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Polymer Lab-on-a-Chip with Functional Nano/Micro Bead-Packed Column for Biochemical Analysis

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ABSTRACT

The objective of this research is to develop new functional nano/micro bead-packed columns on polymer lab-on-a-chips (PLOC) using self-assembly microfabrication technologies for practical on-site biochemical analyses or point-of-care clinical diagnostics.

In order to achieve the goal of this research, new polymer microfabrication methods such as (a) a multi-chip assembly method for polymer chips using pin-hole pair structure and (b) a self-assembly and packing method for nano/micro beads on polymer chips have been newly developed and characterized. The novel microfabrication methods have been applied for the realization of practical polymer lab chips such as (a) sample preparation for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and (b) on-chip capillary electrochromatography with electrochemical detection for on-site clinical analysis.

The state-of-art microfabrication techniques have been demonstrated by development and characterization of high quality disposable polymer capillary electrophoresis (CE) microchips.

To realize polymer lab-on-a-chips in a multilayer format, a new assembly technique using pin-hole pair structure has been developed for the fabrication of multilayered polymer chips. This newly developed technique directly addresses the alignment problem that has been considered as one of the most difficult tasks in the bonding assembly of multiple polymer layers for the multi-layered polymer lab chips.

Functional on-chip bead-packed columns using a slurry packing method and a self-assembly method have been developed and characterized in this work. An on-chip reversed phase chromatography (RPC) column packed with RPC media (SOURCE 15RPC) has been
realized using the slurry packing method, where the geometrical restrictions with precise alignments, which are essential for the packing process, were achieved using the pin-hole pair structure assisted assembly technique.

The RPC column integrated with the sample preparation chip for MALDI-MS has been designed, fabricated, and fully characterized. The integrated column has shown the holding capacity of 48.8 ng of peptide and was applied for the sample preparation for MALDI-MS analysis. 5 µg/ml of Neurotensin sample was filtered using the sample preparation chip and the results analyzed through MALDI-MS were comparable to the results from the commercially available ZipTip products. Small volume of sample (less than 1 µl) and parallel processing capability in the sample preparation are benefits derived from the newly developed sample preparation chip.

A high quality on-chip capillary electrochromatography (CEC) column has been developed using the self-assembly method of silica colloidal beads in microchannels with a depth of 50 µm and a length of 2 cm. Nano/micro beads (0.8 µm ~ 1.98 µm in diameter) were successfully self-assembled in the microchannels (50 µm ~ 100 µm in width), resulting in a hexagonal crystalline structure.

For a point-of-care system for urine sampling and analysis, the self-assembled columns have been developed over the entire channels for both sample injection and separation. This allows the sample to be introduced into the sample injection channel using capillary forces, which eliminates the difficulties associated with the traditional sample introduction techniques such as an external vacuum or applied pressure. In addition, an electrochemical (EC) sensor placed at the end of the separation channel has been implemented and characterized as a CEC
device with EC detection, and uric acid and ascorbic acid have been successfully separated and
detected using the developed chip.

Reproducible results have been achieved and the average migration times from serial
injection and separation were $6 \pm 0.3$ s ($n = 5$) and $10.8 \pm 0.3$ s ($n = 5$) for ascorbic acid and uric
acid, respectively. Linear calibration plots between 0.1 mM to 1 mM concentration for uric acid
($R^2 = 0.991$) and ascorbic acid ($R^2 = 0.998$) have been achieved, which covers the clinical range
of sample concentration from 10-fold dilution of urine samples.

In this research, a new alignment and bonding method for multi-layered polymer chips
and an on-chip nano/micro bead self-assembly method for functional columns have been
successfully developed and fully characterized. These promising microfabrication methods have
been applied for various polymer lab-on-a-chips for biochemical analysis or clinical diagnostics,
which may enable the practical on-site or point-of-care testings using on-chip capillary
electrochromatography.
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<tr>
<td>LOC</td>
<td>Lab-On-A-Chip</td>
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<tr>
<td>POCT</td>
<td>Point-Of-Care Testing</td>
</tr>
<tr>
<td>μTAS</td>
<td>Micro-Total Analysis Systems</td>
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<tr>
<td>CE</td>
<td>Capillary Electrophoresis</td>
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<tr>
<td>CEC</td>
<td>Capillary Electrochromatography</td>
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<tr>
<td>MALDI-MS</td>
<td>Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry</td>
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<td>RPC</td>
<td>Reverse Phased Chromatography</td>
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<td>EC</td>
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<tr>
<td>SEM</td>
<td>Scanning Electron Microscope</td>
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<td>ACN</td>
<td>Acetonitrile</td>
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<td>DI</td>
<td>Deionized</td>
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<tr>
<td>IPA</td>
<td>Isopropyl Alcohol</td>
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<td>PBS</td>
<td>Phosphate Buffered Saline</td>
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<tr>
<td>COC</td>
<td>Cyclic Olefin Copolymer</td>
</tr>
<tr>
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<td>Polycarbonate</td>
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<tr>
<td>PDMS</td>
<td>Poly (dimethylsiloxane)</td>
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<tr>
<td>PEEK</td>
<td>Poly (aryl ether ether ketone)</td>
</tr>
<tr>
<td>PMMA</td>
<td>Poly (methyl methacrylate)</td>
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<tr>
<td>PS</td>
<td>Polystyrene</td>
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<tr>
<td>Si</td>
<td>Silicon</td>
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<tr>
<td>$T_g$</td>
<td>Glass transition temperature of thermoplastic material</td>
</tr>
<tr>
<td>$T_m$</td>
<td>Melting temperature of thermoplastic material</td>
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<tr>
<td>$O_2$</td>
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<tr>
<td>RIE</td>
<td>Reactive Ion Etching</td>
</tr>
<tr>
<td>sccm</td>
<td>Standard cubic centimeters per minute</td>
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<tr>
<td>Ca</td>
<td>Capillary number</td>
</tr>
<tr>
<td>Pa</td>
<td>Pascals</td>
</tr>
<tr>
<td>Ra</td>
<td>Average surface roughness</td>
</tr>
<tr>
<td>Re</td>
<td>Reynolds number</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>LIF</td>
<td>Laser Induced Fluorescence</td>
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CHAPTER 1

INTRODUCTION

1.1. Motivation

The objective of this research is to develop functional nano/micro bead-packed columns on polymer lab-on-a-chips (PLOC) using a self-alignment technology for multilayer polymer chips and a self-assembly method of nano/micro beads for practical on-site biochemical analyses or point-of-care clinical diagnostics. Recently, lab-on-a-chips have emerged as one of the most promising platforms for analyzing biochemical molecules due to its advantages such as low sample volume, fast analysis, high-throughput, low-cost, disposability, and portability [1, 2].

With the introduction and development of new microfabrication technologies for plastics in the 1990s, polymer microchannels with dimensions down to the submicron range were realized using techniques such as hot embossing or imprinting, injection molding, laser ablation, soft lithography, X-ray photolithography, or electron beam lithography [3, 4]. Among them, the injection molding technique has been considered as one of the most manufacturable techniques for polymer microfabrication, allowing mass-production of polymer lab-on-a-chips in low cost. However, though the injection molding technique can produce polymer microstructures in low cost, many extra considerations are required for the assembly and bonding of the replicated polymer wafers when a device contains microchannels and microstructures to be aligned precisely at the wafer level. Furthermore, the complexity of microfluidic systems in lab-on-a-
chips has been rapidly increased as a variety of functions have been integrated into a single microfluidic device or system [5]. Thus, multilayer polymer lab-on-a-chips are desirable, but the assembly of the multiple layers with a high precision of alignment has been considered as one of the most difficult tasks. This alignment issue hinders the development of polymer microchips for practical applications, so a new assembly technique, which is compatible with existing fabrication processes to build complex 3-dimensional polymer chips, is required to build multilayer polymer lab-on-a-chips. In this research work, advanced alignment and assembly techniques for the multilayer polymer chips will be explored and developed to address the fabrication difficulty associated with the mass production of polymer lab-on-a-chips.

While microfabrication technologies and fluid handling techniques in microfluidic devices for biochemical analysis is essential, the integration of functional components to perform complex biochemical analysis is another important trend. Specifically, the integration of on-chip columns with lab chips for sample purification, preconcentration, and separation, has particular significance. Since nano/micro beads provide more active surfaces for biochemical reaction than flat surfaces, the bead-based immunoassays, reactor beds, and chromatography devices have been of great interest to researchers [6, 7]. However, the difficulty of packing portions of a complex microfluidic manifold with beads has hindered the utilization of these ideal reagent delivery vehicles within microfluidic devices. The development of an innovative method of trapping beads on-chip and packing the trapping zones should significantly expand the range of the microfluidic toolbox and extend the practical applications of such devices. Two bead packing techniques, slurry packing and self-assembly packing, are adopted and further explored as fabrication tools to realize the functional on-chip columns. The combination of the new
microchip assembly technique and the functional on-chip column integration can provide new functional lab-on-a-chips for matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and microchip capillary electrochromatography (CEC).

MALDI-MS is one of the most sensitive analytical techniques for the characterization of the primary structure of peptides and proteins. Although it is assumed that MALDI has more tolerance to the presence of contaminants when compared with other ionization techniques such as electrospray ionization (ESI), the incorporation of analytes into growing matrix crystals can be seriously disrupted by the contaminants. When contaminants are present, a bad sample spot is produced that usually results in reduced spectral quality, which is reflected by a lower S/N ratio, resolution and sensitivity. However, in many cases, peptides and proteins can usually be obtained from buffers containing high concentrations of salts, chaotropes or detergents to prevent their precipitation, thus increasing the solubility or extraction yield of peptides from gel matrices after enzymatic or chemical digestion. For this reason, sample preparation techniques for the purification of biological samples prior to sample application on a MALDI target is desirable. An on-chip reverse phase column that can be used for filtering the peptide sample will be integrated in a sample preparation device for MALDI-MS analysis, and the weir structures for slurry packing of beads will be provided by the new assembly technique.

Although capillary electrophoresis (CE) offers high separation efficiency, due to short separation channels, there is a major need for an additional separation mechanism that would manipulate selectivity in the chip-based separation systems. Such selectivity manipulation can be achieved by microchip capillary electrochromatography (CEC). Also, though the conventional CE chip has an excellent analysis capability with extremely low detection limits,
the difficult sampling procedure and long analysis time hinders its practical applications to a point-of-care analysis. The CEC microchip using the electrochemical detection can address the issues arisen from the CE chip, where the sampling can be enhanced by the capillary force through the self-assembled beads in the channels, and the detection can easily be performed using electrochemical methods without needing complex optical systems [8, 9].

In summary, polymer microchips with functional nano/micro bead-packed columns for biochemical analysis fabricated by new microchip assembly techniques will be proposed, implemented, and characterized in this thesis. Actual fabrication and its characterization will show the proposed fabrication techniques can be useful tools to build complex 3D microfluidic network, and functional nano/micro bead packed columns can be integrated and used for various purposes. Finally, experimental results that will be achieved from fabricated devices using developed fabrication techniques will prove that it is feasible to develop practical lab-on-a-chip devices for numerous applications.

1.2. Review of Previous Work

1.2.1. 3D microfluidic networks

The necessity of three-dimensional (3D) structures in a topologically complex microchannel network has been suggested by Whitesides et al. [10] and by Beebe et al. [5], as shown in Figure 1.1. As those two research groups noted, topologically, a 3D fluidic circuit could be constructed by using at least two levels of microchannels, i.e., using at least three layers of plates.
Integration of functional components into a complex microfluidic system requires a new fabrication method for 3D channel geometries. Methods such as stereolithography [11, 12], laser-chemical 3D writing [13], and modular assembly [14] are available for fabricating 3-D features with hard polymer materials, but these processes are expensive and complicated for both prototyping and mass production.

However, the fabrication of 3D channels in PDMS has been considered as one of the inexpensive, simple, and versatile methods for making complex geometries. Neils et al made a combinatorial mixing device with four different flow levels which was constructed by stacking...
nine laser-cut Mylar laminates [15]. Although they demonstrated many devices with three-dimensional channel networks, all materials adopted were limited to PDMS or soft polymer materials. Consequently, these methods provide excellent prototypes, but they are not appropriate for high-throughput fabrication.

Previously, a 3D microfluidic network realized in 3-layer cyclic olefin copolymer (COC) device for combinatorial chemistry was reported by Ahn’s research group, as shown in Figure 1.2 [16]. However, the reproducible results for combinatorial chemistry were unable to be achieved due to poor alignment between layers and chip-to-chip alignment reproducibility.

Figure 1.2 Three-dimensional microfluidic network in 3-layer COC microchip, adapted from [16].
1.2.2. Microchannel packing methods

1.2.2.1. Slurry packing

Previously, beads were packed in capillary electrochromatography (CEC) columns using a frit at the end of the capillary, which was accomplished by heating the stationary phase within the column. The optimization of the heating process to produce the frit requires a great deal of expertise in order to obtain reproducible frits. Unfortunately, fabricating a frit on a microfluidic chip is nearly impossible due to the difficulty in heating the chip appropriately to form a frit. However, the geometry of the chip can be designed to constrain beads within a portion of the microfluidic channel using a series of pillars that are a microfabricated frit as demonstrated by Anderson et al. [17]. Similarly, the height of the microchannel column can be restricted from 100 µm to 10 µm forming a “dam” (Figure 1.3 (a)) as demonstrated by Sato et al. [18 - 20] and Harrison et al. used multi-step glass etching to build two weir structures [21 - 23]. Another approach was introduced by Ceriotti et al., who use a tapered channel in order to trap beads within a microfluidic channel [24]. As shown in Figure 1.3 (b), the tapered channel allowed small beads (3 µm) to be packed into the channel by keystone effect [25], which was then used for the CEC.
1.2.2.2. Self-assembly packing

Traditional packing methods of microspheres usually produce chip-to-chip variations of the packing properties, even though the microspheres are packed using microfabrication techniques. However, since self-assembly techniques for colloidal microspheres provide a crystalline structure with excellent structural reproducibility, there has been a great interest in the self-assembly of colloidal beads (Figure 1.4) for biochemical sensors, catalysts and membranes due to the ease of manufacture as monodispersed samples and the resulting periodic porous structures [21, 26]. Several applications of the partially packed microchannel on a polymer substrate to a capillary electrophoresis (CE) chip and an on-chip blood serum separator using
self-assembly bead packing method have been reported from the BioMEMS group at the University of Cincinnati [27, 28].

1.2.3. Sample preparation for MALDI-MS

MALDI mass spectrometry is one of the most powerful analytical techniques for the characterization of the primary structure of peptides and proteins. The preparation of MALDI samples seems very simple, but it is one of the most critical steps in the overall process for MALDI-MS analysis. In many cases, peptides and proteins can only be obtained from the buffers containing high concentrations of salts, chaotropes or detergents to prevent their precipitation, increasing their solubility or the extraction yield of peptides from gel matrices after enzymatic or chemical digestion. For this reason, sample preparation techniques have been
developed and used for the purification of biological samples prior to sample application on MALDI target.

ZipTip has been the most widely used tool for these sample purification steps [29] and one of the recent advanced chip approaches is CD-type sample preparation device that uses centrifugal force and microfluidic patterns, as shown in Figure 1.5 [30 - 32]. However, these tools require bulky and expensive systems which include robotic arms or a stage for centrifugal spinning of CD type device.

![Figure 1.5 Widely used tools for sample purification: (a) ZipTip and (b) CD type Gyros sample preparation device for peptide cleaning, adapted from [32].](image)

1.2.4. Electrochemical detection for CE

There are several types of detection modes that have been employed to monitor separation in lab-on-a-chip devices. Laser-induced fluorescence (LIF) has been the most popular
mode for microchip CE due to its inherent high sensitivity [33, 34]. However, most compounds are not naturally fluorescent and must be derivatized with a fluorophore to be detected by LIF. Mass spectrometry (MS) has also been employed as a detection mode for miniaturized devices [35, 36]. However, commercially available MS systems are expensive, not inherently portable, and less sensitive than LIF.

Electrochemical (EC) detection is ideally suited to the miniaturized analytical systems and is an attractive alternative mode for microchip CE devices [37 - 41]. Examples in the literature include devices fabricated in poly(methyl methacrylate) (PMMA) and glass, as shown in Figure 1.6. The sensitivity and selectivity of EC detection are comparable to those of LIF detection. There are several advantages of EC detection over other detection modes, including the ability to miniaturize both the detector and control instrumentation and the fact that many compounds can be detected without derivatization. Microelectrodes are fabricated using common photolithographic techniques that are already used to construct microchips. This allows the electrodes to be fabricated directly onto the microchip device, producing a fully integrated system. The electrodes can be miniaturized without loss of sensitivity. This is unlike absorbance detection, which is dependent on Beer’s law. In addition, although microelectrodes generate extremely small currents, the background current is reduced even further, resulting in an increased signal-to-noise ratio and potentially better limits of detection (LODs) [42, 43]. There are three general modes of EC detection: amperometry; conductimetry; and potentiometry and these are well reviewed in Lunte et al and coworker’s review paper [8].
1.3. Research Objectives

The objective of this research is to develop and characterize new functional nano/micro bead-packed columns and to integrate them with polymer lab-on-a-chips for the applications of a sample preparation chip for MALDI MS and a polymer CEC chip with electrochemical detection system. Functional on-chip nano/micro bead-packed columns on polymer lab-on-a-chips, which are fabricated using high throughput self-assembly or slurry-packing techniques, will be developed and fully characterized first. In addition, state-of-the-art self-alignment and bonding techniques using polymer injection-molded pin-hole pair structures will be developed and characterized for the microfabrication of the polymer lab-on-a-chips. Functional nano/micro bead-packed columns will be integrated with the polymer lab-on-a-chips for biochemical analysis systems with the functions of sample preparation and separation. Research emphases are placed on: (1) the development of new self-alignment and bonding techniques using polymer
injection-molded pin-hole pair structures for the polymer lab-on-a-chips; (2) the development of functional nano/micro bead packed columns on polymer chips using self-assembly and slurry packing techniques; (3) the development of a sample preparation chip for MALDI MS analysis; and (4) the development of a polymer CEC device with fully packed columns for on-site or point-of-care clinical analysis.

1.4. Overview of this Thesis

This thesis addresses the challenging issues discussed in the previous introduction section. In this work, Chapter 2 presents the concept of a self-alignment and bonding technique for the development of multilayer polymer microchips. An innovative self-alignment and bonding technique is fully developed and characterized through the demonstration of a microfluidic network which includes a 3-D micromixer and microchip for biochemical analysis.

Chapter 3 focuses on the development and characterization of nano/micro bead-packed columns using slurry packing or self-assembly methods on polymer lab-on-a-chips.

In Chapter 4, a sample preparation chip for MALDI-MS using RPC column is presented. The device is designed, fabricated, and characterized, and the results are compared with the ZipTip, which is a commercially available product.

In Chapter 5, microchip capillary electrochromatography with electrochemical detection for on-site clinical analysis is designed, fabricated, and fully characterized for the point-of-care testing of uric acids.
This thesis concludes with a discussion of the utility and limitations of the described microchip fabrication techniques and fabricated devices in Chapter 6. The potential future work is also suggested.
CHAPTER 2

High Quality Polymer Microchip Fabrication and New Assembly and Bonding Method for Multilayer Polymer Microchip with Alignment Precision

2.1. Introduction

The development of polymer microstructures for the fabrication of disposable microfluidic devices and lab-on-a-chips is becoming increasingly popular as a low cost and high volume production alternative to silicon and glass based microstructures. Micro injection molding technology is ideally suited for the mass fabrication of these devices because of its rapid replication capability at low cost. The micro injection molding technique is combined with a replaceable mold disk technique for rapid fabrication of the replaceable micromold inserts [46].

In this technique, the replaceable mold disk, which contains the high aspect ratio microstructures, is fabricated on a separate circular nickel disk about 3 in diameter and 1.6 mm thick and is inserted into the molding block, as shown in Figure 2.1. This reduces many complexities of mold fabrication such as fabrication cost, turn around time, micromachining, etc. The mold disk can be removed from the molding block and subsequently replaced with other mold disks with different patterns without replacing the entire molding block. The injection molding can be performed using the existing injection molding system, so this replaceable disk
molding technique can be considered as one of the economically viable techniques. The process steps for the fabrication of the micro mold insert are: (1) surface preparation of nickel substrate, where the nickel substrate is lapped flat and parallel and polished using a lapping machine; (2) photolithography using SU-8 photoresist; (3) electroplating of nickel, and (4) photoresist stripping. An additional polishing step can be completed for the reduction of the channel surface roughness.

However, although the injection molding technique can produce polymer microstructures in low cost, many extra efforts are required for the assembly and bonding of the replicated polymer wafers when a device contains microchannels and microstructures to be aligned precisely at the wafer level. Furthermore, the complexity of microfluidic systems in lab-on-a-chips has been
rapidly increased as a variety of functions have been integrated into a single microfluidic device or system [5]. Thus, multilayer polymer lab-on-a-chips are desirable, but the assembly of the multiple layers with a high precision of alignment has been considered as one of the most difficult tasks. New techniques compatible with the existing fabrication processes to build highly complex 3D interconnected microstructures are demanded to build multilayer microfluidic devices.

2.1.1. Alignment Effect in Microfluidic System

A study on the microfluidic behavior in a multilayer microfluidic system was reported in [48], where the probable misalignments in a typical u-bend microfluidic system (Figure 2.2) were modeled and analyzed using CoventorWare version 2003.1. Layer-to-layer misalignments were intentionally added and the corresponding changes in the microfluidic variables were observed.

![Figure 2.2 A u-bend microfluidic structure: a channel with an inlet length of 750 μm and spacing between inlet and outlet of 10 mm, adapted from [48.](image)]
A misalignment of 20 μm was introduced in the flow path of channel diameters of 50 μm and 250 μm as an artifact of tolerance in the assembly process. The same scale of misalignment was introduced to both channels with small diameter (50 μm) and large diameter (250 μm) because tolerances do not scale down or up with changes in the channel dimensions. Table 2.1 presents the effects of misalignment on the various parameters. It can be observed that the degree of change in the various parameters, induced by a misalignment of 20 μm, is greater in the channel with a small diameter while it is smaller in the channel with a large diameter.

Table 2.1 The effect of a 20 μm misalignment on channels with different diameters, adapted from [48].

<table>
<thead>
<tr>
<th>Variables</th>
<th>Dia. 50 (aligned)</th>
<th>Dia. 50 (20 μm misaligned)</th>
<th>% change</th>
<th>Dia. 250 (aligned)</th>
<th>Dia. 250 (20 μm misaligned)</th>
<th>% change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pressure (MPa)</td>
<td>2.077 × 10⁻²</td>
<td>2.24 × 10⁻²</td>
<td>7.847</td>
<td>3.731 × 10⁻⁵</td>
<td>3.736 × 10⁻⁵</td>
<td>0.13</td>
</tr>
<tr>
<td>Velocity (μm/s)</td>
<td>3.2 × 10⁵</td>
<td>4.3 × 10⁵</td>
<td>34.375</td>
<td>1.3 × 10⁴</td>
<td>1.6 × 10⁴</td>
<td>23</td>
</tr>
<tr>
<td>Flow rate (μm²/s)</td>
<td>3.25 × 10⁸</td>
<td>3.565 × 10⁸</td>
<td>9.69</td>
<td>3.676 × 10⁸</td>
<td>3.676 × 10⁸</td>
<td>0</td>
</tr>
</tbody>
</table>

The results confirm that at smaller diameters, micro-fluidic channels need to be aligned precisely and the tolerances of the alignment should be minimized. Thus, new microchip assembly techniques providing precise alignment during fabrication while maintaining high-throughput are highly demanded.
2.2. Design and Fabrication

2.2.1. Double Side Injection Molding

Normally, to build a microfluidic network, channels are patterned on a substrate and then covered with another plain substrate using various bonding techniques: anodic bonding for Si to glass, elastomer bonding for PDMS to glass or PDMS to PDMS, and polymer fusion bonding for thermoplastics. 3D microfluidic networks can be achieved by stacking multiple substrates containing microfluidic patterns, and as the number of substrates increases the complexity of the devices can also be increased.

The double side injection molding technique is beneficial because it can reduce the number of microchannel patterned substrates needed to build a complex 3D microfluidic network by means of patterning on both sides of the substrate. Two replaceable mold disks, which have microfluidic structures designed to be aligned to each other, can be fabricated and used for the double side injection molding process. Figure 2.3 shows the conceptual illustration of this double side process. Once the well-aligned molds have been prepared, these molds are positioned in a mold block while maintaining the alignment accuracy. The injection molding process for the double side technique is essentially the same as the single side injection molding case. However, the nature of the polymer material sticking to the patterned structures on both of the molds should be considered, since ejection of the replicated polymer wafers from the mold cavity is critical for the automatic injection molding process. If the replicated polymer adheres to the stationary mold block instead of the movable molding block which has ejection pins on it, then the injection molding process cannot be performed automatically. For this reason, the
relatively more complex mold, which has higher probability of adhering to the replicated polymer, should be positioned on the movable mold block.

2.2.2. Pin-hole pair structures Assisted Assembly for Multilayer Polymer Microchip

Several approaches to realize the 3D multilayered polymer structures have been reported [5, 10, 49]. In [10], a 3D microchannel in PDMS elastomer was fabricated, where microfluidic structures and alignment keys were patterned in PDMS layers and manually aligned under a low-power stereo microscope. From the finished device, an excellent alignment accuracy of ~15 μm was achieved, but the tedious and time consuming manual alignment process makes it difficult to apply this method for mass production. Furthermore, as the number of stacking substrates increases, it becomes more difficult to achieve the required alignment precision using this alignment technique.
Figure 2.4 displays a schematic illustration of the pin-hole pair structures assisted assembly technique. Well-matched and aligned pin-hole pairs positioned in each layer are mechanically assembled and then thermally bonded. In this manner, we can expand this technique to the multilayered devices, such as the multilayered polymer lab-on-a-chip as shown.
schematically in Figure 2.5. Furthermore, any replica-based polymer microfabrication processes can adopt this new self-assembly technique, demonstrating its suitability as a generic self-assembly technique for most polymer lab-on-a-chips. Figure 2.6 is a summary of the fabrication processes for mold fabrication, polymer replication, mechanical self-assembly, and thermal fusion bonding.

![Figure 2.5 Schematic diagram for the assembly of multilayer chips using pin-hole pair structure assisted assembly technique.](image)

![Figure 2.6 Summary of the fabrication processes to produce pin-hole pairs and their use to assist the assembly of multilayer polymer microfluidic chips.](image)
The shape, number, and size of the pin-hole pair structure should be considered in terms of its height, depth, and diameter. These parameters are related to the methods to pattern the pin or hole structures on the nickel mold and the assembly process of the replicated polymer chips. The pin structure, which is an extruded pattern, can be attained by photolithography and subsequent electroplating step, thus it could have wide range of shapes. However, for the hole structure, further restrictions are imposed due to the limitation from its machining process. The simplest structure that can be patterned on the nickel mold is a round-shaped concave structure since the patterning is achieved using round-shaped drill bits. The male and female structures should be designed to fit with each other so that the structures can produce a pin-hole pair.

The size of the pin-hole structures can be reduced by the selection of the smaller drill bits, but it still has to provide enough mechanical strength to hold two substrates together. With these considerations, the pin-hole pair with 1 mm diameter was chosen. A minimum of two pin-hole pair structures is required, while the addition of more pin-hole pairs may improve the accuracy of the alignment during assembly and even increase the mechanical strength of bonding.

The stability of the alignment will be improved during assembly when the pin structure is taller and the hole structure is deeper. However, the pin structure in the replicated polymer substrate should be shorter than the depth of the hole structure in the other substrate, thus the drilled hole on mold disk should be shorter than the electroplated pin structure on the other mold disk.
2.3. Fundamental Applications

2.3.1. Polymer Capillary Electrophoresis Microchip

2.3.1.1. Design and Fabrication

High quality polymer microchip fabrication techniques are essential for the development of polymer lab-on-a-chips. Using the self-alignment method with the pin-hole pairs and the high quality polymer microfabrication techniques developed in this research, a single lane polymer capillary electrophoresis (CE) microchip has been designed, fabricated and fully characterized as a demonstration of the high quality polymer lab-on-a-chip as well as a separation column.

The CE chips have been fabricated with injection molded poly (methyl methacrylate) (PMMA) and characterized in terms of physical characteristics and analytical performance. The chip performance in terms of different analytical separation parameters and run-to-run and chip-to-chip reproducibility should be evaluated first. Specifically, the reproducibility of the migration time, peak area, peak height, resolution and number of theoretical plates are of importance in peak identification. The fluctuations in analytical parameters are usually associated with the fluctuations in electroosmotic flow (EOF). EOF in CE can be affected by many different parameters including the running buffer composition and pH, the applied voltage, the temperature, and the physical property or chemical nature of the channel surface. Small changes from one of these parameters can result in a large shift in solute migration times.

The schematic layout of the proposed polymer CE chip is shown in Figure 2.7. The distances from the reservoirs to the center of the intersection are 4 mm for SR, BR, SW and 39
mm for BW (separation channel), respectively. The channel width is 100 µm and the depth is 80 µm.

A 3 inch diameter and 1.6 mm thick nickel disk was prepared as the mold insert for micro-injection molding process. The nickel disk was cleaned and then dried in 120 °C oven for 2 hours before the spin coating of SU-8 negative photoresist. An anti-reflection film (OmniCoat) was spin-coated over the nickel disk at 3,000 rpm for 30 seconds and baked on the hot plate at 200 °C for 1 minute in order to improve the adhesion of the thick SU-8 layer on the nickel surface. Then, SU-8 2075 was spin-coated on the baked thin OmniCoat layer at 950 rpm for 45 seconds. This was followed by a soft baking process at 65 °C for 10 min and at 95 °C for 1 hour and 45 min. The soft-baked SU-8 layer on the nickel substrate was then exposed to UV light with a total dose of 250 mJ/cm². After the UV exposure, a post exposure baking process was carried out at 65 °C for 10 min and at 95 °C for 10 min. Then, both SU-8 and OmniCoat layers
were developed by immersing the nickel substrate into the SU-8 developer and the OmniCoat
developer. Next, nickel electroplating process was performed in a nickel electroplating bath at a
current density of 10 mA/cm² for 8 hours to achieve 100 µm of plating height.

The polymer chips were replicated by polymer injection molding. Injection molding
(BOY 22M-A, BOY machines, INC.) was performed with the nickel mold inserted into the
custom-designed molding block. After the injection molding process, a cleaning step (rinsed
with isopropanol (IPA) and distilled/deionized water) was performed to remove the residue or
particles left on the replicated polymer substrate. Then, O₂ plasma surface treatment in RIE was
performed over the polymer substrate by applying O₂ plasma for 2 minutes with 20 sccm of O₂
gas flow, and 30 kHz, 100 W of power (Technics Miro-RIE Series 85). The polymer substrate
with microchannels was bonded with a cover polymer substrate by a thermoplastic fusion
bonding technique using embossing machine (MTP-10, Tetrahedron Associates Inc., San Diego,
CA). The conditions used for the bonding process are 88 °C with 0.4 ton of pressure for 4 chips.
After the completion of the bonding process, reservoirs for loading analytes and applying
operating voltages were made by drilling holes at the ends of the channels. Figure 2.8 is a
picture of the fabricated device. To increase the volume of the reservoirs, pipette tips or glass
tubes of 1/4 inch I.D. were cut into 7 mm pieces and then bonded on the fabricated chips using
epoxy.
Physical Characterization

Knowing the physical characteristics of plastic CE chips is important for proper device function. Several important physical properties of the injection molded PMMA chips were evaluated and compared, which include visual evaluation of fabrication quality (Environmental Scanning Electron Microscope (ESEM) images), surface quality measurements, optical constants and autofluorescence [50], certain thermal/electrical properties (measurement of the current versus applied voltage (I-V) characteristics to determine a voltage range at which electrical failure occurred), and the influence of channel surface treatments.

The chips were inspected using an ESEM, (ESEM XL30, FEI Company, Hillsboro, Oregon). Prior to image recording, the chip surfaces were cleaned and a 10 nm of gold layer was deposited using a Cold Sputter Denton Vacuum (Cherry Hill, NJ, USA). Surface roughness is an important factor in CE because any channel imperfections can cause erratic current or non-uniform sample flow thus working as a source of noise and resulting in poor detection signals.
On the other hand, the surface roughness of the original nickel disk mold was measured using a KLA-Tencor P-10 surface profilometer (KLA-Tencor Corporation, San Jose, CA) with a 10 µm/s scan rate and a 50 Hz sampling rate. The maximum height of the nickel pattern was 80 µm. The measured average roughness \( Ra \) for the nickel disk was 91.63 nm, 161.91 nm for the electroplated pattern and 27.19 nm for the polished electroplated pattern. As indicated in Figure 2.9 and Table 2.2, the surface roughness was greatly improved by the polishing process.

![Figure 2.9 Scanning profilometer images of the microchannel patterns on the nickel molds: (a) before and (b) after lapping and polishing process.](image)

**Table 2.2 Scanning profilometry results showing average roughness (Ra) of nickel disk surface, electroplated pattern, and polished electroplated pattern.**

<table>
<thead>
<tr>
<th></th>
<th>( Ra ) (Average roughness)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ni disk surface</td>
<td>91.63 nm</td>
</tr>
<tr>
<td>Electroplated pattern</td>
<td>161.91 nm</td>
</tr>
<tr>
<td>Polished electroplated pattern</td>
<td>27.19 nm</td>
</tr>
</tbody>
</table>
Good replication has been achieved for the injection molded PMMA chips. ESEM analysis showed that there were no defects in the junction and the channels. Figure 2.10 shows a well-defined cross intersection and channel shape. The well-defined cross intersection is important to control the amount of the injected sample plug during sample loading.

![ESEM pictures of the replicated patterns from the injection molded PMMA CE chips: (a) cross-channel and (b) cut-view.](image)

Figure 2.10 ESEM pictures of the replicated patterns from the injection molded PMMA CE chips: (a) cross-channel and (b) cut-view.

According to Ohm's law, the I-V curve should follow a linear relationship when the resistance is constant. After filling the channels with a running buffer, the stepwise voltage increase of 0.2 kV was applied between the SR and SW reservoirs and between the BR and BW reservoirs. Current readings were taken after an equilibration time interval of 15 seconds for cooling down after each voltage change. Since the channels have uniform cross-section and the buffer filling each channels is the same, the channel resistances should be proportional to the channel lengths. The I-V response showed a linear correlation coefficient of 0.999 up to 3 kV with the voltage applied across the separation channel between reservoirs BR and BW, which indicated no joule-heating effects during the measurement. This voltage corresponds to 0.7
kV/cm within the separation channel. This means that for the 3.9 cm separation distance used in these studies the maximum voltage applicable between the points of injection and detection is 2.7 kV. When the voltage was applied across the injection channel between reservoirs SR and SW, a linear dependence was obtained up to 4 kV.

2.3.1.2. Experimental Result

Reagents and Solutions

PMMA (GE Polymerland) chips were fabricated using injection molding. Polyethylene oxide, PEO was obtained from Sigma-Aldrich and Tris-borate-EDTA (TBE) was obtained from Bio-Rad Laboratories, Hercules, CA. 3% PEO in 10x TBE buffer (890 mM Tris/890 mM borate/20 mM EDTA electrophoresis grade, pH 8.4) was used as stock solution for running buffer, and as electroosmotic flow modifier. The running buffer was made in 0.1% TBE/0.3% PEO buffer prior to use. Running buffer and distilled, deionized (DI) water were degassed prior to experiments. The solutions were then filtered through a 0.45 μm syringe filter (Millipore Corp., Bedford, MA). Fluorescein sodium salt (FL) and Fluorescein isothiocyanate isomer I 90 % (FITC) were obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Fluorescein and FITC stock solutions (1 mM each) were prepared by dissolving appropriate amounts of each in methanol. Mixtures of 25 μM FL and 50 μM FITC were prepared by diluting a 1 mM stock solution into running buffer.

Experimental Setup

All experiments were performed on the system previously described in detail [50]. Briefly, this system consisted of a Nikon TE 2000 epifluorescence microscope equipped with a H6780-20 PMT module (Hamamatsu) and a CoolSnap HQ CCD camera (Roper Scientific). The CCD camera was interfaced with a personal computer through a PCI card and data were
acquired using MetaMorph (Universal Imaging Corporation) software. A Lambda LS xenon arc lamp (Sutter Instrument Company) was used as the light source. High voltage was applied to the microchip through platinum electrodes using an HVS 488 model 3000 (Lab Smith) power supply. Current from PMT was amplified with a low current preamplifier SR570 (Stanford Research) and acquired with 16-bit PCI P6036 DAQ card (National Instruments) at 60 Hz rate. High voltage and signal acquisition was controlled by codes in LabVIEW (National Instruments). The experimental data was exported for further analysis in PeakFit v4.12 (SeaSolve Software Inc.).

**Electrophoresis Procedure (Analytical Performance)**

Analytical performance characteristics of single lane plastic CE separations were evaluated using a simple model system separation: mixtures of dyes fluorescein (FL) and fluorescein isothiocyanate (FITC). The electroosmotic modifier, PEO, was present in the buffer to allow reverse-polarity electrophoresis. It dynamically coats channel surfaces so that the viscosity was high, and surface charge was low enough that EOF would be suppressed in all microchips. If one chip was to be used for many experiments, the chip surface needed to be restored after every run. The conditioning was achieved by flushing with DI water for 5 minutes, then running buffer for 10 minutes.

The fabricated CE chips were tested for capillary electrophoresis separation reproducibility. The running buffer was added to reservoirs labeled SR, BR, and SW (Figure 2.7). The chip was conditioned by applying a vacuum to the BW reservoir before adding a sample to SR. Platinum electrodes were placed in the corresponding reservoirs. A reversed
polarity and electrophoresis scheme was used in all chips which is similar to the common pinched injection scheme [51].

Sample introduction was accomplished by applying 0.25 kV to reservoir BR and 0.65 kV to reservoir SW, while keeping SR and BW at 0 V for 25 s. Sample separation was performed by applying 0.75 kV to SR and SW, 2.5 kV to BW, and 0 V to BR for 45 s. The effective separation field strength was 530 V/cm. Analyte detection was done at 30 mm from the site of injection for all chips. The volume of the running buffer and sample was 70 µl, which is enough to prevent major changes in pH during experiments.

To improve reproducibility, two approaches were used. First, an optimized reversed polarity voltage scheme, which is similar to the common pinched injection, was used in all chips to prevent significant leakage. Another approach was to use 1-2 µl of buffer less in SW than the rest of the reservoirs, because during injection the level of liquid in SR is slightly decreasing and the level of liquid in reservoir SW is slightly increasing. Therefore, the reduced amount of liquid in reservoir SW would partially compensate for the siphoning problem and peak height instability.

Reservoir Material

It was reported that the hydrophilic or hydrophobic properties of the reservoirs had influence on the analytical performance of PDMS microchips [52]. Hydrophilic reservoirs on PDMS chips give more stable performance than hydrophobic reservoirs. So, in order to verify the effect of hydrophilic or hydrophobic properties of the reservoirs on the PMMA chips, the same separation was performed on the injection molded PMMA chips with different reservoir
tips: plastic pipette tips and glass tips. Chips with glass tips exhibited less peak anomalies in peak shape and peak height. The relative standard deviation (RSD) of peak height for the chip with plastic tips within one run was 33 %, as opposed to 3.9 % for the chip with glass reservoirs. This result assures that the use of glass tips is very desirable for achieving the reproducible CE separations. Thus, glass tips were selected and used as the reservoirs for further studies.

Reproducibility Studies

A blind reproducibility study was designed to evaluate the performance of the injection molded PMMA chips with well-defined variations in the chip fabrication procedure. The study also explored the ability to regenerate these chips to determine if they can be reused multiple times. The evaluation included multiple injections for each combination of fabrication processes to examine the analytical characteristics of the chips.

Four different groups of chips were investigated for the evaluation of chip reproducibility. Group 1: O₂ plasma treatment and the holes positioned on the cover substrate; Group 2: O₂ plasma and the holes on the patterned substrate; Group 3: no O₂ plasma and holes on the cover substrate; and Group 4: no O₂ plasma and holes on the patterned substrate. Some concerns were arisen from the idea that if holes were drilled in the patterned portion, debris or deformation could possibly block the channel entrances. For Group 1 and 3, precise alignment was necessary to match the holes in front of the channel.

In order to investigate injection-to-injection reproducibility of the injection molded PMMA chips, sets of 6 repeated injections and separations of FL and FITC were performed. A set of 6 injections and separations was defined as a run. The run-to-run reproducibility of 6 runs,
each with 6 injections was evaluated. All chips were reconditioned after each run. The chip-to-chip reproducibility was also investigated. For each chip, 6 runs were performed. Between each run, the chips were rinsed with DI water and stored in a dry condition. The discussion in this chapter focuses on the migration time of FL and FITC and their ratio, but the same evaluations can be performed for the other parameters.

Figure 2.11 presents a representative electropherogram of the separation of 25 μM FITC and 50 μM FL obtained on a PMMA CE chip when 508.8 V/cm was applied across the separation channel. The insert shows one of these separations.

Figure 2.11 Typical separation of FL and FITC on injection molded PMMA CE chip using glass tips as reservoirs [53].
Figure 2.12 and Table 2.3 show the run stability of FL migration time for all groups of chips. It can be seen that Group 1 and Group 2 (first 8 chips) are more stable than Groups 3 and 4, which indicates that RIE O₂ treatment contributed further to the reproducible migration times. The first group of chips had RSD 1.2 % and 1.1 % for the average migration time of FL. Figure 2.12 also represents a general observation of all other measured parameters.

![Figure 2.12 A graph shows stability (run-to-run reproducibility) of FL migration time for all groups of chips; group 1 and 2 are treated with O₂ plasma and group 3 and 4 are not; group 1 and 3 have holes on the cover wafer and group 2 and 4 have on the patterned wafer.](image-url)
Table 2.3 Stability of average migration time of FL and FITC for all chips.

<table>
<thead>
<tr>
<th>Group 1</th>
<th>% RSD (FL)</th>
<th>% RSD (FITC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(O₂ plasma treatment/ holes on cover substrate)</td>
<td>1.2</td>
<td>1.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 2</th>
<th>% RSD (FL)</th>
<th>% RSD (FITC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(O₂ plasma treatment / holes on patterned substrate)</td>
<td>1.8</td>
<td>1.9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 3</th>
<th>% RSD (FL)</th>
<th>% RSD (FITC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(No O₂ plasma treatment / holes on cover substrate)</td>
<td>12.8</td>
<td>13.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Group 4</th>
<th>% RSD (FL)</th>
<th>% RSD (FITC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(No O₂ plasma treatment / holes on patterned substrate)</td>
<td>11.8</td>
<td>9.2</td>
</tr>
</tbody>
</table>

RSDs of the migration times, peak area, peak height and resolution of FL and FITC as well as their migration time ratio, peak area ratio, peak height ratio for run-to-run studies of individual chips and for groups of chips fabricated differently, are summarized in Table 2.4. The higher values of RSD for the migration times can be attributed to the fact that variations in the position of the detection window after each run is significant compared to the total travel distance, so slight changes from the exact point of detection could become very critical. High values for RSD for peak height and peak area could be attributed to the errors introduced by injection [51].
The results clearly showed that Group 1 with O$_2$ plasma treatment and drilled holes on cover substrate was recommendable among the evaluation sets for the most reproducible CE separation results.

### 2.3.2. 3D micromixer

#### 2.3.2.1. Design and Fabrication

A 3D micromixer was designed and fabricated to characterize the pin-hole pair structures assisted assembly technique. Splitting and recombination (also called lamination) of flow generated in the 3D micromixer will remarkably enhance the mixing efficiency by increasing the interfacial area exponentially over the wide range of Reynolds number ($Re$) [54-56].
Figure 2.13 shows a schematic drawing of the 3D micromixer design. The 3D micromixer is composed of overlapping of F-shape mixing units in two layers, and the overall microchannel path of the arranged mixing units forms a 3D serpentine path. The successive arrangement of F-shape mixing units generates continuous splitting and recombination, and results in the exponential interfacial area growth as shown in Figure 2.13(b). The indicated positions in Figure 2.13(b) correspond to cross-sections in Figure 2.13(a).

Figure 2.13 The 3D micromixer: (a) schematic diagram of the 3D micromixer, and (b) the conceptual cross-sectional mixing behaviors of the ideal lamination at each indicated position.
The 3D micromixer can be easily realized by bonding of two layers containing upper and lower F-shape mixing units and the pin-hole pair structures were patterned along the microfluidic structures to align these two layers. Figure 2.14 shows microscanned images (KLA-Tencor, P10 Profilometer) of the pin and hole structures on nickel master molds.

Figure 2.14 Microscanned images of microfabricated pin-hole pairs: (a) pin, and (b) hole structures from nickel molds.

Figure 2.15(a) is a microphotograph of a pin-hole pair assembled and bonded by fusion bonding. At the same time, a micromixer was formed by connection of F-shape patterns on each layer. To assess the alignment accuracy of the technique, microphotographs of aligned patterns from each wafer of the fabricated device and 10 μm size reference pattern from a Cr mask were taken with the same magnification. The achieved alignment error was evaluated by comparison of the images and calculated using the numbers of pixels counted by conventional graphic software. The results show an alignment error less than ~10 μm has been achieved when it is assumed that the deformation during thermoplastic fusion bonding is negligible. Figure 2.15(b) indicates the precise alignment of two F-shape mixer structures.
2.3.2.2. Experimental Result

In order to demonstrate the utility of this assembly technique for creating complex 3D microfluidic devices, the fabricated micromixer was tested by flowing two fluids such as NaOH and phenolphthalein though the channels driven by syringe pumps. The mixing performance can be evaluated by the color change of phenolphthalein after mixing from colorless to red if pH becomes greater than 8. Only the completely mixed stream turns red, thereby enabling us to measure the mixing performance.

Figure 2.16 shows the experimental setup and a mixing result of the 3D micromixer fabricated using the proposed self-assembly method. The mixing was performed under condition of $Re \approx 4.39$ when a flow rate was maintained as $Q = 50 \mu l/min$. An initial interface between two streams was observed in the vicinity of T-junction entrance followed by a successful mixing while the fluidics is running through the mixer structures. The successful mixing result achieved in this multilayered micromixer confirmed the excellent alignment and utility of the pin-hole pair.
structures assisted assembly technique for complex 3D microfluidics devices. Further detailed numerical mixing simulation and experimental results were reported in [57].

Figure 2.16 Experimental setup and mixing result: (a) experimental setup for mixing performance characterization; enlarged view of the 3D micromixer equipped in the experimental setup. Dark red portion indicates the interface (mixed area) of phenolphthalein and NaOH streams, and (b) experimental result for 3D micromixer using NaOH and phenolphthalein at $Re \approx 4.39$ with a flow rate of $Q = 50 \mu l/min$ [57].
2.3.3. Multilayer Biochip for Blood Analysis

2.3.3.1. Design and Fabrication

The alignment and assembly technique using the pin-hole pair structures also has been applied to fabricate a multilayer lab-on-a-chip for blood analysis. This device is integrated with various functional components such as a metallic needle for sampling, microfluidic channels, solid propellants, and pinch valves for liquid handling, biosensor arrays, and an on-chip pouch holding calibration solution. All the components should be integrated in a lab-on-a-chip which consists of 5 layers of microchannel patterned cyclic olefin copolymer (COC) wafers. The layer-to-layer alignment is critical since the complicated 3D microfluidic system in the biochip could malfunction when there is a significant misalignment [48]. The layer-to-layer error might be accumulated as the number of the layer increases, so the pin-hole pair structures assisted assembly technique is utilized for the multilayer biochip fabrication. Also double side injection molding technique is beneficial to reduce the number of the layers by patterning both sides of the substrate, which also allows more freedom in designing a complex 3D microfluidic system that incorporates other functional components.

Figure 2.17(a) shows a schematic drawing of the device assembly, where three layers (layers 2 – 4) have been achieved from double side injection molding and top and bottom layers are from single side injection molding with replaceable mold disks. Figure 2.17(b) shows a design of the biochip.

The biochip includes a passive microfluidic manipulation system and pinch-valve method, allowing for a programmed set of microfluidic sequencing with on-chip pressure source (AIBN).
The biochip also contains an integrated biosensor array for the simultaneous detection of multiple clinically relevant parameters. Thus, the disposable smart plastic biochip is composed of fully integrated modules with microfluidic control for liquid driving and sequencing, and biochemical sensing. The biochip is inserted into an analyzer unit where the microfluidic sequencing is performed by the control signals from the electronic controller. Utilizing on-chip metallic microneedle, the liquid sample (e.g., blood), is delivered into the microfluidic system and eventually to the biosensor array, and the electrochemical detection is performed to determine the concentrations of the various analytes. As a demonstration vehicle, the biochip has been tested for the detection and identification of several metabolic parameters (e.g., glucose and lactate) from human blood.

Figure 2.17 Multilayer biochip for blood analysis: (a) schematic drawing of the 5-layer biochip assembly, and (b) schematic drawing of the chip design.
2.3.3.2. Experimental Result

For the evaluation of the assembly process, layers 1 and 2 have been assembled and the achieved alignment accuracy is shown in Figure 2.18(a). Each layer which has different depth of microchannels for metallic needle and pinch-valve installation has been achieved from both double side injection molding and single side injection molding. Precise alignment is critical for the appropriate microfluidic control and minimizing dead volume. Assembly of layers 1 through 4 was done by heat and pressure applied fusion bonding and layer 5 has been assembled onto the previously assembled part (layer 1 to 4) using UV adhesive bonding to avoid damaging biosensors integrated in the chip. The pin-hole pair structures provide enough mechanical strength for both fusion bonding and UV adhesive bonding processes. As discussed earlier, a minimum of two pin-hole pairs is required for assembly with precise alignment while the addition of more pin-hole pairs did not significantly influence the alignment result. Finally, the alignment and assembly technique using the pin-hole pair structures has been successfully achieved and utilized for the fully functional 5-layer biochip fabrication and Figure 2.18(b) is the picture of the fabricated biochip.
The actual testing of the developed biochip with blood samples was conducted at The Ohio State University Medical Center, Columbus. Fig. 2.19 shows the test results of the biosensor array with whole blood for glucose and lactate measurement. The results clearly indicate that the sensor array has a very linear response in the normal detection range for both glucose and lactate. The normal physiological limits (daily average) for glucose are 90–120
mg/dl. The integrated glucose sensors can detect the glucose concentrations in the range of 50–
250 mg/dl and the lactate sensor has been successfully tested for 2–12 mg/dl. These results
clearly prove the utility of the developed disposable biochip for clinical diagnostic applications.
Further detailed design, fabrication, and experimental results of the biochip were reported in [58].

Figure 2.19 Measurement results from actual human blood samples: from (a) Glucose and (b)
Lactate sensors [58].
2.4. Conclusion

In this work, the high quality disposable polymer CE chips have been fabricated and characterized for their excellent reproducible CE separation results. Chip-to-chip, run-to-run, and injection-to-injection reproducibility in terms of migration time, peak area, and peak height has been evaluated using the injection molded PMMA CE chips fabricated with O₂ plasma treatment and holes patterned on the cover substrates. Also a new high precision self-assembly technique for multilayer polymer lab-on-a-chips has been successfully developed and characterized by realizing a 3D micromixer and 5-layer biochip for blood analysis with an alignment margin close to ~10 μm for the multilayered polymer lab-on-a-chips. This technique will alleviate the difficulty of assembly of multilayered polymer lab-on-a-chips, providing a mechanical self-assembly technique for the mass production of polymer multilayer chips at low cost. Furthermore, this self-assembly technique can be immediately adopted for most replica-based polymer lab-on-a-chips or other polymer devices which require a precise assembly of multiple layers.
CHAPTER 3

Functional Nano/Micro Bead-Packed Columns on Polymer Lab Chip

3.1. Introduction

While the development of innovative polymer microfabrication technologies for polymer lab-on-a-chips is essential for the realization of point-of-care biochemical analysis, the development of new functional components to perform complex biochemical analysis is also very desirable. Specifically, on-chip functional columns required for sample purification, preconcentration, and separation, have particular significance.

Since nano/micro beads provide more active surfaces for biochemical reaction than flat surfaces, the bead-based immunoassays, reactor beds, and chromatography devices have been of great interest to researchers [6]. Packed-bed chromatography has the benefit of providing low mobile-phase mass transfer resistance, and a wide variety of stationary phases are available [23]. However, the difficulty of packing portions of a complex microfluidic manifold with beads has hindered the utilization of these ideal reagent delivery vehicles within microfluidic devices. The development of an innovative method for trapping beads on-chip and packing the trapping zones should significantly expand the range of the microfluidic toolbox and extend the practical applications of such devices.
3.1.1. Colloidal Microspheres

Colloidal microspheres have long been used as a major component for industrial products such as inks, coatings, papers, cosmetics, photographic films, and rheological fluids [59 – 61]. Colloidal microspheres have been used in photonics for optical communication, optical chips, and optical computers [62-64] as well. In addition, since organized colloidal structures have unique properties, they have been widely used for a variety of diverse applications such as chemical sensors [65].

Colloidal microspheres used in engineering applications are often made of silica [66] or polymers [67, 68] such as poly(methyl methacrylate) (PMMA) or polystyrene (PS). These colloidal microspheres have been manufactured as equally-sized or monodisperse samples in large quantities, and some of the polymer beads can be made to be porous so as to provide more hydrophobic surface condition. The diameter of colloidal microspheres can be adjusted from a nanometer to a micrometer by choosing appropriate synthesis conditions such as variation of the concentration of the additives, process temperature, and pH.

Reverse-phase beads have been extensively used for the chromatography of proteins, peptides, and tryptic digests as well as for other applications of solid phase extraction (SPE) and capillary electrochromatography (CEC) [6, 7]. The surface of the silica colloids is often terminated with silanol group (-Si-OH), which can be ionized to generate a negatively charged interface [69]. Also, the hydrophilic characteristics of the silica beads can be beneficial since it provides strong capillary force and electroosmotic flow (EOF) when packed through channels.
Some distinct applications of silica colloidal microspheres have also been reported, including DNA or RNA purification [70, 71] and immunoassay [72 - 74].

3.1.2. Slurry Packing Method

Slurry packing, which is one of the traditional bead-packing methods, has been used to pack microspheres in a microchip. Slurry packing is a bead packing method by means of designed geometrical restriction that constrains beads within a portion of the microfluidic channel. These geometrical restrictions can be provided by a microfabricated frit [17], a “dam” structure due to height difference [18 - 23], or keystone effect from tapered channel [24, 25].

Previously slurry packing using a “dam” structure made in glass with sequential etching for different heights was demonstrated and beads could be packed into the chamber in a relatively easy procedure [18 - 23]. However, it is considered as a difficult task to built multi-step structure on a single polymer substrate.

3.1.3. Self-Assembly Crystallization of Silica Colloids

Traditional packing methods of microspheres usually produce chip-to-chip variations in packing properties, even though the microspheres are packed using microfabrication techniques. Self-assembly of colloidal microspheres has generated great interest for potential applications to biochemical sensors, catalysts and membranes due to ease of manufacture from monodispersed samples and the resulting periodic porous structures [22].

*Colloidal Self-Assembly Crystallization*
Self-assembly is the concept broadly used over a whole range of fields including physics, chemistry, biology, and engineering. The mechanism of the self-assembly process is driven mainly by competing molecular dynamics among hydrophobic, hydrophilic, gravitational, van der Walls, or Columbic interactions [75]. Self-assembly can be defined as a process in which specific local multiple interactions occur over constrained geometries. During the self-assembly process, components autonomously assemble into the finally desired structure through an exploration of alternative configurations.

Common methods used for self-assembly of colloidal microspheres are based on gravitational [76, 77], electrostatic [78 - 81], magnetic [82, 83], and capillary forces [84 - 86] or physical confinement [87, 88]. Silica colloidal microspheres are typically involved in the sedimentation method using a gravitational field due to their high density. Although the sedimentation method appears simple, it actually involves various couplings of several complex factors such as gravitational settling, translational diffusion, and crystallization. Moreover, the process of sedimentation takes relatively long periods of time (weeks to months) for successful fabrication in good quality [76, 77]. The major disadvantage of the sedimentation method is that it is difficult to control the thickness of the crystalline structure required for some applications such as assembly of colloidal microspheres in a microchannel [89]. The vertical deposition technique using capillary forces shown in Figure 3.1 can be an alternative method for the fabrication of the colloidal microspheres in a microchannel [85, 90 - 92]. For the 2D assembly of colloidal microspheres, self-assembly crystallization is initiated by attractive capillary immersion forces that are mediated in the solvent meniscus between microspheres at the drying front. These capillary forces induce colloidal aggregation. Solvent evaporation from the already
ordered arrays causes a convective particle flux towards the drying microsphere layer from the bulk of the colloidal suspension. The balance between capillary forces and convective particle flux during the solvent evaporation is essential for the monotonic formation of 2D self-assembled structure.

Figure 3.1 Schematic illustration of the mechanism of colloidal self-assembly in the vertical deposition technique: (a) two-dimensional (2D); and (b) three-dimensional (3D) self-assembly structure, adapted from [93].

In the case of 3D self-assembled structure, the process of convective transfer of particles from the bulk of the suspension to the thin wetting film prevails. Colloids, driven to the drying zone of colloidal film, are accumulated towards the crystallized front by the solvent influx that percolates through the cavities of the arrays, thus compensating for the evaporative loss of the solvent. Evaporating solvent from the colloidal drying film forms capillary bridges between
microspheres that exert strong attractive capillary forces that can be responsible for the crack formation in the dried colloidal films.

3.2. Design and Fabrication

Slurry packing

To achieve slurry packing, geometrical restriction is essential to hold beads inside the packing region. To achieve the “dam” structure or weir structure, multi-step etching on glass substrate was used to achieve the desired structure [21-23], but this process is complicated in thermoplastic microchips due to the difficulty of getting double step structure in the mold. Meanwhile, the weir structure can be achieved when two substrates that have different depth of channels for each are assembled. The only concern of this idea is an accurate alignment between two substrates. However, the pin-hole pair structures assisted assembly technique covered in the previous chapter can be a solution to solve the alignment problem.

Figure 3.2 is a schematic illustration explaining the slurry packing steps. Two injection molded COC substrates with channel depths of 60 µm and 15 µm, respectively, are aligned and bonded using the self-assembly technique to form two-step microchannels for slurry packing. Pin-hole structures are patterned in each substrate to assist and ensure the assembly with the desired alignment precision. The final assembly has a pair of weir structures for the slurry packing of 15 µm diameter of beads. The bead slurry was injected at 10 µl/min of flow rate controlled by syringe pump and dried out after finishing the filling process.
Figure 3.2 Schematic illustrations explaining the chip assembly for the slurry packing technique. 15 µm diameter beads are trapped inside the column because of the geometrical restriction which is multi-step channels (15 µm and 60 µm depth) for each top and bottom layer: (a) Top and bottom layer before assembly; (b) assembled structure for slurry packing; and (c) final bead packed column after slurry packing.
**Self-assembly packing**

The microchannels were partially packed with silica colloidal particles by self-assembly packing process which was reported earlier [27, 28]. Figure 3.3 displays the schematic diagram showing the self-assembly bead packing process for simple microchannel. The patterned COC substrate is pretreated with O$_2$ plasma to produce hydrophilicity on the surface of microchannel (March CS 1701 Reactive Ion Etching system, March Plasma Systems, Inc.).

![Figure 3.3 Dipping process for self-assembly of colloidal suspension in a microchannel for fully packed CEC device. Silica beads fill the hydrophilic treated plastic microchannels by capillary force, and 3D periodic array of uniform colloidal silica suspensions are formed while water is evaporated.](image-url)

Figure 3.3 Dipping process for self-assembly of colloidal suspension in a microchannel for fully packed CEC device. Silica beads fill the hydrophilic treated plastic microchannels by capillary force, and 3D periodic array of uniform colloidal silica suspensions are formed while water is evaporated.
The colloidal silica solution is heated and stirred to prevent slow precipitation of the aggregated silica particles. The room temperature and humidity should be controlled during the self-assembly bead packing process to ensure repeatable results. The end of the channel was dipped cautiously in the suspension by holding with a jig. Pretreated open microchannels have enough hydrophilicity to drive the silica colloidal suspension to the end of each channel by capillary action. Once the colloidal solution reached the end of the channel, spontaneous three-dimensional packing of the silica particles started from the end of the microchannels due to the slow evaporation of water. The self-assembly packing process continued toward the end of empty microchannel at the bottom. The packing speed was controlled by the opening of the beaker and the packing process was stopped when the channels were fully packed. The packed chip should be washed very gently and cautiously wiped using the clean room wipes soaked with deionized (DI) water to remove extra silica particles at dipped area and dried completely at room temperature.

3.3. Experimental Result

**Slurry packing**

This slurry packing process is relatively easy and straightforward after the chip assembly. As the sequential pictures shown in Figure 3.4, the beads start to fill the chamber from the end of the column near the outlet and keep filling until they completely fill the column and slurry inlet. The bead slurry was injected at 10 µl/min flow rate, which was controlled by syringe pump, and dried out after finishing the filling process. 15 µm diameter beads are only packed inside the column due to the physical restriction from the height of channels positioned at the
opposite ends of the column, which are less than 15 µm. The speed of the bead loading/packing process is extremely rapid (less than 2 min).

**Figure 3.4** Captured images from slurry packing method showing assembly in the microchannel.

**Self-assembly packing**

This self-assembly process should be characterized and optimized since the combination of the width of the microchannel, size of bead, and the target length is critical for the quality of the bead assembly.
Simple microchannel patterned COC chips were prepared and tested for the self-assembly of 0.8, 1.98, and 3.56 µm diameter silica beads. Each microchannel has a length of 2 cm and a depth of 50 µm with 5 different widths (50, 100, 200, 300, 400 µm), as shown in Figure 3.5.

The patterned COC substrate is pretreated with O₂ plasma for 2 minutes with 20 sccm of O₂ gas flow and 200 W of power to give hydrophilicity to microchannel surface. The colloidal silica solutions (0.1 wt%) were heated up to 60 °C in a beaker with gentle stirring (80 rpm) to prevent slow precipitation of the aggregated silica particles. The room temperature and humidity were controlled during the self-assembly bead packing process as 68 °F (20 °C) and 46 %.
respectively. The chip was removed from the solution after 3 hours. This experiment was repeated for 3 times for each case and Table 3.1 shows the result.

Table 3.1 Bead packing result: 0.8, 1.98, and 3.56 µm diameter of silica bead packing in 50, 100, 200, 300, and 400 µm width of channels.

<table>
<thead>
<tr>
<th>Bead size (µm)</th>
<th>Width (µm)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>50</td>
</tr>
<tr>
<td>0.8</td>
<td>3/3</td>
</tr>
<tr>
<td>1.98</td>
<td>3/3</td>
</tr>
<tr>
<td>3.56</td>
<td>2/3</td>
</tr>
</tbody>
</table>

As shown in Table 3.1, as the channel width and the bead size increase, it is more difficult to get a good self-assembled structure. This size-effect is demonstrated in Figure 3.6, which shows self-assembly of 0.8 µm silica beads in 50 and 200 µm wide channels. Uniform hexagonal closed packed structure with 25.95 % of porosity [94] was achieved using 50 µm wide, 50 µm deep, 2 cm long channel as shown in Figure 3.6(a).

The capillary suction and the water evaporation play important roles as well. The capillary force ($\Delta P$) can be defined as following,

$$\Delta P = \frac{2\gamma \cos \theta_c}{r},$$

(1)
where r, γ_{LG}, and θ_c are the channel width, the surface tension of the liquid and the contact angle, respectively. It is clear that the wider channel gives the lower capillary force, which weakens the self-assembly process, resulting in voids and dislocations (Figure 3.6 (b)).

Figure 3.6 SEM image of bead packed structure using self-assembly method: (a) Uniform hexagonal closed packing structure (0.8 µm beads in 50 µm channel), and (b) voids and dislocations (0.8 µm beads in 200 µm channel).

### 3.4. Conclusion

In this work, the fabrication tools for nano/micro bead-packed columns for a lab-on-a-chip have been developed and optimized for the applications that will be covered in the following chapters. A well known slurry packing method was employed for the on-chip RPC column in polymer lab-on-a-chip while geometrical restriction, which is essential for slurry packing, was realized by means of pin-hole pair structures assisted self-assembly technique. The designed column could be packed in a relatively easy and rapid manner, and it took less than 2 minutes for the bead packing process to be completed. The self-assembly of various sizes of colloidal silica
beads was characterized and optimized for packing in various widths of microchannels. It was found that smaller beads in narrower channels are desirable for reliable bead self-assembly. Reproducible and uniform self-assembly packing of 0.8 µm diameter silica beads in 50 µm width, 50 µm depth, and 2 cm long microchannel was achieved, whereas fabrication yield decreases when the bead size and the channel width are increased.
CHAPTER 4

Sample Preparation Polymer Lab Chips for MALDI-MS

4.1. Introduction

Proteomics is the study of the expression, function and interaction of proteins in health and disease, and is a steadily growing field of interest for applications such as drug discovery. Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) is widely used for the characterization of the primary structure of peptides and proteins [95, 96]. MALDI-MS employs a soft ionization method which is typically applied to the analysis of solid phase analyte cocrystallized with an energy-absorbing matrix material on the surface of a supporting target plate. The acronym MALDI was introduced by Karas et al. [97] in 1985 when it was observed that the amino acid alanine could be easily ionized with a pulsed 266 nm laser when the sample was mixed with tryptophan, which acted as a matrix component to efficiently absorb the laser energy. To date, a variety of MALDI sample preparation recipes have been reported, among which the most frequently used is the dried-droplet method [96], which allows improved tolerance towards involatile contaminants. Meanwhile, sample purification is considered as one of the critical steps of the sample preparation process for MALDI-MS since the incorporation of analytes into growing matrix crystals can be seriously disrupted by the presence of contaminants such as high concentrations of salts, chaotropes or detergents [98]. These steps often are time-
consuming and require repetitive pipetting of reagents onto MALDI sample targets, leading to sample loss and dilution as well as sample contamination by unwanted impurities. A microfluidic device which can handle multiple samples and reagents in small volume is essential for the sample preparation steps, especially when the protein amount is low. Also the sample loss can be minimized when a reverse phase chromatography (RPC) column is integrated to hold the protein sample during the washing step.

A new RPC column integrated with the sample preparation chip for MALDI-MS described in this thesis is aiming to achieve sample preconcentration and cleaning to provide easier sample preparation steps for MALDI-MS. Pressure driven fluidics are controlled by a syringe pump to regulate fluid flow on the chip. Specifically, for serial or stepwise processing, individual samples or small groups of samples are processed in consecutive steps, yielding completed samples from same processing set over a period of time. With parallel processing all samples are treated as a group, and are equal at each processing step, thus removing time effects and guaranteeing consistent and equal preparation of samples. It should be noted that although MALDI-MS itself is a serial process, its high duty cycle enables reasonably high-throughput for a large number of prepared samples.

4.2. Design and Fabrication

4.2.1. Design

The sample preparation chip for MALDI MS was designed and fabricated according to the specific need of performing all sample preparation steps, including preconcentration and cleaning of protein or peptide samples, after loading of the chip. The column integrated into the
chip holds the protein or peptide samples, and preconcentrated samples are cleaned to remove salts, detergents, and other additives. Then the cleaned samples, which are ready for MALDI-MS, can be eluted and mixed with matrix solution resulting in the crystallization on MALDI target.

Figure 4.1(a) is the schematic drawing of the proposed device. It consists of microfluidic patterns, weir structures as geometrical restrictions for slurry packing, on-chip MALDI target, and pin-hole structures for assembly, which will be explained in further details. Patterned polymer substrates were achieved by injection molding process using cyclic olefin copolymer (COC, Topas 5013) material. COC is suitable as a chip material since the surface condition can be modified to avoid biological molecular absorption and it is chemically resistant to a wide variety of liquids used in sample manipulation [58]. The designated microchannel was packed with SOURCE 15RPC (reverse phase chromatography media, Amersham Biosciences Corp) to create an on-chip column after chip assembly.

Figure 4.1(b) shows the fabricated device which has the on-chip RPC column as well as the microfluidic network for sample and reagent measuring and dispensing. For each step, liquids (L1-L5) are measured and introduced through the designated channels following the sample preparation protocol (Table 4.1). The designed volume for each channel is 1 µl with the exception of L4 (washing solution, 5 µl).
4.2.2. Microchip Assembly and Slurry Packing of Functional Polymer Beads

For the completed device, a bead-packed column was prepared with slurry packing method for the purification of peptide samples. As covered in the previous chapter, to achieve the slurry packing a pair of weir structures is essential to hold beads inside the packing region. In this work, the weir structure is achieved by assembly of two substrates which have different depths of micro patterns. The alignment of each pattern is critical to achieve the desired microfluidic structures, so the developed pin-hole pair structures assisted the self-assembly and bonding technique is also utilized in this fabrication to secure the alignment. SOURCE 15RPC,
based on polystyrene/divinyl benzene monodisperse beads was injected at 10 µl/min flow rate, which was controlled by a syringe pump, and dried out after finishing the filling process. 15 µm diameter beads are only packed inside the column due to physical restriction from the height of channels (less than 15 µm) positioned at the opposite ends of the column. The bead loading/packing process is extremely rapid (less than 2 min). Figure 4.2 shows a fabricated device and a microphotograph of the column holding FITC labeled IgG.

Figure 4.2 Microphotograph of the fabricated device and a microphotograph of bead packed column holding FITC labeled IgG.
4.3. Experimental Result

4.3.1. Chip Running Protocol

A basic test for a proof-of-concept of this research was performed by injection and elution of the 250 µg/ml of FITC labeled IgG sample to show the column’s functionality. The sample preparation protocol for MALDI MS starts from injecting wetting solution and is then followed by equilibration solution, sample solution, washing solution, and then finally elution solution. As shown in Figure 4.2, the column is holding the FITC labeled protein samples and emitting fluorescent light. Afterwards it is eluted using 1% TFA elution solution, which is preceded by a washing step following the protocol.

The sample has to be changed to FITC labeled peptide (sample info) for further study since the molecular weight of the protein sample is over the range for MALDI MS analysis. The sample solution is prepared by 25-fold dilution of 50 µg/ml concentration of FITC labeled peptide. To determine the column’s functionality, the maximum mass-loading amount can be determined by observing the electropherogram recorded while the chip is being loaded with controlled flow rate. The actual mass of sample delivered to the column can be calculated by knowing the concentration of the sample and flow rate. Also the chip will be evaluated by observing the electropherogram while the chip is manipulated by the sample preparation protocol for MALDI MS: ~1 µl of wetting solution (50 % ACN); ~1 µl of equilibration solution (0.1 % TFA); ~1 µl of sample solution; ~5 µl of washing solution (0.1 % TFA); and ~1 µl of elution solution (1 % TFA). Appropriate column functionality is shown in Figure 4.3. The column was saturated after 13 minutes of sample flow at 2 µl/min flow rate, resulting in ~50 ng of maximum
peptide sample that could be captured in the column. Also the captured peptide during washing step was not lost and finally elution was possible.

Table 4.1 Peptide sample preparation protocol using the sample preparation chip.

<table>
<thead>
<tr>
<th>Step</th>
<th>Solution / volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>L1  Wetting</td>
<td>50% ACN / 1 µl</td>
</tr>
<tr>
<td>L2  Equilibration</td>
<td>0.1% TFA / 1µl</td>
</tr>
<tr>
<td>L3  Sample loading</td>
<td>Peptide sample solution / 1µl</td>
</tr>
<tr>
<td>L4  Washing</td>
<td>0.1% TFA / 5 µl</td>
</tr>
<tr>
<td>L5  Elution</td>
<td>50 % ACN and 0.1 % TFA / 1µl</td>
</tr>
</tbody>
</table>

Figure 4.3 Expected electropherogram from the outstream and real electropherogram recorded from the device.
4.3.2. Column Characterization

Fluorescence detection was employed to determine the nominal mass loading column capacity. The column’s holding capacity was evaluated by observing electropherogram detected from out stream immediately after the column while injecting sample solution (2 µg/ml, FITC-LC-EMP17, 2485 Da, Anaspec) with 10 µl/min flow rate. As shown in Figure 4.4, which is a magnified view of the dotted box from Figure 4.3, the intensity remained steady and began to increase after the column was saturated. The holding capacity of the column can be calculated with the time taken for the column saturation point, sample concentration, and flow rate. In this typical case it took 12.2 minutes for column saturation, which resulted in a total of 48.8 ng of sample binding to the column.

![Electropherogram](image)

Figure 4.4 Resulting electropherogram of sample loading: 2 µg/mL FITC conjugated peptide sample with 2 µl/min of flow rate for 12.2 min. Calculated column’s holding capacity is 48.8 ng.
4.3.3. MALDI-MS Result

A comparison between the on-chip and ZipTip\textsubscript{C18} desalting was chosen to determine the analytical performance of this device. Liquids collected from the out stream after each step were analyzed by MALDI-MS (Reflex IV, Brunker Daltonics). The MALDI-MS results using 5 µg/ml of Neurotensin (N6383, 1672.9 Da, Sigma) with artificially added salt (5 µg/ml NaCl solution) are shown in Figure 4.5: (a) using microfluidic chip and (b) a magnified view of the dotted area from (a), (c) using ZipTip. Meanwhile, it was not possible to see any distinguishable peaks when the salty sample was used for MS analysis without any cleaning process.

The developed sample preparation chip for MALDI-MS has been successfully demonstrated for its functionality for liquid handling and sample cleaning. The microfluidic chip provides an acceptable resolution and mass accuracy when it is compared to the result from ZipTip. However, any direct quantitative comparison was not made since the type, size, and volume of packed beads were different for each case. Furthermore, ZipTip allows more flexible liquid handling such as repeating pushing and pulling, which allows more sample binding and washing capabilities. On the other hand, single-pass processing is available for the sample preparation chip developed in this work. So, although it is difficult to conclude the superior performance of the developed chip to the ZipTip, there is significant room for further improvement of the performance of the chip by integration of a circulation system, or by varying the type and volume of beads assembled through the on-chip column.
A slurry packed RPC column integrated sample preparation chip for MALDI-MS has been developed and fully characterized. The integrated column has 48.8 ng of peptide holding capacity and the fabricated device was used for peptide sample cleaning prior to MALDI-MS analysis. 5 µg/ml of Neurotensin sample was cleaned using the device and the analysis results of

4.4. Conclusion

Figure 4.5 MALDI-MS results: (a) and (b) using the sample preparation chip developed in this thesis; and (c) using ZipTip. (b) and (c) are the magnified views at the sample peaks.
MALDI-MS were comparable to the commercially available ZipTip product while any meaningful peaks were not shown when salty peptide sample was used. Better sample and liquid handling with small volume of sample (1 µl) and parallel processing capability are benefits of the developed device.
CHAPTER 5

Polymer Lab Chips for Capillary Electrochromatography with Electrochemical Detection for Point-of-Care Clinical Diagnostics

5.1. Introduction

Most point-of-care clinical diagnostics and on-site environmental analyses require a portable or hand-held analyzer with smart lab-on-a-chips, which have sampling and detection capabilities for the target molecules. Although the analyzer can control the sampling and analysis sequences, most practical problems usually come from the liquid handling and detection methods. On-chip capillary electrophoresis (CE) has been a popular tool for various biochemical analyses, but several hurdles related to its natural difficulties at the initial sample injection still remain, retarding its practical application for the point-of-care testing (POCT) or on-site analysis. A vacuum or pressure pump is usually required for the initial liquid introduction to the CE chip, which hinders its applications to the point-of-care analysis. Also the problems of water head caused by the height different between reservoirs, improper leveling, and external shocks or vibration needs to be addressed to achieve reproducible results.

Furthermore, CE chips use the laser induced fluorescence (LIF) method for detection, which is difficult to integrate with a hand-held analyzer system due to its bulky laser source. As
a result, there is demand for the development of a new CE chip that can address the sampling difficulty while using an alternative detection method to the LIF.

In consideration of the problems associated with using CE chips for POCT, microchip capillary electrochromatography with electrochemical detection (CEC-EC) can be a great alternative to CE chips. The primary advantage of CEC-EC microchips is the easy liquid and chip handling using the microchannels fully packed with submicron silica beads. Proper leveling of the solution between the reservoirs is not required, and fully packed channels also can serve as built-in submicron filters for the sample and buffer solutions, while producing the capillary forces to pull the buffer solution and the liquid sample into the channel as well. The channels are free from clogging by particulates, so any extra-filtering process is not required. The channel can be filled with buffer solution only by the capillary action of the packed channel and it is free from air bubble trapping problems. In addition, the electrochemical detection using on-chip electrochemical sensors can allow the replacement of the bulky laser source with electronic circuits such as potentiometer.

Numbers of reviews about CEC have been published [99 - 103, 6] in recent years covering aspects such as application range, packing material and procedure, but the new polymer CEC-EC chips using the polymer microfabrication technologies and the self-assembled bead-packed columns have been designed, fabricated and characterized for the analysis of uric acids.

5.1.1. Uric Acid Test

In humans, the degradation of endogenous nucleic acids (adenosine and guanosine) is the major source of purines expelled as uric acid in urine [104]. Clinical studies have shown that
monitoring uric acid levels in urine and blood serum can be used to diagnose several diseases [105]. Two such disorders are hyperuricemia and hypouricemia. Hyperuricemia can result from either an increased production of uric acid or a decreased renal excretion of uric acid and has been linked to primary gout, Lesh-Nyhan syndrome, and chronic renal disease [106]. Hypouricemia can result from a decreased synthesis of proteins or a defect in renal tubular reabsorption of uric acid. These abnormalities have been linked respectively to severe liver disease and Fanconi’s syndrome [106]. Several methods for the detection of uric acid have been reported, which include chromatography [107 - 110], electrophoresis [111, 112], mass spectroscopy [113], enzymatic and colorimetric methods [114, 115].

5.1.2. Capillary Electrochromatography

Microchip-based electrochromatography is a combined method of the microchip capillary electrophoresis (CE) and the chip-based liquid chromatography (LC), adopting the favorable characteristics from both methods for a new analytical tool. The separation mechanism of CE is based on the difference between mobilities of solutes, while LC is based on the differences of partition coefficients between two phases. Combining CE with LC creates a powerful analytical tool capable of separating both ionic and neutral compounds. Furthermore, the plug-like electroosmotic flow (EOF) profile results in a reduced dispersion of the analyte zone, which improves the efficiency of the separation column.

5.1.3. Electrochemical Detection

Among several different types of detection mechanisms for biochips, laser-induced fluorescence (LIF) has been the most popular method for the microchip CE due to its high
sensitivity [116, 117]. However, most compounds including uric acids are not naturally fluorescent and must be derivatized with a fluorophore to be detected by LIF. Furthermore, it is necessary to select the excitation wavelength that is specific for the particular fluorophore. Mass spectrometry (MS) has also been employed as a detection mode for miniaturized devices [118, 119]. The primary advantage of coupling MS with microchip CE devices is to improve the analysis throughput. Unfortunately, the commercially available MS systems are costly and not inherently portable. Conventional measurement methods of uric acids are based on the conversion of urate to allantoin via uricase [106]. Colorimetric techniques based on this reaction are, however, temperature-dependent, expensive, and require labile reagents. An additional obstacle in monitoring uric acids is the interference from other compounds such as ascorbic acid and dopamine [120]. This complication has led to the development of various electrochemical (EC) detection methods and separation schemes for the selective determination of uric acid [121-123].

Since EC detection methods offer an analytical platform which can exhibit a higher selectivity and higher sensitivity than other commonly employed methods, and has the inherent advantages such as lower cost, rapid sensing time, and reduced size of the system, they are well-suited to the miniaturized analytical systems and are attractive alternatives for microchip CE devices [37-41]. The sensitivity and selectivity of EC detection are comparable to those of LIF detection. Additionally, there are several advantages of the EC detection over other detection modes, including the ability to miniaturize both the detector and control instrumentation and the fact that many compounds can be detected without derivatization. Microelectrodes are fabricated using well-developed photolithographic techniques that allow the electrodes to be
fabricated directly onto the microchip device, producing a fully integrated system. Unlike absorbance detection, which is dependent on Beer’s Law, the electrodes can be miniaturized without loss of sensitivity. In addition, although microelectrodes can generate extremely small currents, the background current is reduced even further, resulting in an increased signal-to-noise ratio and potentially better limits of detection (LODs) [42, 43].

There are three general modes of EC detection: amperometry, conductimetry, and potentiometry. Amperometry is the most widely reported EC detection method for chip-based separations [37, 38, 124 - 127]. It is accomplished by applying a constant potential to the working electrode and measuring the resulting current [128]. In the conventional three-electrode setup, a reference and counter (or auxiliary) electrode are also present. The current response is directly proportional to the concentration of analyte oxidized or reduced at the electrode surface as described by Faraday’s law:

\[
\frac{dQ}{dt} = i_t = dF \frac{dN}{dt},
\]

where \(i_t\) is the current generated at the electrode surface at time \(t\), \(Q\) is the charge at the electrode surface, \(t\) is time, \(n\) is the number of moles of electrons transferred per mole of analyte, \(N\) is the number of moles of analyte oxidized or reduced, and \(F\) is the Faraday constant (96,485 C/mol). Selectivity is achieved through the judicious choice of detection potential, with the optimal detection potential normally being determined by hydrodynamic voltammetry or cyclic voltammetry [129].
Several different detection methods have been developed for the amperometric detection such as end-channel, in-channel, and off-channel detection. Martin et al. [125] introduced an in-channel amperometric detection method to eliminate the band broadening that is characteristic of the end-channel detection method. The in-channel method involves the placement of the working electrode directly within the separation channel which was made possible by the development of the electrically isolated (“floating”) potentiostat. This “floating” potentiostat draws its power from a 9 V battery and transmits data via optical isolators. Thus, there is no electrical path to ground that could damage the potentiostat. The in-channel configuration also helps eliminate some of the negative separation performance characteristics encountered with an end-channel configuration, especially with respect to the alignment of the working electrode at the end of the separation channel.

5.2. Design and Fabrication

5.2.1. Materials and Solutions

Cyclic olefin copolymer (COC, Topas 5013, 8007) was chosen as the substrate material. Colloidal silica beads (Bangs Laboratories, Inc.) with diameter of 0.8 µm were selected as the beads to be self-assembled. Silver plating solution (Silver Cyless RTU; Technic, Inc.) and potassium chloride solution were used for Ag/AgCl plating. Negative photoresist (NanoTM SU-8 2007), OmniCoat and OmniCoat developer (Microposit MF 319), SU-8 stripper (Remover PG), Positive photoresist (Shipley 1818), 351 developer were purchased from Microchem Corp. (Newton, MA, USA). Running buffer solution (10 mM of phosphate buffer, 2.7 mM of potassium chloride and 137 mM of sodium chloride, pH 7.4 at 25 °C) was freshly prepared with
one phosphate buffered saline (PBS) tablet (Sigma-Aldrich) dissolved in 200 ml of deionized water. pH 7.4 was employed for the selective determination of uric acid and ascorbic acid. Uric acid (MW 168.11) and ascorbic acid (MW 176.12) were purchased from Sigma-Aldrich. Solutions used in experiments were directly used as received without any further purification or degassing steps.

5.2.2. Design

The conceptual drawing of the device design is shown in Figure 5.1, where 0.5 cm of short channels and 1.5 cm of separation channels are patterned in 2 cm x 3.5 cm size of device. All patterned channels have 50 µm width and 50 µm depth and are fully packed with the silica beads of 0.8 µm diameter by following the self-assembly bead packing steps developed in Chap. 3. Holes of 2.37 mm diameter were drilled at the end of each channel and then additional reservoirs were glued over the holes to ensure adequate reservoir volume for solutions. Holes of 1 mm diameter were drilled on the bead-packed substrate aligned to the electrodes on the cover wafer and filled with silver epoxy (Circuit Specialists Inc.) to create the electrical connection after assembly.

Sensor electrodes patterned on the cover substrate have 100 µm width and 100 µm spacing between each electrode, and the 3 electrode electrochemical sensor cell is positioned 12 mm away from the channel intersection of the chip as described in Figure 5.1.
Figure 5.1 Design of the CEC-EC microchip for on-site analysis. The short channels are 0.5 cm and the separation channel is 1.5 cm in length.

Figure 5.2 shows a photograph of the fabricated CEC-EC microchip with SEM images of the bead packed structure and microphotograph of the integrated sensor.
Figure 5.2 Images of the fabricated CEC-EC microchip: 0.8 um diameter bead packed (hexagonal close packing) channels and integrated 3 electrode EC sensor.
5.2.3. Microchannel patterned Polymer Replication

Figure 5.3 summarizes the fabrication steps for the microchannel patterned polymer substrate by replica injection molding process and the self-assembly bead-packing in the microchannels.

Figure 5.3 Microchannel patterned substrate (COC, Topas 5013, T_g = 136 °C) was prepared by injection molding using Ni master mold. Patterned microchannels were fully packed with 0.8 µm diameter silica beads using self-assembly packing method.
For the micro-injection molding process, a nickel mold insert was fabricated by SU-8 process followed by nickel electroplating on the nickel disk. The same fabrication techniques developed and described in the previous chapters have been used for the fabrication of the chips. Briefly, microstructures were fabricated using the UV-LIGA process. The master mold for microchip replication was fabricated on a nickel (Ni) mold disk 3 inch diameter and 1.6 mm thick. The nickel disk was lapped flat and thick photoresist (SU-8 2075 negative photoresist) processing was performed on the disk followed by nickel electroplating. 80 µm of plating height was achieved after 8 hours of electroplating in a nickel electroplating bath with a 10 mA/cm² of current density.

Since the surface roughness and flatness of the chips are critical not only for the self-assembly of silica beads but also for uniform liquid flow in microfluidic devices, the development of the flat and smooth channel surface of the nickel disk mold is necessary. So, the 80 µm thick electroplated nickel microstructures on the nickel substrate were lapped and polished to obtain an appropriate height with smooth and flat surface. As mentioned in the previous chapters, the final surface condition has been greatly improved (average surface roughness, Ra = 26.67 nm) when compared with the condition before the lapping and polishing process (Ra = 159.11 nm).

Finally, a nickel master mold which has 50 µm width and 50 µm height microchannel patterns was prepared for injection molding after stripping the residual SU-8 layer by immersing in the SU-8 stripper solution. Microchannel patterned COC substrates were replicated though micro-injection molding process (BOY 22M-A, BOY machines, INC.) using prepared nickel mold insert, with cycle times under one minute.
5.2.4. **Self-Assembly Crystallization of Silica Colloidal**

The microchannels were packed with silica colloidal particles by the self-assembly packing process which was reported earlier [27, 28]. Figure 5.5 is the schematic diagram showing the self-assembly bead packing process for CE microchip. It is a simple variation of the previously characterized self-assembly bead packing method developed for a straight microchannel. The dipping angle was tilted for 45°. Since the CE microchip has cross channels, when the chip was vertically dipped as with the straight microchannel, the horizontally positioned channels were not packed properly, which was due to the channel close to the cross section was dried out more quickly than the end of the channels. This blocked the continuation of the self-assembly along the channels. However, the tilted dipping with 45° addressed the problem, achieving the self-assembly along the whole channels without defects. The patterned COC substrate was pretreated with O$_2$ plasma for 2 mins with 20 sccm of O$_2$ gas flow and 200 W of power to produce hydrophilic condition on the surface of microchannel (March CS 1701 Reactive Ion Etching system, March Plasma Systems, Inc.).
The colloidal silica solution (0.8 µm diameter, 0.1 wt%) was heated to 60 °C in a beaker with gentle stirring (80 rpm) to prevent slow precipitation of the aggregated silica particles. The room temperature and humidity were controlled during the self-assembly bead packing process at 68 °F (20 °C) and 46 % respectively. The end of the separation channel was dipped cautiously into the suspension by holding with a jig. Pretreated open microchannels showed enough hydrophilicity to drive the silica colloidal suspension to the end of each channel by capillary action. Once the colloidal solution reached the end of the capillary channels, spontaneous three-dimensional packing of the silica particles started from the end of the microchannels due to the

Figure 5.5 Dipping process for self-assembly of colloidal suspension in a microchannel for fully packed CEC device. Silica beads are filling through hydrophilic treated plastic microchannels by capillary force, and 3D periodic array of uniform colloidal silica suspensions are formed while water is evaporated.
slow evaporation of water. The self-assembly packing process continued toward the end of the empty microchannel at the bottom of the chip. The water evaporation rate was controlled by the opening of the beaker and the packing process was stopped when channels were fully packed. The packed chip was washed very gently and cautiously with a clean room paper soaked with deionized (DI) water to remove extra silica particles at the dipped area and dried completely at room temperature. The resulting uniform hexagonal closed packed structure with 25.95 % of porosity [130] is shown in Figure 5.2.

5.2.5. Electrochemical Detection System

The sensor electrodes including working, counter, and reference electrodes were patterned on a blank COC substrate that was fabricated using lower glass transition temperature material (Topas 8007, Tg = 80 °C) than the substrate containing the microchannel (Topas 5013, Tg = 136 °C). Figure 5.6 shows brief fabrication steps for the sensor patterning process. First, positive photoresist (Shipley 1818) was spin-coated at 3,000 rpm on 1,000 Å thick Au film deposited COC substrate, and then baked in the 60 °C oven for 30 minutes. The baked photoresist was exposed under UV light (300 nm ~ 460 nm wavelength, ~8 mW/cm²) for 12 seconds and followed by developing (1:5 dilution of 351 developer solution). Ag and AgCl were sequentially electroplated on one of the Au electrodes to form reference electrode. Each electrode has 100 µm width and the distance between adjacent electrodes is also 100 µm.
Figure 5.6 Au electrodes were patterned on cover substrate (COC, Topas 8007, $T_g = 80 \, ^\circ C$) through photolithography and subsequently the reference electrode was prepared by Ag/AgCl electroplating.

5.2.6. Microchip Assembly

Figure 5.7 is the schematic illustration of the microchip assembly. Again, the microchannel patterned COC substrate was made with resin having high $T_g$ (Topas 5013, $T_g = 136 \, ^\circ C$). After packing with submicron silica particles, the packed substrate was covered with a blank COC plate having low $T_g$ (Topas 8007, $T_g = 80 \, ^\circ C$) and bonded by fusion bonding technique using heat and pressure applied by embossing machine (MTP-10, Tetrahedron
Associates Inc., San Diego, CA). To avoid the destruction of bead-packed structure by pressure, the temperature in the hot embossing machine was maintained at 85 °C for 20 minutes without pressure, allowing the cover plate to soften since that temperature is higher than its T_g. The softened cover plate was gently pressed for another 20 minutes and then cooled down while holding the pressure constant. However, the deformation of the substrate during this process was significant enough to disconnect the thin micropatterned electrodes as shown in Figure 5.8(a). We observed these problems at the electrodes on top of the bead-packed microchannels. Optimized bead packing conditions prevent the electrodes from being damaged due to the gap between the meniscus from the top surface of the bead-packed structure and the cover substrate. Figure 5.8(b) shows the sensor electrodes without damage after assembly. In order to achieve a precise alignment between the separation column and the sensing electrodes, the pin-hole pair structures assisted alignment and assembly method is also adopted in the fabrication of the chips as well.
Figure 5.7 Schematic diagram of microchip assembly: microchannel patterned substrate packed with beads (COC, Topas 5013, T_g = 136 °C) and sensor patterned substrate (COC, Topas 8007, T_g = 80 °C) were bonded by heat and pressure applied fusion bonding: 85 °C, 20 mins with gentle pressure after softening the low T_g substrate for 20 mins, then cool down to room temperature while keeping the pressure.

Figure 5.8 Microphotographs of the sensor electrodes after assembly: (a) damaged and disconnected, and (b) without damage or disconnection after optimization of the bead packing and following bonding conditions.
The ultimate goal of this effort is to develop miniaturized clinical analyzers by coupling easy liquid and chip handling including self-conditioning without filtration or degassing step, and integrated electrochemical detection system for capillary electrochromatography on a microchip platform. Such an attractive combination is demonstrated below for the separation and detection of uric acid and ascorbic acid.

5.3. Experimental Result

5.3.1. Experimental Setup

Figure 5.9 shows a schematic diagram of the experimental set up. The fabricated CEC-EC microchip was mounted on a holding jig to aid chip handling and connected to a mTK Microfluidic Tool Kit (Alberta Microelectronics, Alberta, Canada), which was controlled by a LabVIEW (National Instruments, Austin, TX, USA) program written by the Alberta Microelectronics Corporation. The microchip was also connected to a battery-powered PalmSens handheld electrochemical sensor interface (Palm Instruments BV, Netherlands), which was kept electrically isolated for in-channel amperometric detection. The acquired signal was transferred to a handheld device through wireless data transfer.
5.3.2. Self-Conditioning

As mentioned previously, one of the major advantages of this device is self-conditioning since it allows the device to be conditioned without using extra equipment like vacuum pump, making it appropriate for on-site analysis. Figure 5.10 shows images taken from video clips of the self-conditioning process recorded at a position 2 mm downstream from the cross-channel; the images were taken with 1 frame/s rate. The device was completely filled with PBS running buffer solution in 15 minutes using only the capillary force without air bubbles. Also, the bead-packed microchannels work as a built-in nanofilter, thus purification or degassing steps for solutions were not necessary.
5.3.3. Cyclic Voltammetry

Electrochemical oxidation has been widely used for the detection of uric acid in solution [131 - 133]. However, ascorbic acid oxidation interferes with this process, making it difficult to achieve the selective determination of uric acid in the presence of ascorbic acid. Both uric acid and ascorbic acid exist as anions beyond pH 5.4 in the alkaline range [132]. For this reason, cyclic voltammetry has been performed to understand their behavior for the selective determination of uric acid in the presence of ascorbic at neutral pH.

Cyclic voltammetry is a type of potentiodynamic electrochemical measurement. A voltage is applied to a working electrode for forward scan and subsequent reverse scan while the working electrode remains in contact with the solution, and current flowing at the working electrode is recorded versus the applied voltage. Cyclic voltammetry can be used to study the electrochemical properties of species in solution at the electrode/electrolyte interface.
It has been known that uric acid is oxidized via a two electrons and two protons process (Figure 5.11), followed by a hydrolyzation reaction to produce allantoin [134].

![Chemical structure of uric acid and ascorbic acid](image)

**Figure 5.11 Oxidation mechanisms of uric acid (a) and ascorbic acid (b), adapted from [134].**

The electrochemical properties of uric acid and ascorbic acid in the designed sensor system and buffer solution were investigated through the use of cyclic voltammetry. For cyclic voltammetry, the potential was scanned from -0.3 V to 0.8 V for the forward scan and the opposite for the reverse scan. 0.005 V steps and 0.05 V/s scan rate were used for both forward and reverse scan. Figure 5.12 depicts the cyclic voltammetric responses from the electrochemical oxidation of 1 mM uric acid and ascorbic acid in pH 7.4 of PBS buffer solution. It shows that 0.65 V of detection potential would be appropriate for electrochemical detection since it is high enough for the oxidation of the uric acid while it allows low and flat baseline from the PBS buffer solution. As shown in Figure 5.12, unexpected peaks are appeared at the
lower scanning voltage. It is mainly due to the silver ions released from the reference electrode that travel toward the working electrode by diffusion during the cyclic voltammetry measurement. However, in the CEC-EC microchip, since the reference electrode is positioned at the end of the separation channel, the working electrode should not be influenced by the released silver ions from the reference electrodes. The released sliver ions should flow forward toward the end of the channel, allowing no chance to diffuse to the working electrodes.

Figure 5.12 Cyclic voltammetry of 10 mM ph 7.4 of PBS buffer solution, and 1 mM uric acid and ascorbic acid in PBS buffer solution.
5.3.4. Separation and Detection

Microchip CEC Condition

For chip operation, 0.4 kV was applied across the sample reservoir (SR) and sample waste reservoir (SW) for 40 seconds during the injection step, and 0.6 kV was applied across the buffer reservoir (BR) and buffer waste reservoir (BW) for 60 seconds during the separation step while keeping the other reservoirs at floating potential for each step (Table 5.1).

Before detection, the channels were filled with buffer solution through the self-conditioning process which will be explained in a later section. PBS buffer solution with pH 7.4 was used as a running buffer solution, and the uric acid and ascorbic acid stock solutions (10 mM for each) were prepared by mixing with DI water, and further diluted with buffer solution to the required concentration. The buffer solution in the SR was replaced with a mixture of 1 mM uric acid and 1 mM ascorbic acid after conditioning while other reservoirs remained filled with running buffer solution.

Table 5.1 Voltage setting for injection and separation.

<table>
<thead>
<tr>
<th></th>
<th>SR</th>
<th>BR</th>
<th>SW</th>
<th>BW</th>
</tr>
</thead>
<tbody>
<tr>
<td>Injection (40 sec)</td>
<td>0.4 kV</td>
<td>Flt</td>
<td>Gnd</td>
<td>Flt</td>
</tr>
<tr>
<td>Separation (60 sec)</td>
<td>Flt</td>
<td>0.6 kV</td>
<td>Flt</td>
<td>Gnd</td>
</tr>
</tbody>
</table>
Separation and Detection

Separations of uric acid and ascorbic acid were carried out in a pH 7.4 PBS buffer solution. Figure 5.13(a) shows the reproducible results from serial injection study. Typical electropherograms showing the separation of 0.1 mM, 0.5 mM, and 1 mM of uric acid and ascorbic acid were shown in Figure 5.13(b). The same voltage scheme mentioned previously was used for both cases. The average migration times from serial injection and separation were $6 \pm 0.3 \text{ s} \ (n = 5)$ and $10.8 \pm 0.3 \text{ s} \ (n = 5)$ for ascorbic acid and uric acid, respectively.
Figure 5.13 Electropherograms from CEC-EC microchip: (a) Reproducible results from serial injection study, and (b) typical electropherograms showing successful separation and detection of 0.1 mM, 0.5 mM, and 1 mM of uric acid and ascorbic acid: 0.4 kV, 40 s for injection, 0.6 kV, 60 s for separation.
Detection Range

The concentration dependence was examined for increasing levels of uric acid and ascorbic acid (Figure 5.14). Highly linear calibration plots were observed for both uric acid and ascorbic acid from 0.1 mM to 1 mM of concentration range, with slopes of 24.59 and 17.99 nA/mM (and correlation coefficients \( r \) of 0.991 and 0.998, respectively).

Typical uric acid concentration levels in urine for healthy person and gout disease patient \((2.30 \times 10^{-4} \text{ g/ml} \text{ and } 0.85 \times 10^{-3} \text{ g/ml, respectively})\) [135] are well covered by the calibration plot when the sample is 10-fold diluted.

![Image](image-url)

**Figure 5.14** Linear calibration plots of uric acid and ascorbic acid. Concentration range is from 0.1 mM to 1 mM \((R^2 = 0.991 \text{ and } 0.998)\).
5.4. Conclusion

A bead packed CEC device integrated with electrochemical detection system has been designed, fabricated, and fully characterized. Smooth and flat microchannels-patterned COC substrate was replicated from the nickel mold which has 26.67 nm of average surface roughness after lapping and polishing process. Self-assembly silica bead packing was optimized for full packing of microchannels with small meniscus at the top surface of the bead packing structure. Micropatterned sensor integration for electrochemical detection has also been realized in this research. Bonding conditions for the assembly of sensor electrodes patterned on blank COC substrate (T_g = 80 °C) and bead packed COC substrate (T_g = 136 °C) without damaging bead packing structure and sensor electrodes were optimized.

The quick and easy chip preparation manner with self-conditioning, which takes less than 15 minutes, makes on-site analysis possible and, furthermore, filtration or degassing steps were not necessary during preparation of solutions for the analysis. The electrochemical property of uric acid was studied by cyclic voltammetry and 0.65 V of detection potential was selected for electrochemical detection. Uric acid and ascorbic acid were successfully separated and detected in 100 s using fabricated CEC device. The developed CEC device was fully characterized and showed reproducible results in terms of migration time and peak height from serial injection study. The linear calibration result for the uric acid ranged from 0.1 mM to 1 mM, which includes the practical clinical range of uric acid concentration.

This device provides numerous advantages when compared with conventional capillary electrophoresis (CE) devices: fast and easy chip conditioning; improved liquid and chip
handling, better performance in terms of analysis speed and reproducibility; and compact size of
detection system.
CHAPTER 6

CONCLUSION

6.1. Summary

In this research, new polymer lab-on-a-chips with functional nano/micro bead-packed columns have been designed, fabricated, and characterized for biochemical analysis.

High quality disposable polymer capillary electrophoresis (CE) microchips have been developed and fully characterized as a demonstration of the state-of-art microfabrication techniques. In addition to the currently available techniques, double side injection molding with replaceable mold disk and a new self-alignment and assembly technique using pin-hole pair structures have been developed for 3D microfluidics and multilayer polymer lab-on-a-chips. Thus, the alignment problem associated with multilayer polymer devices, which has been considered as one of the most difficult tasks in the development of multilayered polymer lab-on-a-chips, has been well addressed. Alignment accuracy close to 10 µm has been achieved and successfully applied for the fabrication of 3D micromixer and 5-layer biochip for blood analysis.

Also functional on-chip bead-packed columns using a slurry packing method and a self-assembly method have been developed and characterized,
An on-chip reverse phase chromatography (RPC) column packed with RPC media (SOURCE 15RPC) has been realized using the slurry packing method, where the geometrical restrictions with precise alignments, which are essential for the packing process, were achieved using the pin-hole pair structures assisted assembly technique.

The RPC column integrated with the sample preparation device for MALDI-MS has been designed, fabricated, and fully characterized. The integrated column has shown the holding capacity of 48.8 ng of peptide and was applied to the sample preparation for MALDI-MS analysis. 5 µg/ml of Neurotensin sample was filtered using the sample preparation chip and the analyzed results through MALDI-MS were comparable to the results from the commercially available ZipTip products. Small volume of sample (less than 1 µl) and parallel processing capability for the sample preparation are benefits derived from the newly developed sample preparation chip.

A high quality on-chip capillary electrochromatography (CEC) column has been developed using the self-assembly method of silica colloidal beads in the microchannels with a depth of 50 µm and a length of 2 cm. Nano/micro beads (0.8 µm ~ 1.98 µm in diameters) were successfully self-assembled in the microchannels (50 µm ~ 100 µm in width) with a hexagonal crystalline structure.

For a point-of-care urine sampling and analysis, the self-assembled columns have been developed over the entire channels for both sample injection and separation, so the sample is introduced into the sample injection channel using the capillary forces, which eliminates the difficulties associated with the traditional sample introduction techniques such as using an
external vacuum or pressure. In addition, an electrochemical (EC) sensor placed at the end of the separation channel has been implemented and characterized as a CEC device with EC detection, and uric acid and ascorbic acid have been successfully separated and detected from the developed chip.

Reproducible results have been achieved and the average migration times from serial injection and separation were $6 \pm 0.3$ s ($n = 5$) and $10.8 \pm 0.3$ s ($n = 5$) for ascorbic acid and uric acid, respectively. Linear calibration plot between 0.1 mM to 1 mM of concentration of uric acid ($R^2 = 0.991$) and ascorbic acid ($R^2 = 0.998$) have been achieved and it covers clinical range of sample concentration from 10-fold dilution of urine sample.

This device provides numerous advantages when compared with conventional capillary electrophoresis (CE) devices, including fast and easy chip conditioning, improved liquid and chip handling, better performance in terms of speed and reproducibility, and compact size of detection system.

Finally, the developed fabrication techniques and on-chip column preparation methods have been successfully applied to fabricate various biochemical analysis devices in a lab-on-a-chip platform, and the developed biochemical analysis devices have been characterized to show their functionality.

In conclusion, in this research a new alignment and bonding method for multi-stacked polymer chips and an on-chip nano/micro bead self-assembly method for the functional columns have been successfully developed and fully characterized, and then applied for various polymer
lab-on-a-chips for biochemical analysis or clinical diagnostics, which can enable the practical on-site or point-of-care testings using on-chip capillary electrochromatography.

### 6.2. Suggestion for Future Work

The sample preparation device for MALDI-MS has been successfully applied for the purpose of peptide sample cleaning. However the eluted peptide sample was collected from the device for crystallization on off-chip MALDI target instead of using the chip as an on-chip MALDI target. It was simply due to the limitation of the design of the cartridge that holds MALDI targets to be inserted into MALDI-MS equipment. Thus on-chip sample preparation and MALDI analysis using the on-chip target would be possible when the cartridge is modified for the microchips. Also, parallel sample preparation would be possible if microchip design is changed for arrays. Finally, the application can be extended for various purposes with the combination of appropriate functionality of the columns and microfluidic platform.

The capillary electrochromatography microchip with electrochemical detection system can be further developed toward fully miniaturized clinical analyzers for on-site analysis by integrating on-chip electrodes to apply high voltage using a portable high-voltage power supply for separation. Since it has not yet been fully applied for human urine test, such an experiment would be very interesting as a point-of-care clinical diagnostics application.

Also, the bead-packed channels can be used for the separation of various analytes, such as catecholamines, amino acids, drugs and metabolites, peptides, etc, since it has ability to separate not only mobile phase but also stationary phase, and neutral solutes in addition to charged solutes. An electrochemical detection system incorporated with capillary electrochromatography could
be applied for the separation and detection of various compounds while appropriate separation mechanisms and electrodes for targeting molecules are used.
References


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