mass transport is larger than rate of reaction. A modulus of zero indicates the highest digestion efficiency since the reaction is limited by the enzyme kinetics.

Figure 4.5 Evaluation of % sequence coverage for the test protein, cytochrome c, as a function of flow rate in µl/min for different micro-reactor geometries. ▲ represents open chamber geometry of dimensions 35 mm x 230 µm x 100 µm without microposts, ♦ represents posted micro-reactor chamber of geometry similar to the open channel reactor and ♦ represents open chamber geometry with 50 µm channel width but same length and depth as the other chambers described above.
An open-channel reactor with a 50 µm width (~5 times reduction in channel width compared to the micro-reactors discussed above) but the same length and depth was also tested for digestion efficiency. The results from this geometry indicated a 20% reduction in sequence coverage compared to the large geometry micro-reactor with micro-posts, but a 40 to 60% enhancement compared to the large geometry without micro-posts. These results are consistent with lower mass transport and trypsin load in the case of the large geometry without micro-posts and also indicate that the micro-posted chamber provided higher mass transport and surface area for loading trypsin with unrestricted enzyme access to the protein in solution.

The effects of flow rates were investigated by flowing protein solutions through the reactor chamber at varying flow rates. Figure 4.6 shows a plot of flow rate and residence time as a function of sequence coverage for the model protein, myoglobin. As indicated in this plot, short residence times afforded higher sequence coverage and therefore, improved protein identification efficiency compared to longer residence times. The high surface area-to-volume ratio architecture of the reactor chamber was used since it minimized the diffusion paths and allowed increased substrate-enzyme encounters. At high flow rates, peptide fragments generated inside the chamber were rapidly flowed out to the exit reservoir spending less time in the chamber. The adsorption of peptide fragments onto the microstructures or fluidic wall was minimized at high flow rates. Indeed according to stokes law, the hydrodynamic shear forces (F) experienced by the fragments was directly proportional to the fluid stream linear velocity (v), \( F \propto v \).

At high flow rates, the strength of the hydrodynamic shear forces were greater than the strength of adsorption forces. Addressing the reactor chamber in a flow-through format at high flow rates allowed
operation such that the substrate residence time, $t_r$, was greater than the initial substrate-
trypsin complex formation rate, $k_1$, and turnover rates, $k_{cat}$, which typically occur in the
millisecond time scale ($k_{cat} + k_1 \ll t_r$). However, the rate of mass transport was higher than
turnover rate in the reactor chamber and therefore the digestion efficiency was controlled by
the enzyme kinetics.

![Figure 4.6 A plot of percent sequence coverage for a model protein myoglobin as a function of flow rate in µl/min and residence time in sec. At high flow rates (short residence time) the sequence coverage was high compared to low flow rates.](image)

At lower flow rates, the sequence coverage was reduced due to increased peptide
adsorption onto the microstructures. The flow through format alleviated the digestion
inefficiency typical of solution phase protocols that result from chemical modifications,\textsuperscript{4, 7, 9, 10, 14, 15} or possible inhibition of trypsin activity by the generated peptide fragments.\textsuperscript{7, 8, 11-13}
Such factors in addition to fragments adhering onto microstructures could account for the reduced sequence coverage that was observed at lower flow rates or longer residence times in the reactor chamber.

Two types of PMMA material were investigated for these studies, gray PMMA and clear PMMA. Structurally, the two types of PMMA materials are the same however, they exhibit different physical properties. Gray PMMA has a higher wettability (contact angle of $27^0 \pm 2^0$) and thus is more hydrophilic compared to clear PMMA (contact angle of $73^0 \pm 3^0$). Further, the optical clarity of the two materials is different, gray PMMA absorbs more UV light than clear PMMA. Figure 4.7 shows the effects of the substrate on sequence coverage as a function of flow rate.

![Graph showing sequence coverage as a function of flow rate.](image)

**Figure 4.7** A plot of percent sequence coverage for the model protein myoglobin as a function of flow rate in µl/min for two PMMA materials. ■ represents the more hydrophilic gray PMMA while ● represents the more hydrophobic clear PMMA.
At higher flow rates, the differences in sequence coverage for both materials appeared to be negligible, however at lower flow rates, there appeared to be a more pronounced difference with the gray PMMA showing higher sequence coverage compared to the clear PMMA. A possible explanation for this phenomenon lies in the differences of the material properties. Because gray PMMA absorbed more UV radiation than clear PMMA,\textsuperscript{58} this could result in more carboxylic groups on the surface.\textsuperscript{59} In turn, more enzyme was therefore loaded onto the microstructures for gray PMMA compared to clear PMMA, resulting in differences in enzyme activity per unit area and hence the sequence coverage. At lower flow rates, the enzyme activity per unit area was controlled by the rate of mass transport while at higher flow rates it was controlled by the enzyme kinetics.

Although the contact angle slight decreases upon exposure to UV light, rinsing the surface removes the monolayer of photochemically modified low molecular weight polymer, restoring the contact angle close to the original value and introducing an increase in surface roughness.\textsuperscript{59-61} According to Young’s equation, the difference in contact angles of the PMMA materials indicate that gray PMMA had more surface energy than clear PMMA.\textsuperscript{62} Since the wettability of a surface is governed by the surface energy and roughness,\textsuperscript{63, 64} the propensity of generated peptides to adhere to the microstructures was high for the hydrophobic low surface energy clear PMMA. However, at higher flow rates, the substrate effects were negligible because the hydrodynamic shear forces experienced by the generated peptide fragments at high velocities would remove adsorbed peptides within the micro-reactor.

The model proteins were all water soluble however they varied in MW from 12 kDa to 69 kDa and in $pI$ from 5.3 to 9.6 as shown in Table 4.1. At the shortest residence time of
3.8 s, the model proteins demonstrated a molecular weight dependency on the sequence coverage. Smaller proteins, like cytochrome c, with a MW of 12,348 Da showed 100% sequence coverage while for larger proteins like BSA, with a MW of 69,294 Da, lower sequence coverage was observed. This is an indication that the ease of digestion is also dependent on other properties of the protein. For example, the molecular weight, structure and isoelectric point of the protein in question. Structurally, these proteins are also different with cytochrome c having predominantly a secondary structure while BSA has a tertiary structure. Tertiary structures are stabilized by disulphide bonds making cleavage sites inaccessible by proteolytic enzymes and are therefore difficult to digest.

Table 4.1 A list of test proteins with their MW and the computed percent sequence coverage at the same residence time inside the micro-posted chamber.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Molecular weight (Mw, Da)</th>
<th>Isoelectric Point (pI)</th>
<th>Residence Time (sec)</th>
<th>Sequence Coverage (Percent, %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c</td>
<td>12348</td>
<td>9.6</td>
<td>3.85</td>
<td>100</td>
</tr>
<tr>
<td>Myoglobin</td>
<td>16954</td>
<td>7.4</td>
<td>3.85</td>
<td>90</td>
</tr>
<tr>
<td>β-casein</td>
<td>25108</td>
<td>5.3</td>
<td>3.85</td>
<td>60</td>
</tr>
<tr>
<td>BSA</td>
<td>69294</td>
<td>5.8</td>
<td>3.85</td>
<td>50</td>
</tr>
</tbody>
</table>

4.4 Conclusions

Solid-phase flow through digestion of proteins has been demonstrated in a micro-reactor that provided high digestion efficiency and allowed for improved protein identification efficiencies without performing tandem MS on the peptide fragment peaks.
The digestion was accomplished using a high surface area-to-volume ratio chamber with surface immobilized trypsin on microstructures. The chamber afforded fast digestion (4 s) of model proteins with sequence coverage that was found to depend on the molecular weight. For example, with ~ 4 s digestion of cytochrome c, which has a molecular weight of 12,348 Da, the peptide map generated 100 % sequence coverage information. The geometry of the chamber was found to significantly affect the efficiency of digestion. At the same time, the material properties of the chamber were found to affect the sequence information depending on the residence time. Physical properties like MW, pI and structure of the model proteins were also found to affect the efficiency of digestion.

4.5 References


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5.1 An Integrated Sample Processing Microfluidic System for Protein Analysis

As mentioned in Chapter 1, we are developing an integrated sample processing system for protein analysis that can be interfaced to a variety of different biological mass spectrometers (MS), such as MALDI or ESI. The system was comprised of a protein extraction and pre-concentration unit connected to a fluidic network for two-dimensional separations of proteins extracted from the first unit. The two-dimensional protein separation unit was connected to another module consisting of a series of solid phase micro-reactors arrayed parallel to each other for proteolytic cleavage of individual protein bands separated from the two-dimensional unit. The reactors were connected to a downstream separation unit for sorting peptide fragments that are finally introduced into a mass spectrometer for protein identification. A schematic diagram for the integrated sample processing system is shown in Figure 5.1.

**Figure 5.1** A schematic diagram illustrating an integrated sample processing system with four units; unit I performs protein extraction and pre-concentration; unit II is for two-dimensional separation of proteins; unit II does proteolytic digestion of individual protein; and unit IV separates peptide fragments before they are introduced into the MS for protein identification.
The system will be tested by introducing whole cells that were lysed and the protein components extracted and pre-concentrated from cellular matrix using nanopillars decorated with a thermally-responsive polymer, poly (N-isopropylacrylamide) (pNIPAAm). This polymer exhibited reversible wettability and protein adsorption properties that can be exploited to capture and release proteins from solution. Above its liquid critical solution temperature (LCST), the polymer is hydrophobic and below its LCST, the polymer is extremely hydrophilic, therefore acting as an on-off switch to pre-concentrate extracted proteins in the channel. Extracted proteins were then be injected to the two-dimensional separation unit. In this unit, microchip gel electrophoresis and micellar electrokinetic chromatography will be used for protein separation. Two conductivity sensors will be used to monitor separations in this unit. Protein bands eluting from the second dimension were injected into individual solid-phase micro-reactors for digestion. The solid phase micro-reactors will consist of trypsin immobilized onto high surface area micro-posts for rapid digestion. Peptide fragments generated from these micro-reactors will be further separated downstream using microchip electrochromatography or free solution electrophoresis. The fragments detected using MALDI MS could be used for proteins identification using peptide mass fingerprinting.

5.2 Experimental Section

To produce the prerequisite structures, PMMA was hot-embossed from a brass mold master fabricated using high-precision micromilling. The nano-reactors and solid-phase extraction bed contained nanopillar arrays situated within a micro-channel and were fabricated using a nano-templating technique. A patterned anodic alumina oxide (AAO) template supported by aluminum, which was shadow printed via Ar ion sputtering of an Al
target into the appropriate locations within the fluidic network, was ultra-sonicated followed by filling the nanopores with methyl methacrylate (MMA) monomer containing benzoin methyl ether and 1% PMMA powder. The polymerization occurred under UV-254 nm light with the sacrificial AAO template removed using phosphoric acid and a freezing drying technique. Trypsin was surface-immobilized onto the nanopillar structures following UV-254 nm exposure and incubation with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC). For protein capture, the nanopillars were dynamically coated with poly(N-isopropylacrylamide), pNIPPAm. Meso-scale pNIPPAm films have contact angles of 71° above its liquid critical solution temperature (LCST) and 50° below.

5.3 Results and Discussion

To affect isolation of a series of proteins from whole cell lysates, nanopillar arrays (see Figure 5.2A) were dynamically coated with pNIPPAm, which showed super-hydrophobicity at a temperature above its LCST (contact angle ~90°) and super-hydrophilicity below its LCST (contact angle ~0°), which served as a highly efficient solid-phase capture-bed for hydrophobic proteins (see Figure 5.2B).

Figure 5.2 (A) Micrographs of high aspect ratio (>340) nanopillars fabricated by nano-templating using AAO. The pillars were 60 µm in height and 175 nm in diameter with a 285 nm edge-to-edge spacing, (B) Nanopillars that were dynamically coated with pNIPPAm and used to capture avidin that was labeled with a fluorescent dye. The first image shows the material above its LCST, in which the protein is captured and the second micrograph shows the release of avidin by dropping the temperature below the LCST.
Following protein isolation, a 2D SDS µ-CGE x MEKC separation was performed, which produced an efficiency, resolution and peak capacity of $>10^5$ plates, $>4$ and $\sim897$, respectively (see Figure 5.3A). The sorted protein components were then digested using a nanopillar-based solid-phase proteolytic reactor. The proteolytic nano-reactor was tested with several proteins to evaluate its performance, which indicated high protein identification efficiencies due to fast (<4 s digestion times) and efficient (>80% sequence coverage) proteolytic digestions (see Figure 5.3B).

![Figure 5.3](image)

**Figure 5.3** (A) 2D SDS µ-CGE x MEKC electrophoretic separation of proteins. The detection was accomplished using laser-induced fluorescence of proteins labeled with Alexa-Fluor 633. (B) Mass spectrum showing the MALDI-TOF-MS analyses of peptides fragments generated from cytochrome C that were subjected to solid-phase proteolytic digestion using trypsin immobilized onto nanopillars.

The generated peptides from the proteolytic digestion could be separated using µ-CEC of the peptide fragments by coating the PMMA surface with a C18 hydrophobic phase following UV-activation of the PMMA surface to create a functional scaffold of carboxylate groups. Since there could be more than one protein in each band from the second dimension that is injected into the nano-reactors, a peptide separation step that simplifies the fragment mixture is necessary in order to improve the protein identification process. The automated
analysis could be completed by interfacing the microfluidic system to MALDI MS via a rotating ball interface. Peptide mass maps obtained from MALDI MS could be used to identify the proteins present in the sample.

5.4 Conclusions

We are evaluating the design and performance of the integrated polymer-based microfluidic system consisting of mixed-scale structures for analyzing proteins in an automated fashion. The system can be multiplexed for high-throughput analyses and has the potential for accelerating biomarker discovery and performing diagnostics.

5.5 References


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VITA

Harrison Kyallo Musyimi was born in Machakos, Kenya, on December 8, 1976, to Georgina and Bernard Musyimi Nthuli. He attended primary school at Machakos and Kasunguni Primary Schools and thereafter was admitted to a public high school at Makueni Boys High School. After graduating from high school in 1994, he attended a computer and electronics college at Kenya Christian Industrial Training Institute in Nairobi, Kenya, for a year, where he was graduated with a diploma in both courses. Harrison was later admitted to Jomo Kenyatta University of Agriculture and Technology (JKUAT) in Nairobi, Kenya, for undergraduate studies in March 1996. He graduated with a Bachelor of Science degree in chemistry (First Class Honors) in March 2000. Immediately following his graduation, Harrison started a master’s program in chemistry at JKUAT and had just finished his coursework before he was accepted to Louisiana State University to pursue his doctoral studies in August 2001. He joined the Department of Chemistry in the analytical chemistry division under the supervision of Professor Steven A. Soper and Professor Kermit K. Murray. His dissertation research focused on integration of micro-scale separations to matrix assisted laser desorption and ionization mass spectrometry for protein analysis. He is currently a doctoral candidate in analytical chemistry, which will be awarded at the December 2006 Commencement.