On-chip Multi-dimensional Biomolecule Separation Using Multi-layer Microfabricated Valves

by

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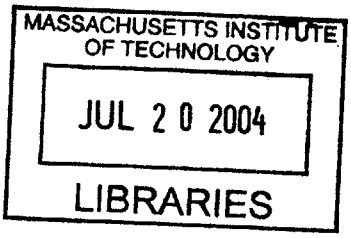

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ABSTRACT

Recent proteomics researches require a sensitive, high-speed, and automatic protein separation tool that far exceeds the capability of traditional two dimensional (2D) protein gel electrophoresis. Methods are described to achieve multi-dimensional biomolecule separation in a microfluidic channel. The new methods couple isoelectric focusing (IEF) with high ionic strength capillary electrophoresis (CE) by active micro valve control in a microchip. Several experiments demonstrating independent 2D separation were performed, and critical parameters for better chip performance were identified; including channel passivation, electroosmosis control, IEF linearity control, and detection enhancement. The result can be used for the filtration of high-abundance proteins, which used to be done by affinity columns. Also, it can be used for much possible integration between different heterogeneous separation/ analysis techniques such as IEF, polyacrylamide gel electrophoresis (PAGE), CE, reverse-phase chromatography, and mass spectrometry (MS).

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Chapter 1 Introduction

1.1 Background

In this post human genome era, uncovering mysteries of human proteome and protein-protein interaction became the most important task in biology. The impact of proteomics will bring radical changes to drug industries and medical treatments. For the study of protein functionality within cells or serum, multi-dimensional protein separation is an essential tool. A typical cell or serum sample could contain about 10,000 proteins. Moreover, it is estimated that 300,000 to 400,000 proteins might present inside human proteome, which makes protein identification more difficult to achieve. To make things more complex, the three-dimensional protein structures and protein-protein interactions make their shapes highly unpredictable and therefore hard to identify. Even though we know the genetic codes for all genes, we still need to understand the protein concentrations and profiles to fully understand the complex biological systems such as cell. However, proteome is much harder to determine when compared with genome(Service 2001). Genetic codes of a human do not change appreciably over the course of a person's life , but his/her proteome is changing constantly due to environmental factors, such as the influence of disease or drinking. Therefore, tracking human proteome is significantly more challenging than the study of human genome..

1.2 2D Gel Electrophoresis

Nowadays, the most commonly used protein identification and quantification method is two-dimensional (2D) gel electrophoresis coupling immobilized pH gradient (IPG) IEF strips and sodium-dodecyl-sulfate (SDS) polyacrylamide gel electrophoresis (PAGE), also called SDS-PAGE, followed by mass spectroscopy (MS) analysis. In 2D gel electrophoresis, proteins are first sorted by their isoelectric point (pI), the net electric charge, and then by their molecular size in the SDS-PAGE step. By coupling IEF and SDS-PAGE, one can achieve separation between protein species that are not resolvable by either technique only. Two-dimensional gel electrophoresis has been very useful in proteomics for decades, mainly because it is the only way to resolved thousands of proteins simultaneously. However, even if it provides very high peak capacity (~1000), it is still limited in the analysis of complex biomolecule samples such as serum. First, the gel IEF and SDS-PAGE coupling is rather laborious and the manual protein fixing, labeling, and buffer exchanging procedures make the peak resolution from the first dimension IEF not being fully maintained. Second, its large physical scale (50 cm by 50 cm in general, 25 cm by 25 cm for Mini-PAGE) requires high operation voltages and long separation time. These practical problems become more significant in the analysis of information-rich proteins in 'real' biological samples such as serum. Resolving low-abundance proteins is extremely difficult in gel electrophoresis, because the majorities of proteins in cells (representing more than 80% of total protein contents) diffuse and cover their adjacent low-abundance proteins. In these samples, hundreds of these information-rich proteins are effected by high abundant proteins and therefore hard to detect by traditional 2D gel electrophoresis. Typically, majority proteins like albumin

and globulins have mM concentrations, while their adjacent information-rich proteins presenting only in nM range. In order to detect low abundance proteins, it is necessary to load excessive amount of samples, which would sometimes overwhelm the separation column and make the detection even harder to achieve. To overcome these problems, a microfluidic 2D protein separation system with rapid analysis time, sharp peak band, and clear isolation would be highly desirable.

1.3 Microfluidics for Protein Separation

During the last decade, microfluidics technology has been extensibly applied to the analysis and separation of biomolecules, with a lot of success. Microfluidic biomolecule analysis devices can improve the speed and sensitivity of proteomics analyses and are superior to traditional tools in many aspects. For instance, microfluidic analysis devices have ultra fast separation time and require only micro liters of sample. Furthermore, it is potentially an automated and portable tool that is very important in many application areas, such as chemical / biological agent detection or remote health monitoring. A full overview of the wide field of microfluidic 'lab-on-a-chip" can be found elsewhere. (Auroux, Iossifidis et al. 2002; Reyes, Iossifidis et al. 2002)

While it is highly desirable to implement multi-dimensional protein separation techniques on a microchip that can substitute the bulky, time-consuming slab gel currently used, integration of two different separation techniques on a microfluidic chip has been challenging. So far, microfluidic 2D separation has been achieved without active sample peak control or isolation between different separation columns. Ramsey's group developed a two-dimensional separation system coupling micellar electrokinetic chromatography (MEKC) with CE (Rocklin, Ramsey et al. 2000). By doing so, estimated 500-1000 peak capacity can be achieved. Also, Herr et al. demonstrated another 2D separation microfluidic device, where IEF coupling with ampholyte based CE was achieved. (Herr, Molho et al. 2003) However, these separations are coupling intrinsically the same separation techniques. (For example, combination of neutral charge CE caused by surfactants in the ambient with free solution CE, or ampholyte based IEF with

ampholyte based CE.) The limitation here is mixing between interconnected fludic channels. Moreover, MEKC requires surfactants such as SDS in the CE buffer that make the further connection with MS more difficult to achieve. More recently a microfluidic device that couples IEF and SDS-PAGE has been demonstrated, but without active peak control. (Li, Bush et al. 2004)

We used microfluidic valves to achieve the integration of heterogeneous components. There are many valve designs developed for microfluidic applications (Schasfoort, Schlautmann et al. 1999; Beebe, Moore et al. 2000; Xie, Yang et al. 2001; Yu, Bauer et al. 2001). Among different previous valves designs, we adopted the design by Quake et al. (Unger, Chou et al. 2000) since it is simple to make, have a low dead volume which is important in molecular separation.

The microfluidic valves here are the key components in this device that can prevent intermixing between two separation buffers (ampholyte and CE buffer). The valves also isolate a group of IEF-focused proteins from the rest of the IEF columns, preventing the problems of peak inter-diffusion and band dispersion during the buffer exchange process. Finally, the trapped target proteins in the peak transfer region were sent into second dimension capillary channel for the second dimension separation of choice, in this case capillary electrophoresis. This new strategy could allow the integration of many different and heterogeneous separation techniques (hydrophobic separation, affinity based separation, etc) to be used in an integrated fashion, which could greatly enhance the efficiency of the biomolecule analysis of serum or cell extract.

1.3.1 The Main Fields of Application

Proteins, peptides, sugars, nucleic acids, amino acids or anything with charges can be separated by electrophoretic methods. The areas of applications are listed below:

1) Direct application: proteomics, glycomics, metabolomics, pharmacology, forensic and clinical investigations (where molecules are so diverse and really needs multi-dimensional separation)

2) Indirect application: This is a good example of integrating two different microfluidic components, which could be important for developing integrated microfluidic molecular analysis and detection systems(Burns, Johnson et al. 1998; Fan, Ricco et al. 2003)

With the progress in proteomics, the ability to carry out and couple appropriate electrophoresis techniques for specific separation problems, the goal of this thesis, will become increasingly important.

1.4 Thesis Overview

In this thesis, we focus mainly on the coupling between isoelectric focusing (IEF), the only way to resolve proteins only by their net charges; and capillary electrophoresis (CE), a rapid and compact way to resolve mixtures by differences in mobility under the influence of electric fields. Capillary electrophoresis is a weighty analytical tool, because its ability to do fully automated analysis for large amount of samples that always involved in protein analysis. To implement IEF and CE, we developed a new method to integrate heterogeneous separation components with incompatible buffer requirements together. The new method integrates IEF, a charge based separation technique, and any other heterogeneous microfluidic separation techniques in a microfluidic system. The significance of this approach is to facilitate an integration of two different techniques even with heterogeneous, incompatible buffer systems. This is a very universal, current problem in the field of microfluidics and μTAS . Many individual biomolecule separation and manipulation techniques have been implemented on a microchip with impressive performance characteristics as promised. However, integration of multiple steps of biomolecule analysis and characterization – which is critical in the biological research and in biotechnology – has not been progressing as expected.

Chapter 2 Theoretical Overview

Proteins perform various functions in biological systems, as enzymes, hormones (chemical messengers), antibodies and structural materials. Even though many efforts have been devoted into the research of protein biological function, the function and working mechanism of most proteins have not yet been identified. Because most proteins exist with a very small concentration in a cell, it is not surprising that one of the major difficulties in biological analysis comes from the purification of the target biomolecules, especially proteins. (Unlike DNA, proteins do not have an amplification technique (such as polymerase chain reaction, PCR) to increase the number of target molecules, which makes the analysis of protein even more challenging.) Also, proteins can be characterized by various properties, such as electrical charge, hydrophobicity, binding affinity and others. Therefore, one needs various separation techniques designed to differentiate these molecular properties. To have better understanding and controlling over the separation tools, the basic principles have to be studied. In this chapter, the principles and mathematical models of the most widely used purification techniques, IEF and CE, are discussed. These are the techniques that can be readily applicable to the microfluidic separation.

2.1 Electrophoresis, Zeta Potential and Electroosmosis

To know the theory of isoelectric focusing, we first have to understand electrophoresis and electroosmosis. Electrophoresis separation techniques are widely used in purification methods, because of its high separation efficiency in separating molecules and the simplicity in experimental setup. In short, electrophoresis is a motion of charged particles in an aqueous solution under the influence of electric field, and the mobility of charged particles is determined by the interaction between electrophoresis and electroosmosis flow. For any charged spherical particle in a liquid solution, it has an electrophoretic velocity U when applied electrical field E_o: (Grodzinsky 2003)

$$U = \left[\frac{\frac{\varepsilon \xi}{\eta}}{1 + (\frac{\varepsilon \xi \sigma_m}{\eta \sigma R})} \right] E_o \qquad \text{Equation 1}$$

Other than electrical field, the velocity depends on the zeta potential ξ, the mobile charge in the double layer σ_m, dielectric constant ε, dynamic viscosity η, and particle radius R.

To explain the zeta potential ξ and how forces exert on charged particles, the concept of electrical double layer has to be introduced. Based on Poisson's equation where ρ_u is the volume charge density:

$$\nabla^2\Phi = \frac{-\rho_u}{\varepsilon}$$ Equation 2

In a space near the surface with certain surface charge density, counterions to the surface charge will gather around near the surface and shield the surface charge. Mathematically, from the equation 2, one can use the Boltzmann equation to relate the charge density ρ_u to the potential Φ, and integrate the equation to obtain the (exponential-like) potential profile shown in the Figure 1(Grodzinsky 2003).

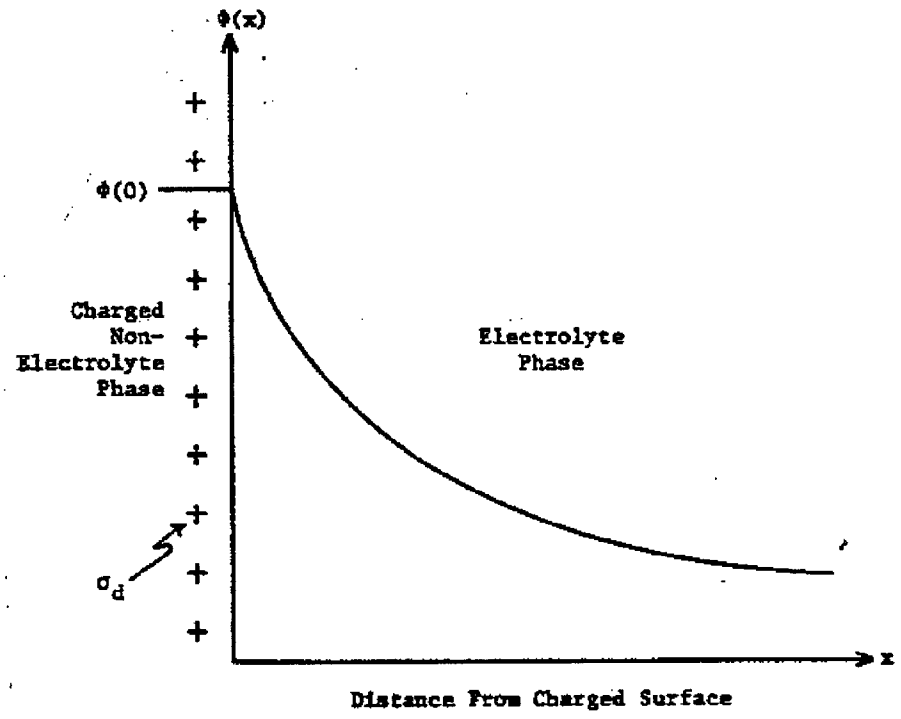

Figure 1: Plane-parallel potential distribution model around electrolyte

If we take a deeper look into the charge distribution, we can define the ions into two different layers. The first is the Stern layer where counter-ions are under strong Coulomb

force and firmly attached onto the charged surface, while the second one is diffuse-double layer where the rest of counter-ions are located. Inside the diffuse-double layer, ions are in dynamic equilibrium between the repulsion from the Stern layer and the attraction from the charged surface. The charge concentration (also potential) decay in diffuse double layer is due to the diffusion and convection of co-ions and counter-ions. Shown in the following figure. The double layer is formed to neutralize the charged particle where the thickness of it depends on the concentration and dielectric constant of the ambient.

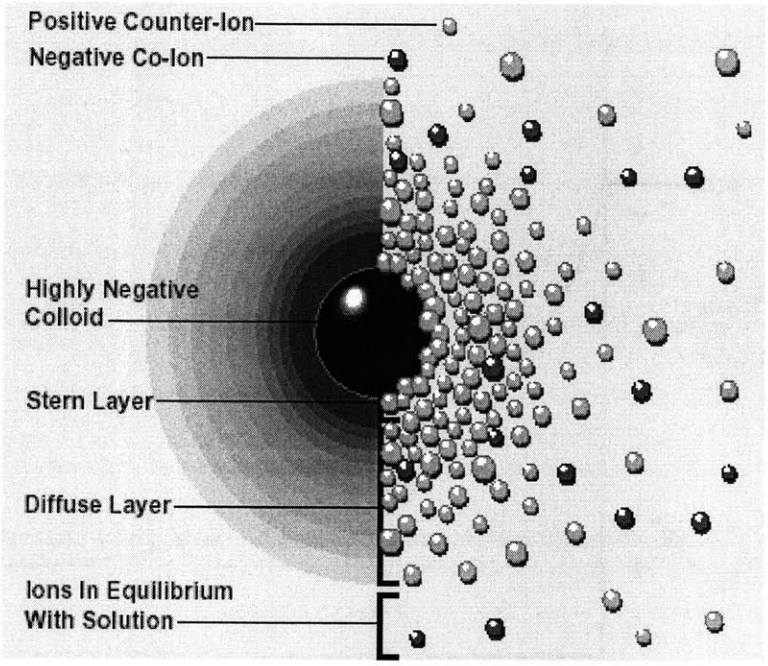

Figure 2: Schematic Drawing of the Double Layer from a single charge particle in a solution

Outside the double layer, charged particles are virtually electrically neutral. However, when under a voltage field, they will move with a fixed velocity since the charges in diffuse double layer are movable. This phenomenon is called electroosmosis. The moving

boundary is located between the Stern and diffuse double layer (Usually in a distance of about two or three molecular diameters from the surface). Thus, we can regard the this moving boundary as a fixed non-moving wall, and the potential at this boundary (defined as zeta potential ζ) could be treated as an "effective" surface potential for the purpose of anlaysing electroosmotic phenomena. In the field of electrophoresis and electroosmosis, zeta potential is more significant than the actual surface potential, because the latter cannot be easily measured without ambiguity.

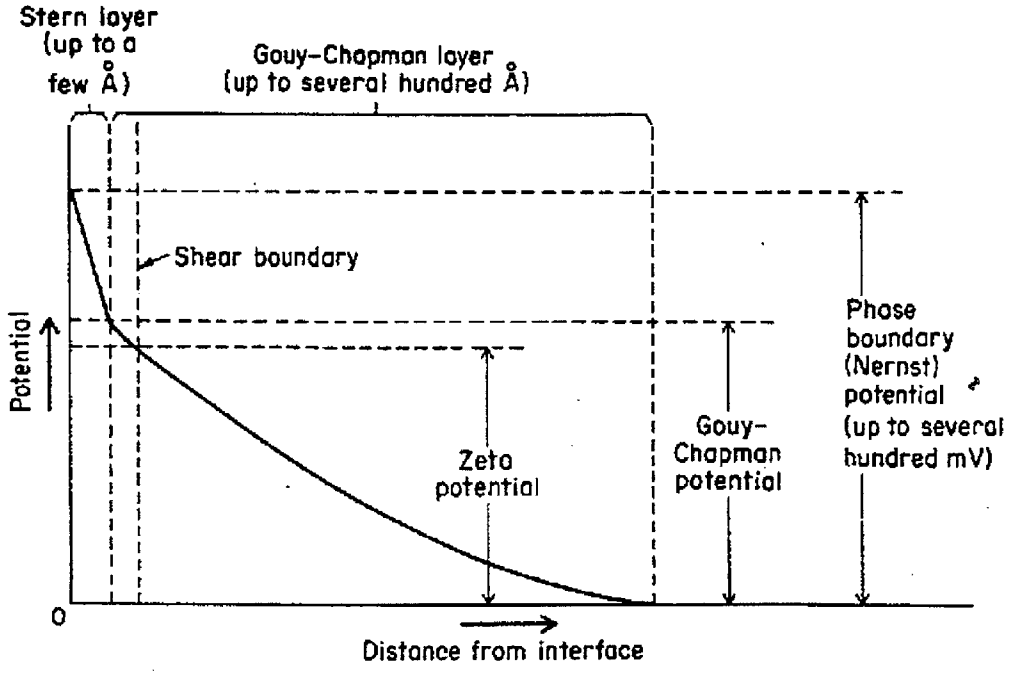

Figure 3: Gouy-Chapman-Stern Length Scales Model
(Jain 1972)

Electroosmotic flow (EOF) is known to be an electromechanically-induced flow due to the viscous and electric shear stresses applied on the electrical double layer. In a circular tube with inner surface zeta potential ζ, and the tube length l_p and external

potential drop Ψ_p, we can have the electroosmotic flow velocity written as(Grodzinsky 2003):

$$U_z(r) = \left(\frac{\varepsilon[\xi - \Phi(r)]}{l_p \eta} \right) [\Psi_p] \qquad \text{Equation 3}$$

The thickness of the double layer is typically a few hundreds of nm depending on the buffer concentration and surface property. No EOF is expected if the pipe radius is large enough to make Reynolds number relatively large. In high Reynolds number case, convention of the fluid will eliminate the EOF. This is the one reason why electrophoresis has to be carried out in paper, gel, or capillary tubes (in a low-Reynolds number limit).

In the observer reference flame, the motions of charged particles are the net effects of electrophoresis and electroosmosis. It is known that zeta potential changes by both the surface charge and ion concentration. In order to have a predictable and repeatable charged particle migration behavior, the zeta potential must be carefully controlled. Because from equation 1 and equation 3, electrophoresis is in first order relation to the zeta potential while the electroosmosis is in hyperbolic relation to the zeta potential. Therefore, charged particles will move along or against the electroosmosis flow with different mobility depends on the external voltage, fluid viscosity, and buffer concentration, and a particular location in the tube or microfluidic channel. By controlling these parameters, one can minimize the dispersion in the separation processes. Low Reynolds number flow condition in microfluidics is ideal for minimizing convection-related sample dispersion, it is still important that both electrophoresis and

electroosmosis have to be carefully considered in a microfluidics environment to have a predictable, non-dispersive flow or separation.

2.2 Isoelectric Focusing

Isoelectric focusing is the only electrophoretic separation (focusing) technique that can separate biomolecules based on their isoelectric points (pI). Thus, it is one of the most important electrophoretic separation techniques in analytical biochemistry.

2.2.1 Ampholyte

pK value of amphoteric molecules

Proteins are amphoteric molecules, which means they can be characterized as either an acid (a proton donor) or a base (a proton acceptor). Whether they behave as acid or base depends on the pH value they are sitting in. In other words, they could carry either positive, negative, or zero net charges depending on the pH. Therefore, by controlling the pH of the solution, we can change the electrophoretic mobility of proteins. This characteristic comes from the titration properties of amino acid side chains and amino- and carboxyl-terminals. Take one of proteins' basic amino acids, glycine, for example. It has one carboxyl and one amino group on it (Shown in figure 4). Presented in a medium pH environment, charged groups of this molecule are the unprotonated carboxyl group and the protonated amino group, because the pK value (25°C) of the carboxyl group is 2.4, and for the amino group is 9.8.

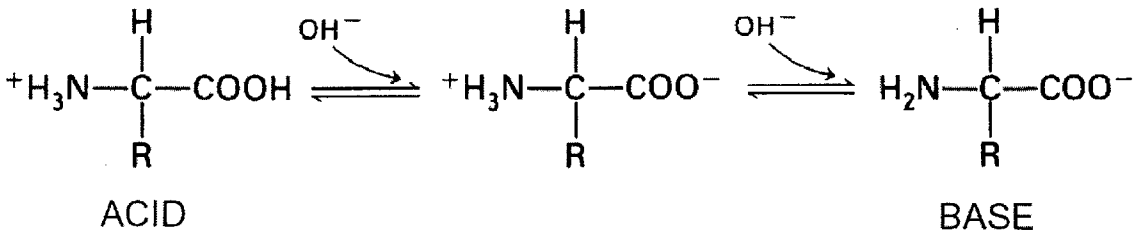

Figure 4: Titration of an amino acid

As a result, glycine amino acid has a positive net charge when surrounded by strong acid buffer. In contrast, in the vicinity of strong base buffer, these groups will be titrated and the amino acid will become electrically neutral and then negative charged. The transit electrical neutral state is called the isoelectric point (pI) of the amphoteric molecules. Proteins and peptides with many different charged groups would have a unique pI values, and these values can be useful in identifying and characterizing such molecules.

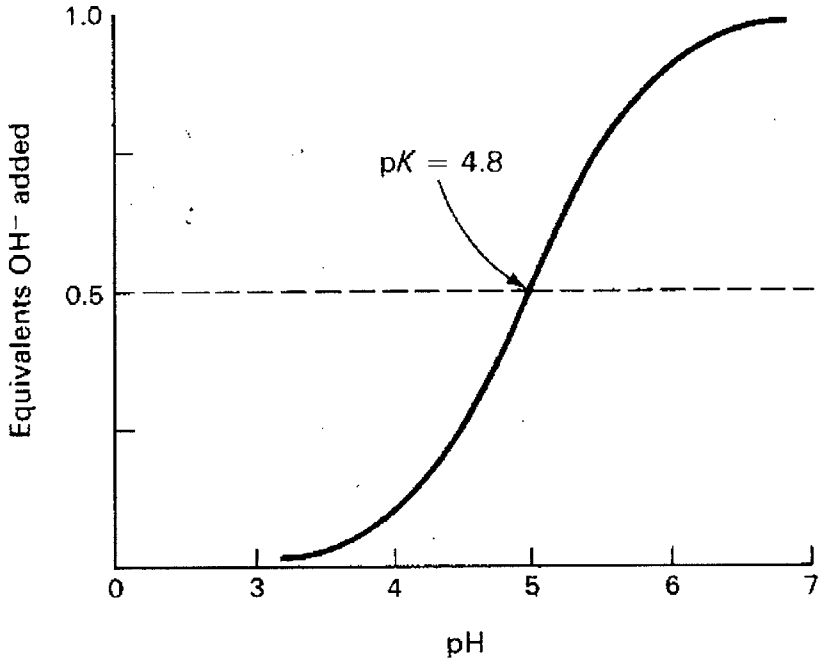

Figure 5: Titration Curve of Acetic Acid(Berg, Tymoczko et al. 2002)

The isoelectric focusing is an electrophoretic method that separates proteins according to their isoelectric points (pI). The strategy used here is based on the amphoteric nature of proteins. Because proteins are amphoteric molecules, the net charges on them change in the vicinity of acid or base buffers. Therefore, if we establish a pH gradient that can cover all the isoelectric points of proteins, by applying an external electrical field across the pH gradient, proteins will initially move toward the electrode with the opposite charge and either lose or obtain protons. By arranging the anode in acid buffer and cathode in base buffer, proteins will lose their net charges during this migration and eventually reach a steady equilibrium in their pI value. Electrophoretic driving force will help maintain proteins focused in a sharp band, because once they diffuse into a pH region higher than their pI value, they will acquire negative charges based on their titration properties and forced back into their pI region.

In a similar fashion, they will be protonated and forced toward the cathode if diffused into region closer to the anode from the focused point. In this mechanism, we can resolve proteins according to their pI which is a unique and irreplaceable in analytical and preparative purposes. Moreover, it is an extremely powerful tool after combing with electrophoresis called two-dimensional separation technique. In gel format isoelectric focusing, proteins with 0.001 unit differences in pI value have been separated(Bjellqvist, Ek et al. 1982).

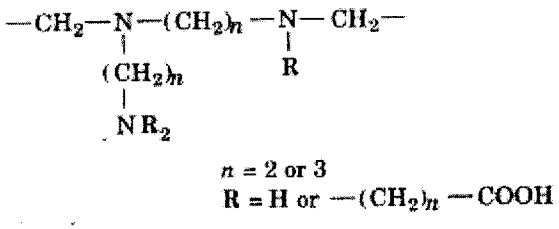

Figure 6: The general formula of the polyampholytes used in isoelectric focusing

As a result, the most important factor in isoelectric focusing is establishing a stable and high-resolution pH gradient. Although one can easily realize a pH gradient by mixing two solutions with difference pH together, this kind of pH gradient is not stable enough due to local convections and ion immigration in the electrical field, and cannot support a significant ion current that is required for sample focusing. Rather, ampholytes, a complex mixture with hundreds of different small amphoteric molecules ranging from 300 to 600 Dalton were used as the pH gradient stabilizer. These ampholytes are polymers with different ratios in amino groups and carboxyl groups. Once the electrical field is applied, they act as a pH gradient buffer that maintains the gradient.

2.1.3 Theoretical Calculation of IEF

A. Calculation of the pH Gradient

For

$[H^+] = h$

activity coefficient of monovalent ion = f

degree of dissociation = α

total concentration of the acid = C

c = the unit concentration of 1 mole/liter

pH = - log h/c

pK = - log K/c

Known from electroneutrality condition of solution :

$hf(h/f - C\alpha) = K_w^2$ and $hf\alpha = K(1 - \alpha)$

Therefore, the concentration of acid regarding the association rate:

$$C = (\frac{h^2}{K} + \frac{h}{f})(1 - \frac{Kw^2}{h}) \qquad \text{Equation 4}$$

Multiply Equation 4 with the differentiation of pH, we can have the pH dependence on concentration:

$$\frac{d(pH)}{d(C/c)} = \frac{-\log e}{(2h^2/Kc)+(h/fc)(1+Kw^2/h^2)}$$ Equation 5

B. Resolving Power of IEF

Effected by pH, column coordinate x, mobility u, and concentration C of the protein. The resolution of IEF can defined by the smallest difference in isoelectric point that can be detected as:

$$\Delta(pI) = 3(\frac{D(dpH/dx)}{E(-du/dpH)})^{1/2}$$ Equation 6

The detail derivation and discussion can be found from the *Isoelectric Focusing* written by Catsimpoolas(Catsimpoolas 1976).

2.1.4 The Balance Between EOF and EP

As described in the previous session, once the electrical field is applied, proteins will focus into a sharp region where the pH values equals to their pI values. Shown in Figure 7 is the schematic drawing of one species of proteins during its focusing process in a pH gradient established by external electrical field. Proteins sitting closer to cathode or anode have more charges and therefore move faster than those in the center region because more base or acidic buffer are presenting in outer region. Therefore, a symmetric condensing behavior shown in the drawing should be expected.

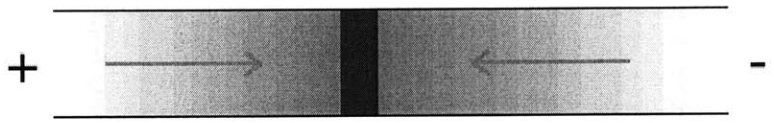

Figure 7: Ideal IEF without EOF

However, in real case, the isoelectric focusing is always accompanied by electroosmosis. Because the electroosmotic flow heads toward cathode everywhere in the channel, it can have either a positive or negative net effect to the IEF. For proteins sitting in pH region higher than their pI, the focusing is a process fighting with the EOF while they accelerated by the EOF sitting in the low pH region. Therefore, proteins in lower pH region focus faster than that in high region ones. Because the viscosity and zeta potential are affecting the mobility in different orders, by changing either one of them, we can have different net motions. For example, if higher viscous solution is used, proteins will have

relatively smaller EOF compared to low viscous ones and focus to the center of the channel faster. We performed a serious of experiment with different viscosity present the same result as predicted.

Under the influence of EOF toward the cathode, proteins in high pH region have to move against the EOF and therefore take more time to be focused. Moreover, in high pH environment, the protonation makes the wall more negatively charged which induce higher EOF than low pH environment. By controlling the channel length, solution viscosity, and electrical fields strength in the experiment, we can control the balance between EOF and electrophoresis. Typically observed protein focusing behaviors shown in Figure 8.

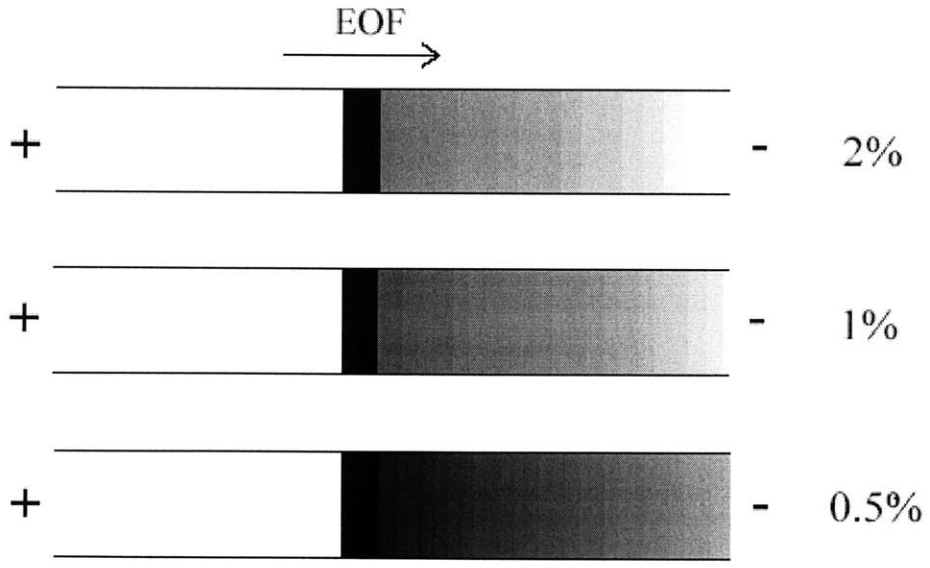

Figure 8: IEF under different viscosity and electrical field strength.

In high viscous case where 2 % of methylcellose were added, the EOF were suppressed and therefore less protein were trapped in catholic session

2.3 Capillary Electrophoresis

Both gel electrophoresis and capillary electrophoresis are designed to provide a convention free, low Reynolds number environment for a successful electrophoretic separation. Compared to gel electrophoresis where solid gel with small pores were used to eliminate the conventional flow, capillary electrophoresis uses very thin capillary with 25 to 300 μm inner diameter (I.D.) as a synthetic pore to provide small Reynolds number environment. The better heat dissipation property the capillary permits higher electrical field (typically 100 to 500 V/cm) that can reduce the separation time to several minutes. The rapid separation not only can save time but also minimizes the peak broadening that yields extremely sharp separation bands. In this session, empirical models and different CE techniques were discussed.

2.3.1 Separation in Free Solution

The success of proteins separation lays on the ability to control motilities of proteins. The prediction of electrophoretic mobility allows us to understand and furthermore to optimize separation conditions of electrophoresis. Based on the titration (charge vs. pH) property discussed above, the electrophoretic migration mobility can be obtained for their mass (size) and charge characteristics by semi-empirical equation(Offord 1966):

$$\mu = k \cdot Z \cdot M^{-\frac{2}{3}} \qquad \text{Equation 7}$$

This charge to size relation can be used as a simplest model to predict protein mobility in capillary electrophoresis with constant pH, also called capillary zone electrophoresis (CZE). Because every protein has distinct titration property, the selection of pH and buffer concentration is very important in having a nice separation. Moreover, because EOF increases as pH increases, to increase the detection sensitivity, EOF control is also an important issue.

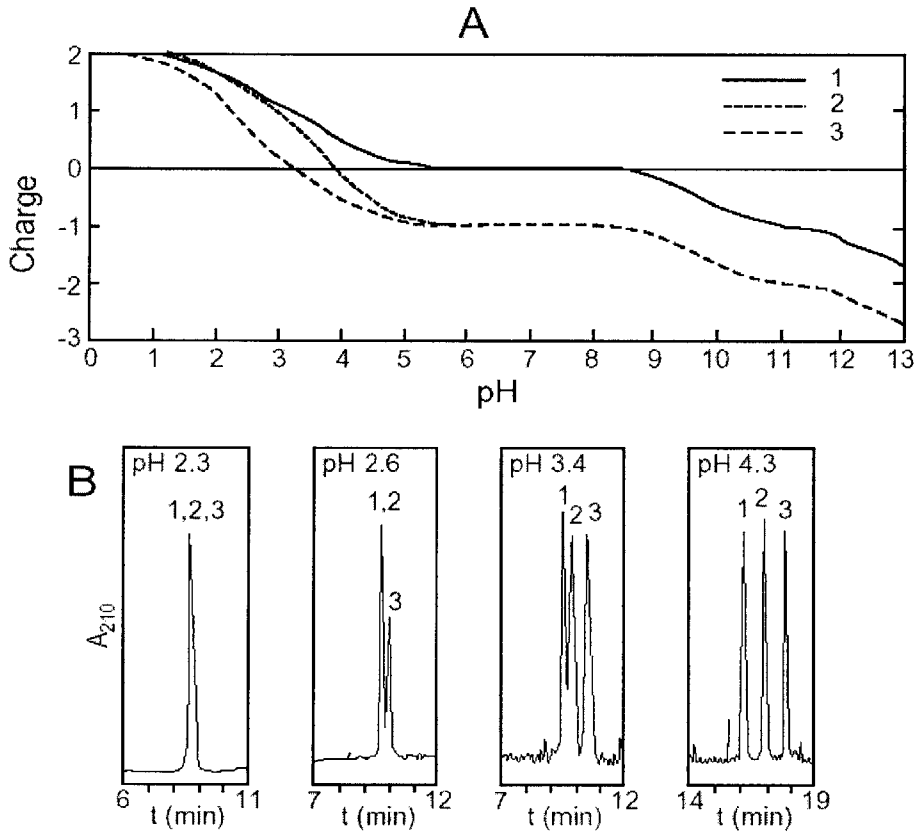

Figure 9: Titration curve of synthetic peptides and electropherograms of them:
(A) Titration curve of synthetic model peptides; (B) electropherograms at varying pH (25-mM Na_2HPO_4 /H_3PO_4 running buffer)(Bongers, Lambros et al. 1992)

In figure 9, polypeptide, a chain of amino acids connecting to each other by peptide

chains, was used to demonstrate the significance of mobility control in protein separation. The reason peptides were used is that they are smaller and easier to predict than proteins. Most peptide chains have molecular weights between 5500 and 220,000 while most proteins have molecular weights (also named Dalton, the unit weight of hydrogen atom) between 10,000 to 300,000 daltons.

Other than the pH, the buffer concentration is also an important factor to consider in capillary electrophoresis. Higher concentration can decrease the thickness of shielding layer, and therefore decrease the zeta potential, which means the EOF is small in this case. Smaller EOF can help obtain sharper peak and brings in the stacking effect. However, high conductivity buffer makes the joule heating and electrolysis (bubble generation)a serious problem and should be handled with extra cares.

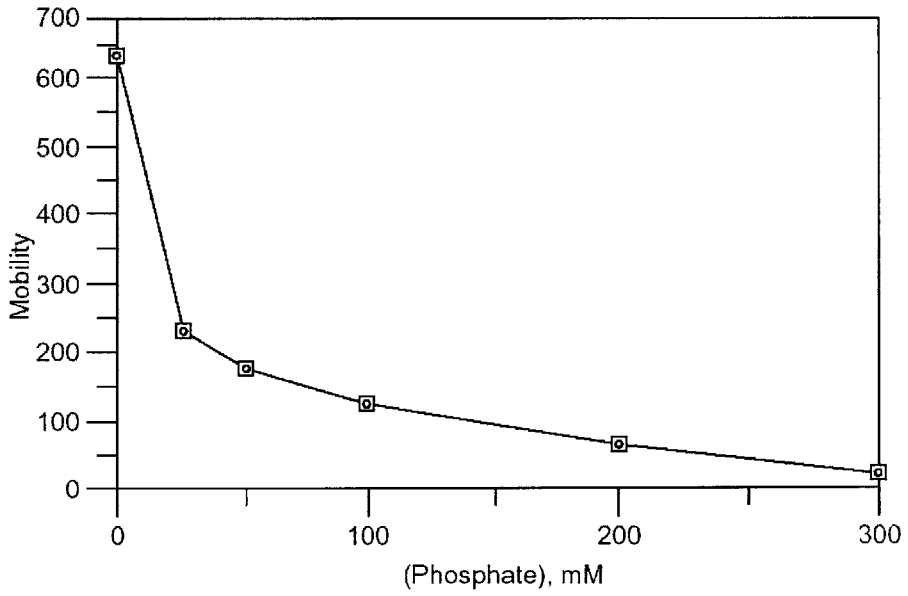

Figure 10: Mobility of Benzyl Alcohol vs. Buffer Concentration
(Schwartz and Pritchett 1995)

2.3.2 Protein adsorption and Strategies to prevent it

Proteins are functioning as enzymes and messengers inside bodies. They have a tendency to bind various materials, such as metals, plastics, and glassware (including the so-called biocompatible instrumentation), nonspecifically. In most cases, the negatively charged silanol on the wall adsorb negative protein with a rate k_a while desorbing at a rate k_d. The net result of this is the dynamic equilibrium, which affects the bandwidth and even more the detection limit of protein separation. Also, the hydrophobic forces between proteins and the wall are important factors for some hydrophobic proteins. Except in some hydrophobic separation, like C-18 column protein separation where proteins adsorbs to and desorbs from the wall selectively as a separation mechanism, the protein adsorption is highly undesirable and developing strategies to prevent it is extremely important in capillary electrophoresis. Most of the time in untreated fused-silica capillaries, the k_a is much larger than the k_d that no protein separation can be detected.

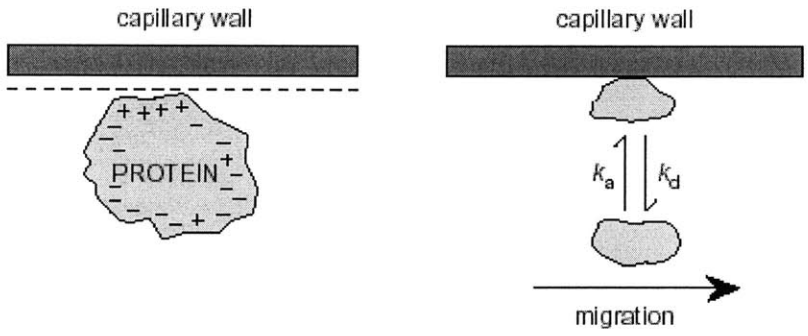

Figure 11: Schematic Drawing of the electrostatic interaction of proteins with the wall (Schwartz and Pritchett 1995)

Extreme pH buffer condition

The easiest but sometimes not feasible way to prevent protein adsorption is using extremely high or low pH buffer. At high pH condition, both the charged groups on the capillary wall (silanol groups) and proteins (amino and carboxyl groups) are negatively charged based on the titration properties discussed before. Therefore, the electrostatic adsorption will be minimized due to the electrical repulsion force. However, at high pH, strong EOF makes both detection and stable separation very difficult to achieve. Alternatively, when using extremely low pH buffer, both the wall and proteins are protonated and having net positive charges. In this care, the electrical repulsion force between positive charges helps diminish the protein adsorption. Although extreme pH conditions are easy to produce, problems like wall dissolving, protein denaturing, deactivation, and titration make the extensive use of it impossible.

Surface Treatment:

Permanent (Static) Coating

In 1985, Hjerten et al develop a surface modification by monomolecular layer of polyacrylamide through Si-O-Si-C chain on the wall(Hjerten 1985). It is the most well-known and widely-used coating method among researchers. For this, 0.4% of silane was first used as an adhesion promoter between glass and polyacrylamide. Then, acrylamide solution containing catalyst was induced with 30 mins reaction time. By doing so, both the electroosmosis and adsorption of solutes can be eliminated. Possibly, the coating reverses the surface charges and repelled out solutes from sticking. Other than polyacrylamide, other hydrophilic polymers like polyethyleneglycol or polyether have been used as coating molecules to suppress EOF and minimize adsorption. In some

special application, other types of surface modification using positively charged polymers like polyamine is adopted. In this case, the surface will be given cationic charges so that the direction of EOF will be reversed. By reversing charge, some basicproteins (positively charged) will be dispelled from the surface.

Dynamic Coating

An alternative way to suppress protein interaction is using dynamic coating, in which additives were added to buffer solution to disturb the protein-wall ion-exchange mechanism. Unlike previous mentioned extreme pH condition case, high ionic strength cationic amines or non-ionic surfactants were used to suppress solute-capillary wall interaction in this case. The latter ones were especially useful when analyzing hydrophobic proteins. Suppressors like ethylene glycol or cellulose derivatives can decrease EOF in both high concentration and low concentration case. In low concentration case (0.01 to 0.03 %), they can decrease the zeta potential, which is the key factor that determines EOF. In high-concentration case (0.5% methyl cellulose or 10% to 30% ethylene glycol), the viscosity and molecular sieving effect will be observed. Another significant way to reduce adsorption of cationic proteins is by using charge reversal reagents. In this case, nonpolar chains with form a bilayer by hydrophobic interaction and therefore change the charge property of the wall.

2.3.3 Protein Modification

Since most of the difficulty in proteins separation comes from the complexity of their structures. A promising way to separate them is modifying protein structures before the separation. Agents like ionic surfactants and urea will break the covalent structures of proteins. For example surfactants like SDS binds to protein in a constant ratio of 1 to 1.4 grams per gram of proteins and yield a rod-shaped complex. This rod shape structure covered by ionic surfactants has a linear charge density proportional to its the molecular weight. This reversible SDS binding can further increase the difference in electrophoresis motilities and therefore increase the separation resolution. The other frequently used additive is urea (4 to 8M), which can prevent aggregation and increase precision in migration times and peak heights.

2.3.4 Sample Stacking

Besides protein modification and channel wall treatment, sample-stacking techniques are often being used in CE separation to improve sensitivity and resolution. From equation 1, we can have the relation between electrical field and electrophoresis mobility. Therefore, the stacking mechanism happens when samples ions moving toward the adjacent separation buffer zone, to the higher conductivity buffer from the low conductivity buffer. Ions in low conductivity zone have much higher mobility because most electrical field strength located in low conductivity zone. Due to the sharp decrease in the electrophoretic velocity, ions will stack into a narrow plug shape also known as a pre-concentration step in CE separation. Other than buffer conductivity, different buffer ionic strength will affect the separation result under similar mechanism(Camilleri 1998).

2.3.5 Various Modes of Capillary Electrophoresis

Different from paper electrophoresis and gel electrophoresis, capillary electrophoresis uses capillary tubes with very small inner diameters to eliminate peak broadening caused by local convection. Although microfluidic chips have different exterior appearance with fused silica capillary, their separation capillaries have similar diameter (50~100 μm) and material. Among different capillary electrophoresis techniques, the most important ones are capillary electrophoresis, SDS capillary gel electrophoresis (SDS CGE), capillary IEF (CIEF), and affinity capillary electrophoresis (ACE)(Camilleri 1998).

2.4 Minimization of IEF and Capillary Electrophoresis

Having an automated, portable device with rapid analysis time is always the goal while developing analysis tools. Among all the possible approaches, the minimization of microchip is a promising way to improve the performance of microfluidic separation chips. By minimizing IEF and CE separation channels, we can build not only a portable IEF and CE devices in chip format, but also make them easier to integrate with other separation methods or a mass spectrometer. Also, the shorter overall length makes the imaging of all column image an easier job to accomplish.

Moreover, more efficient Joule heat dissipation in small diameter capillaries enable the use of higher electrical field and therefore speed up the focusing process. It is observed in the experiment that by reducing the IEF channel length from 5 cm to 1 cm, we can

decrease the separation time from 10 minutes to 1 minute with the same external potential applied. Because the same pH gradients are establishing in both short and long channel, shaper and more concentrated peaks can be expected inside smaller scale capillaries(Mao and Pawliszyn 1999; Mao and Pawliszyn 1999; Yao, Anex et al. 1999; Wen, Lin et al. 2000; Bousse, Mouradian et al. 2001; Raisi, Belgrader et al. 2001; Wu, Sze et al. 2001; Han and Singh 2002; Wu and Pawliszyn 2002; Herr, Molho et al. 2003)..

Chapter 3 Gel Polymerization in Porous Micro Fluidic Channel

3.1 Why Gels in Micro Fluid Channel

Micro-machined capillary electrophoresis devices have outstanding flexibility in arranging channel layout, such as locations of reservoirs, separation channels, PCR chambers, detection region, and so on. However, microdevice has been limited by the difficulty in the integration of various component, such as heaters, detection sensors, reaction chambers, and valves.

With the progress in microfabrication, the use of photosensitive material enables in-situ polymerizations that are easy being loaded into channels. Materials like acryamide and bisacryamide or poly(ethylene glycol) and diacrylate can be polymerized into cross linked gel matrix with the presenting of photoinitiators like 2,2-Dimethoxy-1,2-diphenylethan-1-one or 2-hydroxy-2-methylpropiophenone (Irgacure651 or HOMPP). These kind of functional gels can be used as biomolecule separation material, flow control valve(Beebe, Moore et al. 2000), microreactors or microsensors (Zhan, Seong et al. 2002), and so on. Also, they are relatively inexpensive to implement, and can be made to have different pore sizes for different applications.

3.1.1 Microchip Polyacrylamide Gel Electrophoresis (Bio molecular Separation)

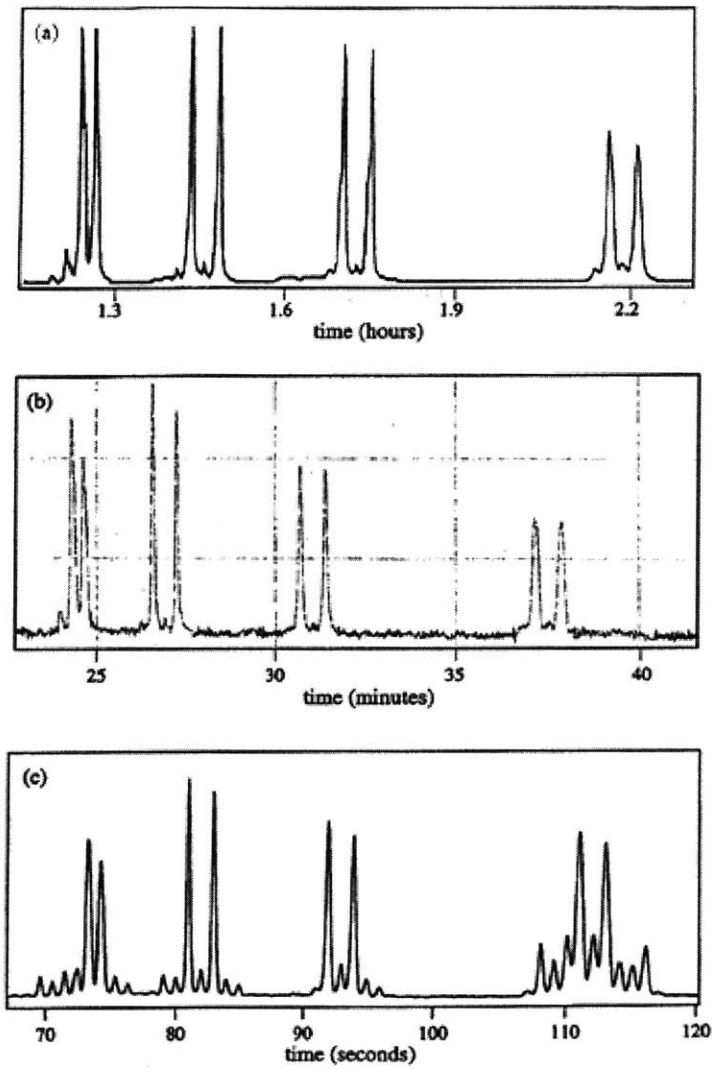

Figure 12: Comparison between gel electrophoresis, capillary gel electrophoresis and microchip electrophoresis:
(a) the gel plate separation, 2.3 hours has been spent with 28 Watts power consumption; (b) capillary separation with 38 cm effective length and 200 V/cm, the whole separation process took 37 minutes; (c) PAG chip separation with 26 mm channel and 200 V/cm

electrical field strength, separation done in 2 minutes.

By the use of polyacryamide gel inside microfabricated channel, one can achieve the same level of resolution with two orders of magnitude less time compare to slab gel electrophoresis in very short separation distance. Shown in the figure 12 is the experiment done by Daniel et al.(Schmalzing, Koutny et al. 1997) using polyacrylamide solution. The overall length of the microfabricated channel is 26 mm with 4% polyacrylamide solution cured inside. The comparison between PAG plate, PAG capillary, and PAG chip is shown. Even better solution can be achieved by solid polyacrylamide gel. (Brahmasandra and Ugaz 2001; Han and Singh 2002).

3.1.2 Flow control

Reported by Crooks et al. (Seong, Zhan et al. 2002) is the use of hydrogel as isolation valves for reaction chamber. By using in-situ UV polymerization, the pre-injected gel solution will be hardened and therefore make isolated chambers required for biochemical analyses assay.

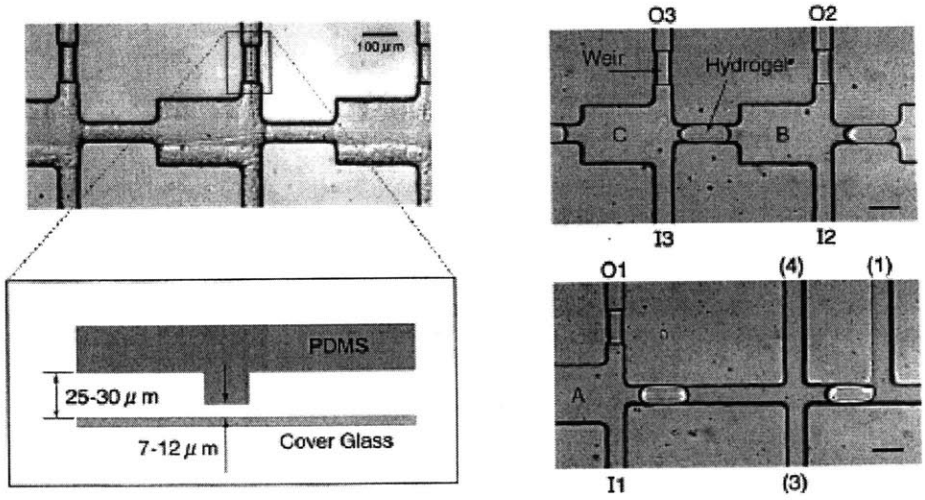

Figure 13: UV-cross-linked hydrogels and photolithographically:

Defined weirs as the flow control valve, the three microchannels are later demonstrated by capture and release complementary DNA targets from a complex mixture.

3.1.3 Microreactors or Microsensors

The other useful application of UV curable polymer solution is microreactors or microsensors. By immobilized biological materials like enzymes onto the gel, it is possible to sense cells, proteins, or DNA in a more effective way (Greenway, Haswell et al. 1999; Guenat, Ghiglione et al. 2001; Ikuta, Ikeda et al. 2001; Lu, Schmidt et al. 2001; Yamamoto, Nojima et al. 2001)

3.2 Free Radical Polymerization

Synthetic polymer gels above-mentioned are made by UV sensitive reactions, termed "free radical polymerization", one of the most common and useful reaction making polymers. In these reactions, we initially create free radicals by breaking a pair of electrons into two initiator fragments. This first initiation step is launched by mixing catalysts like tetramethyl ethylene diamine (TEMED) and ammonium persulfate (APS) (chemical initiation), or high energy UV light that can break the vinyl bond in monomers of photoinitiator molecules (UV-photoinitiation). Each of these initiator fragments can create a chain reaction until terminated by either the other unpaired electron or oxygen quenching phenomenon. In other words, free radical reactions are vinyl monomer reactions, small molecule containing carbon-carbon double bonds, that make polymers by constantly grabbing free electron form other species. There are varieties of polymers making by free radical polymerization, including polystyrene, poly(methyl methacrylate), poly(vinyl acetate) and branched polyethylene. We can easily find these materials in items such as grocery bags, toys and cups.

3.2.1 Free Radical Polymerization of Acrylamide gel

Polyacrylamide gel is the most commonly used sieving matrix in proteomics. The polymer matrix of it is prepared by acrylamide and bisacrylamide linking through aminocarbonyl groups. In general case, it takes about 30 minutes to prepare a polyacrylamide gel for gel electrophoresis (in the scale of 30 cm). Depends on the pore size required, one can adjust the acrylamide to bisacrylamide ratio from 60:1 to 29:1.

Although it is a well-established process in gel electrophoresis, it encounters serious oxygen quenching problem when we tried to do the polymerization in micro scale channel made by oxygen-permeable materials. PDMS is particulary problematic since the material is highly permeable to oxygen and other gases.

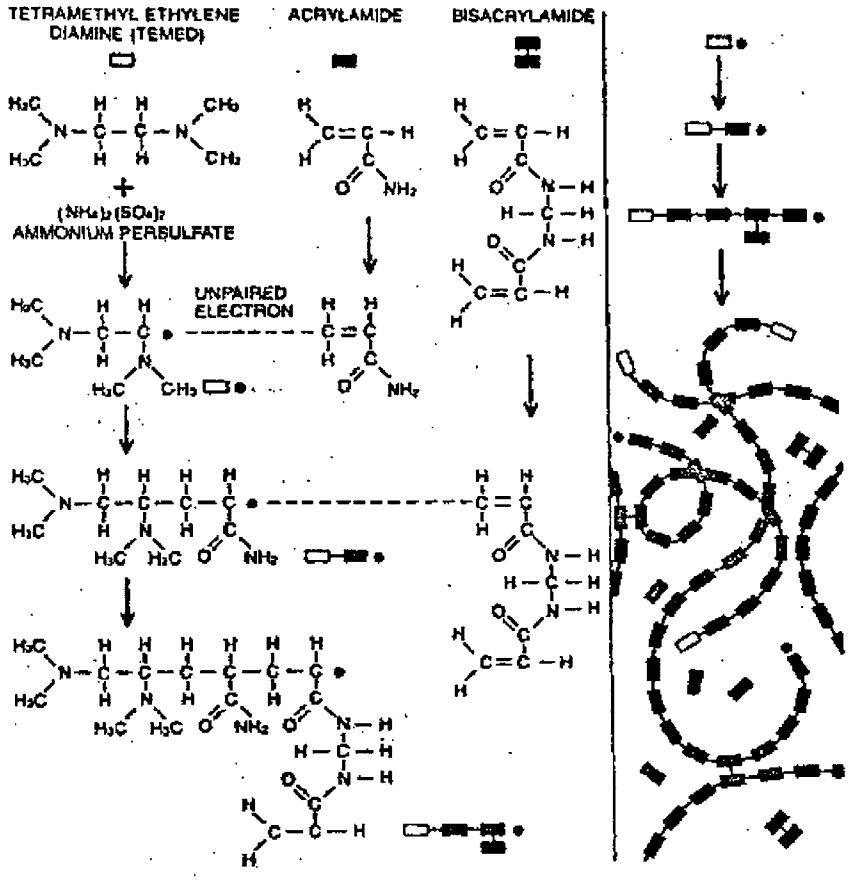

Figure 14: Polymerization of Polyacrylamide Gel:

Proceed by way of a chain reaction. Steps including initiation activated by TEMED and APS, propagaion by free end shifting through $-CONH_2$ groups, and termination by interconnections. (Tanaka 1981)

Table 1 Oxygen Permeability

(Zheng, Mastrangelo et al. 2002)

Oxygen Permeaility (g/cm-s-toor)	
Polycarbonate	10^{-11}
Parylenes (Plastic Material)	10^{-10}
Glass	10^{-16}

3.2.2 Free Radical PEG-DA Polymerization or Methacrylate

Polymerization

One possible solution to make gel in microfluidic channel is using less oxygen sensitive monomers like poly(ethyleneglycol) diacrylate (PEG-DA) or methacrylate. As discussed above, researchers can make PEG-DA or 2-(Dimethylamino)ethyl methacrylate even within a PDMS channel. Without the presenting of amino side chain, the initiator fragments are known to be less reactive and therefore have less damaged by oxygen quenching. By the use of acrylate gels, one can make gel in 100 μm channels without difficulty. However, the polymerization in 10 μm thickness channel is still a challenge.

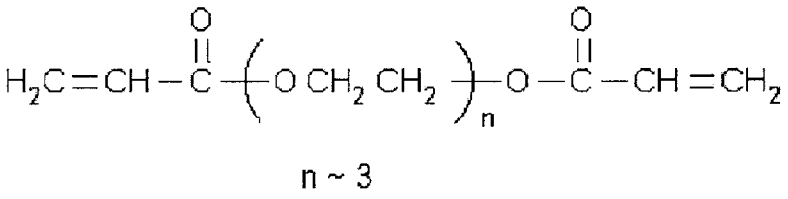

Figure 15: PEG-DA

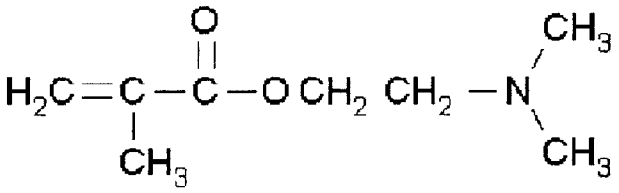

Figure 16: 2-(Dimethylamino)ethyl methacrylate

3.2.3 Solution toward Oxygen Quenching Problem

The difficulties of polymerizing in micro-scale channel come from the oxygen diffused from the ambient. Therefore, gels can be obtained by either using silicon based channel (Ugaz, Burke et al. 2002) or coating the channel with some oxygen barrier like parylene, alumina, or silicon oxide(Zheng, Mastrangelo et al. 2002). Known from Table 2 and 3, glass or parylene have oxygen permeability four to six orders of magnitude smaller than PDMS that both of them can be chose as coating materials.

Table 2 Permeability Comparison between Parylene and PDMS

Oxygen Permeability (cc-mil/100 in^2-24 hours)	
Parylene C	5
Epoxies	5-10
PDMS (Silicones)	50,000
Urethanes	200

The other possible solution to achieve polymerization of polyacrylamide gel is using nitrogen purging to decrease oxygen concentration. It is possible to decrease oxygen concentration by several orders with a well-fabricated nitrogen-purging chamber.

3.3 Polymerization within oxygen-free environment

By using a homemade nitrogen purging chamber shown in Figure 17 with a high power UV light source (1 W/cm^2). We can accelerate the reaction by six orders of magnitude and therefore polymerize the gel inside PDMS channel without passivation.

Figure 17: Nitrogen Purging Chamber Coupling with UV fiber

The nitrogen-purging chamber we have has a three-way valve on it, which connects the desiccators, nitrogen tank, and a vacuum pump together. Compared with polymerization in atmosphere by typical UV lamp system, that is able to polymerize bulk gel. The UV chamber system has 1000 times stronger light source and 1000 times lower oxygen concentration. Therefore, polymerization of polyacrylamide is possible inside the UV chamber system.

Figure 18: UV lamp with 10mW/cm^2 intensity

Table 3 Gel Composition Used in Purging Experiment

Gel Composition	
VA-086 Initiator 5 %	50 mg
Acrylamide, bis-acrylamide solution 16%	400 μl
DI Water	350 μl
Tris-Cl 0.375 M	250 μl

Shown in Picture 19 are the pictures of partially polymerized polyacrylamide gel in non-passivated PDMS channel. In the upper picture, there still some unpolymerized solution around the boundary. This purging process is still under development, perhaps by simply a CYTOP or parylene coating we can cured acrylamide gel inside PDMS channel.

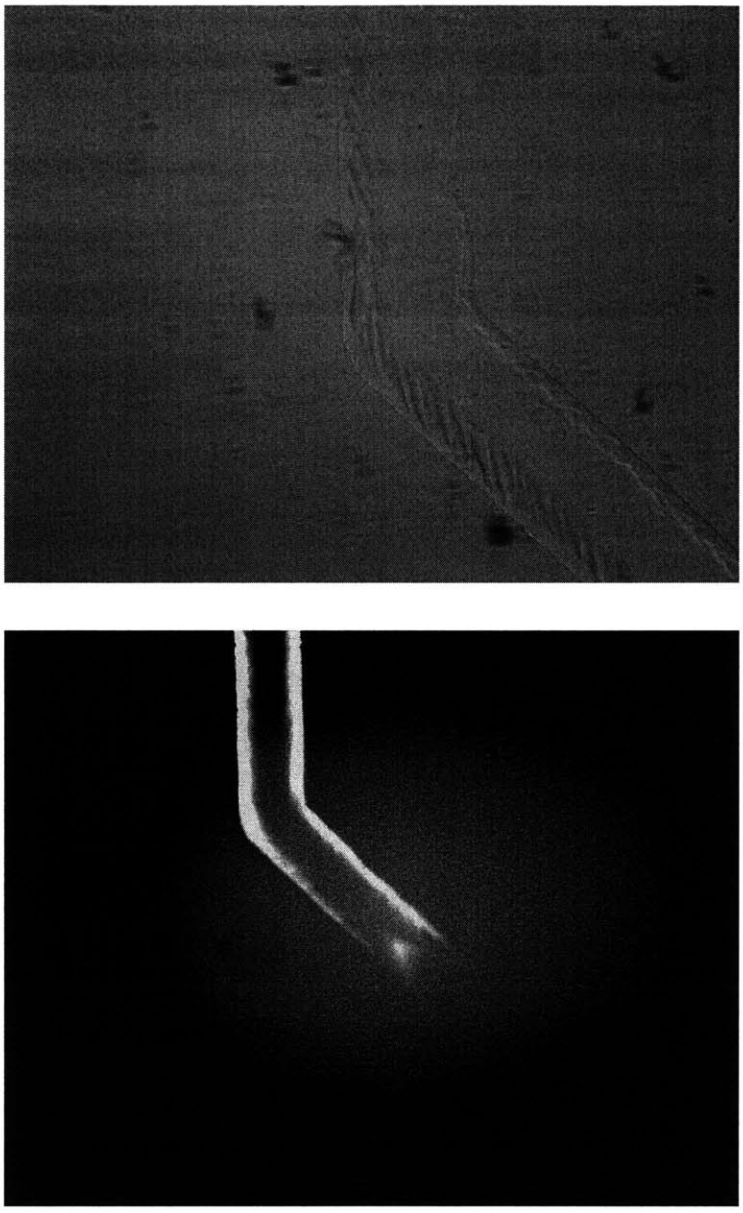

Figure 19: PAG in PDMS channel and its electrophoretic protein separation:
The unpolymerized boundary caused leaking while doing fluorescent electrophoresis (top: optical micrograph, bottom: fluorescence micrograph with labeled proteins)

Chapter 4 On-Chip Protein Separation Coupling IEF and CE

4.1 Design Principle

The need to couple different separation technique comes from the complexity of protein samples. Due to the diversity of proteins, there is no single isolation method that can be used to distinguish all of them. As a result, more than one separation steps are required, to pre-separate as well as to pre-concentrate, before protein identification processes such as mass spectrometry can be done. Such pre-separation can be carried out with various methods like centrifugation, electrophoresis, or chromatography. Depending on the identification (detection) techniques to be used, different combinations of separation techniques should be chosen. For instance, ultra-filtration and affinity chromatography were used to remove high abundant proteins as a pre-separation process. However, the increasing in complexity and sample loss from additional purification steps make them less preferable.

Unlike previous IEF and CE coupling experiment(Herr, Molho et al. 2003; Li, Bush et al. 2004), our design focus on isolation between incompatible buffers. Without active valve control, the resolution form first dimension IEF is hard to maintain. Even more, high ionic strength CE buffer in free solution CE will disturb the focusing of ampholytes. No

focusing could be detected if coupling IEF and free solution CE without an isolation valve. In our experiment, we chose Quake's valve design, because it has very small dead volume, which is very important feature to prevent proteins form inter contaminations.

4.2 Materials and Reagents

The microchip was made out of PDMS (polydimethylsiloxane), as described earlier (Duffy, McDonald et al. 1998). The Silicone Elastomer Kit (Serial No. Sylgard 184, Dow Corning) was mixed at 10:1 ratio and degassed in a desiccator under about 300 Torr vacuum before pouring or spinning onto master molds. After curing in 65 °C conventional oven for at least two hours, different layers of PDMS were treated with oxygen plasma in a plasma cleaner (Serials No. PDC-001, Harrick Scientific Corporation) connecting with oil-sealed vacuum pumps (Serial No. TRIVAC E, Leybold vacuum) before the bonding. Master molds were fabricated by photolithography with two different photo resist. A positive-tone photoresist (Serial No. AZ-4620, Clariant Corporation) was used to form 12 μm wide fluid channel patterns using standard photolithography, then the patterns were heated to 150 Degree for 30 mins to make the channel to have a round-shaped profiles. A negative-tone photoresist (Serial No. SU-8 50, Newton) was used to for the 40 μm thick channel for the valve control. The ampholyte sample mixture was made of 2 to 3% ampholyte (Serial No. eCAP SDS 14-200 Kit, Beckman Coulter), 10 % Glycerol (Catalog No. 161-0763, BioRad), 1% methyl cellulose (Catalog No. 27,442-9, Sigma-Aldrich), and protein samples in aqueous solution. To have a better mixing in the peak transfer region, same concentration of methyl cellulose was used in all solutions including separation gel (90% eCAP SDS 14-200 Gel Buffer and 1 % methyl cellulose), anolyte solution (acetic acid: 0.1 M, pH 2.5), and catholyte

solution (ammonium hydroxide 0.5%, pH 10.5). As for the CE experiment, the buffer is mixed by For the free solution CE experiment, buffer solution contains 0.375 M Tris-Cl (pH 8.6) was used. The imaging of fluorescent signal was achieved by an inverted epifluorescence microscope with a build in 100W mercury lamp (Model No. IX-71, Olympus) though a standard FITC filter set (Omega Optical Inc.). In this experiment, thermoelectrically cooled CCD camera (SensiCam, Cooke co.) was used for fluorescence imaging through the side port of the microscope. Sequencing images were taken and analysed by IPLab 3.6 for Windows (Scanalytics) at a rate of 1 Hz. Protein samples used here were one naturally fluorescent green fluorescent protein (GFP) and three fluorescein- or Alexa- (Alexa Fluor 488, Molecular Probes) labelled proteins: ovalbumin, low density lipoprotein, and trypsin inhibitor were used as pI and MW markers. All protein pI markers for the pI distribution experiment and other chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

4.2.1 Characterization of Material and Reagent

Comparing to other frequently used silicone elastomer kit (Serial No. RTV 615, General Electric) with tensile strength 6.3 MPa and elongation rate 120%, Sylgard 184 has tensile strength 7.1 MPa and elongation rate 140%, that makes it a better candidate for devices with hydraulic valves. In strong electrical filed applications like capillary electrophoresis, high-pressure valve operation is especially important. Because by increasing the pressure from 30 psi to 100 psi, we can increase the valve resistance from 688 MW to GW range. The high resistance is not measurable by lab-use multimeter. Under this resistance (both fluid and electrical), no protein inter contamination was observed even

after thirty minutes. The 1 fL dead volume can be further reduced by better alignment system instead of the stereomicroscopy currently used.

4.3 Chip Fabrication

To isolate and control channels, multi-layer microfluidic valve systems reported by Quake et al. were used to fabricate the control valve for the fluidic channel. The fabrication includes three major processes: 1) master fabrication, 2) PDMS shaping, and 3) PDMS multi-layer bonding.

The master fabrication requires both positive tone and negative tone photoresist, because of different requirement in separation and control channels. We found that the best valve operation regarding the pressure applied and resistance acquired is having a 150 μm separation channel with a 300 μm control channel. In the separation channel, positive tone photoresist must be used because it has to be reflowed into round shape to provide lower mechanical stiffness. Without the 95 °C, 30 minutes baking, the channel is extremely hard to be sealed. As for the control channel, in order to obtain at least 30 μm height (PDMS is known to have 10:1 aspect ration), negative tone SU-8 serious must be used(Odom, Love et al. 2002). The 12 μm thick separation channel mold and 40 μm thick control channel mold were treated with silane vapor for 30 minutes to prevent adhesion with silicone rubber.

After the silane treatment, mixed PDMS was spun onto the bottom separation channel mold under 2,000 rpm with a target thickness 70 μm. In the same time, the top control channel was poured with about 8 mm thick PDMS and cured in 65°C oven together for two hours. Before the final bonding process, a metal syringe needle (Serial No. 91016, Hamilton Company) with 1/16" OD was used to punch holes though the end of control

channels for future connection with 1/16" Teflon tubing as pressure control interface. From our experiment, the elasticity of PDMS itself is the best way to seal the tubing comparing with epoxy, PDMS or other silicone glues. By doing so, we can apply more than 100psi without leaking.

At last, the oxygen plasma bonding reported by Beebe et al.(Jo, Lerberghe et al. 2000) was used to bond first the 8mm control channel layer on to 70 μm separation channel and then together on to the other bottom PDMS membranes. By having a homogeneous channel, we can minimize uneven electroosmotic flow (EOF), which might cause excessive band dispersion. After bonding together under microscopy inspection, the device should be left overnight (at least two hours) to have the maximum bonding strength. Because PDMS is gas permeable, the top layer control channels must be filled with water before the first use to prevent air diffused in separation channels under high-pressure operation. Otherwise, the operation of valve will drive air bubbles into the channel, which is catastrophic in separation. The perspective view of the device is shown in Figure 1-(a).

Surface Treatment

PDMS is sometimes considered unsuitable material for molecular separation device, because of its non-uniformly charged, protein adsorptive surface(Liu, Fanguy et al.). In order to have analytical and repeatable experimental result, it is essential to prevent protein adsorption problem and decrease uneven EOF in either in PDMS or glass microfluidic protein separation devices. Compared to glass channels, PDMS has lower EOF and less adsorption(Ocvirk, Munroe et al. 2000). However, certain PDMS channel

coating is still required to have better separation.

In our experiment, we used polyacrylamide coating, which has been well developed in glass channel(Hjerten 1985) to prevent protein adsorption. To grow acrylamide polymer on PDMS surface, we first coated PDMS channel with 3-(Trimethoxysilyl)propyl methacrylate as adhesion promoter for polyacrylamide. This silanization has no adverse effect on samples subjected to isoelectric focusing as described in manufacture date sheet. Then, polyacrylamide solution was mixed with tetramethyl ethylene diamine (TEMED) and ammonium persulfate (APS) before introduced into the microfluidic channel for polymerization chain reaction. Right after the mixing, it was put in a home made vacuum chamber with nitrogen purging valve to eliminate oxygen quenching problem in free radical polymerization(Zheng, Mastrangelo et al. 2002). After thirty minutes reaction time, 0.5% methylcellulose solution was used as pre-treatment coating material to interfere with protein-wall ion-exchange mechanism. Comparing to detectable adsorption with only dynamic methylcellulose coating, no adsorption was observed with current detection system after coated the channel with this process. However, no further quantitative experiment has been taken yet.

4.3.1 Chip Operation.

The major operation involves two sets of valves (top control layer in Figure 20-(a)) designed to isolate different separation media and to transfer IEF focused peaks in the transfer region. By closing valves with 80 psi pressure in different sequence, a 2D separation without cross-contamination can be achieved. The 2D separation has four major steps including CE separation media injection, ampholyte and sample mixture injection, IEF focusing, and CE separation. The CE separation media can be either liquid gel made of agarose polymer or free solution with target pH value. First, the right valve control line was pressured to close the fluid channel below it. By clogging the channel, we can inject liquid gel without contaminating our IEF channel, which is very sensitive to higher concentration of ions (Figure 20-(b)). The operation pressure for separation media injection is 20 psi for free solution and 30 psi for gel solution depends on the solution viscosity. Pressure up to 60 psi could be applied to the channel without affecting the closure of the valve.

Then, the left valves (valve #1 and 2) were pressurized to isolate liquid gel. After closing left valves, right valves were opened for the injection of ampholyte and sample mixture (Figure 20-(c)), followed by replacing the reservoir solutions with catholyte and anolyte solutions. IEF separation was achieved by applying an electric potential between anode and cathode reservoirs. Due to the electroosmotic flow generated in the IEF channel, peaks are slowly mobilized from anolyte to catholyte reservoirs, and protein peaks will enter and leave the central peak exchange channels sequentially based on their pI values. After the IEF of proteins was established, one can isolate any pI region of interest from

the other peaks by closing the valves (valve #3 and 4). When valve #1 and 2 are open, isolated protein peaks focused within this region will reacquire charges and be sent to the second dimension separation channel by electrophoresis (Figure 20-(d)). Since protein IEF is maintained until the very last moment of peak isolations, the resolution obtained from the IEF separation is maintained, and the interdiffusion and dispersion of peaks caused by coupling two separation techniques was minimized. During this migration, proteins will be separated based on the separation column chosen for the second dimension (charge-to-size ratio for CE column, for example)

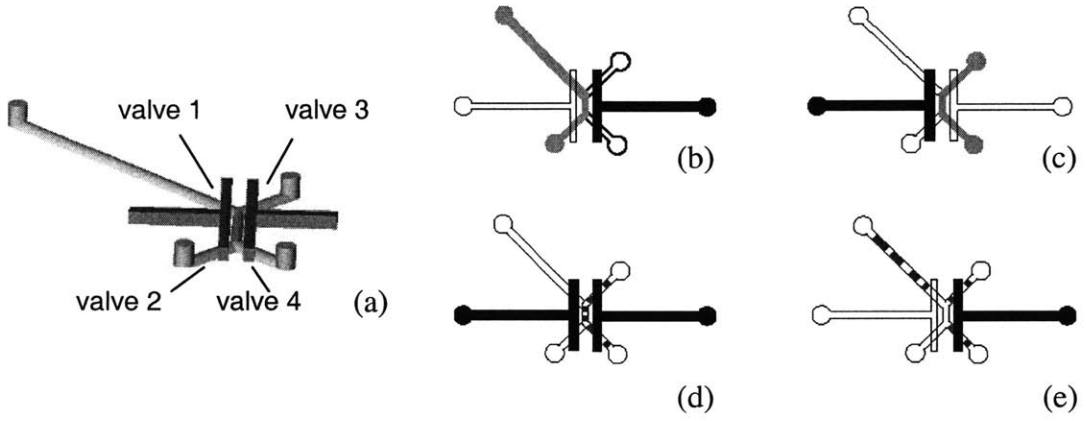

Figure 20: Schhematic drawing of double layer PDMS channel and the four step separation process

After the focusing, we trap target proteins, open left valves to mix proteins with distinct separation media. Then, electrical fields are applied to initiate second dimension CGE. In this step, the stacking of protein sample can happen since low conductivity ampholyte solution is being mixed with 0.4M high-conductivity Tris-Cl CE or CGE buffer. As a result, proteins mixtures in certain narrow pI range were separated again based on

different charge to mass ratio (Figure 20(e)). The whole separation process takes 10 minutes.

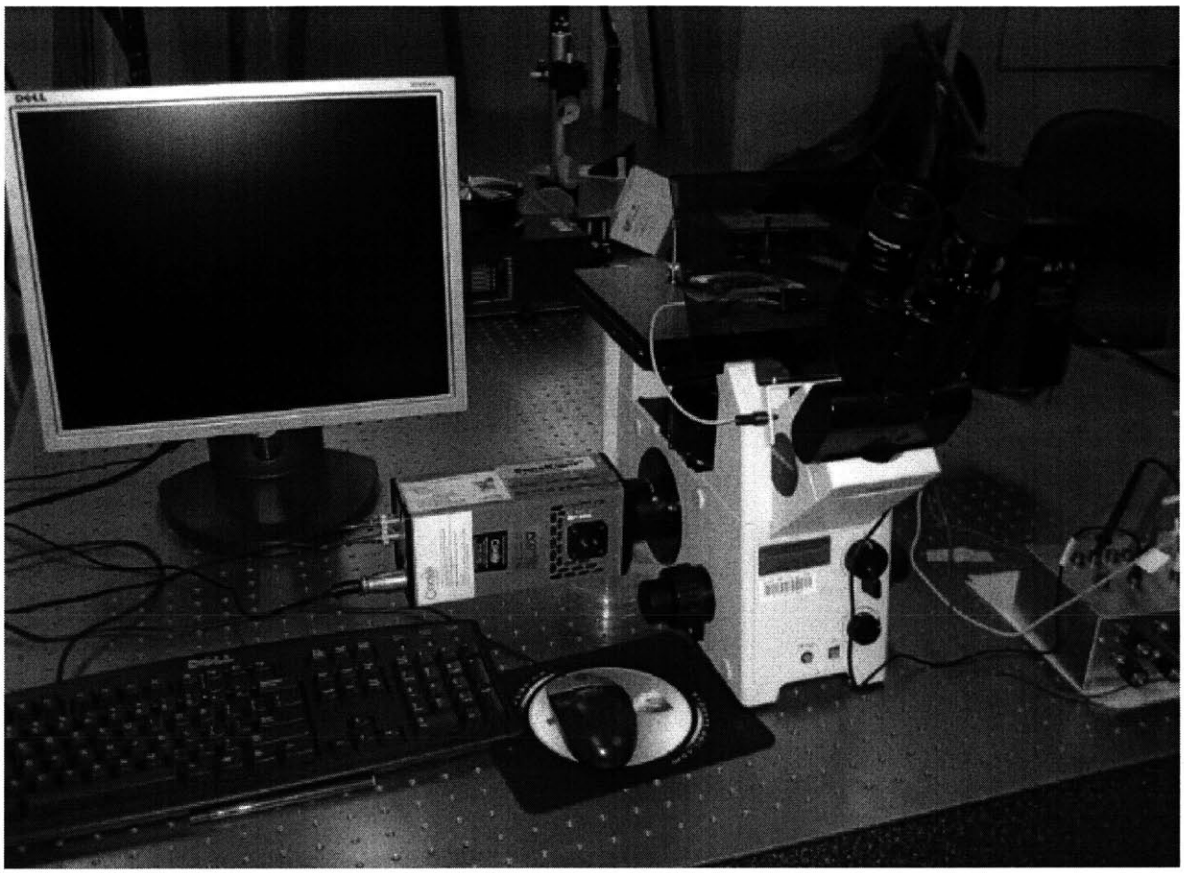

Figure 21: Experiment Setup:

Including CCD camera, inverted fluorescent microscope, and power supply. (Syringe holders not shown in this pictures)

4.4 Results

In this section, we first present the linearity of the first dimension IEF and ways to achieve better separation. Then, on-chip 2D separation including IEF-CGE and IEF-CE are shown and compared. Moreover, several concept-proof experiments are presented to demonstrate both the ability to choose target protein in IEF and the distinct mobility of second dimension CE form the first dimension separation. Last, relation between IEF and CE are discussed to have better understanding about 2D on-chip separation system.

4.4.1 IEF of Proteins in an Ultra-short PDMS Channels

In this linearity experiment, eight IEF-Makers from fluka with pI 3.0, 4.5, 5.1, 6.2, 7.2, 8.1, 9.0, 10.3 with 10 μg/ml concentration were used to help identify the dynamic pH gradient established in the PDMS channel. Except the markers, 10% glycerol, 3% methylcellose, and 4% ampholyte from BioRad were mixed together in the sample mixture. These IEF-Markers have excitation wavelength from 330 to 340 nm and emission wavelength from 415 to 500 nm. Therefore, An ultrahigh-vacuum mercury burner (280 nm to 600 nm) coupling filter cube with specified bandwidth (Serials No. XF 1001, 550WB300, Omega Optical) were used for the epi-fluorescent detection.

As shown in Figure 22, IEF markers were used to test the ability establishing a wide pH gradient. It shows a convincible evidence of having a relatively high resolution dynamic gradient from pH 3 to pH 10. The whole column image was taken after applying 500 V/cm electrical fields for 30 seconds and IEF markers were used to visualize the pH

gradient. The overall 10 mm length of the channel makes the voltage required across it small compare to 20 to 30 kV in capillary format CIEF. Moreover, the focusing time required in short IEF is two orders faster than typical capillary isoelectric focusing (CIEF) (30 to 40 minutes), where proteins have to travel long (10~30cm) capillary. Conventionally, pH gradient in a gel use either amphoteric buffers containing carrier ampholytes or immobilized pH gradient gel containing immobilized solutions to establish and maintain the gradient. In the carrier ampholyte case, the amphoteric buffers provide a quick and easy way to establish dynamic gradient, however, it has many disadvantages listed in Table 4. Although much improved resolution (ultra narrow, up to 0.001 ΔpH) can be done by using immobilized pH gradient gel(Bjellqvist, Ek et al. 1982), the casting and forming immobilized gel is technically challenging in either chip or capillary format IEF.

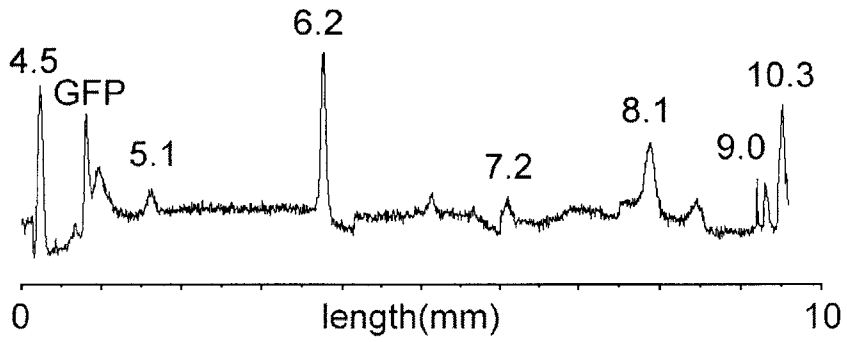

Figure 22: Intensity plot of GFP and fluorescent markers:
Focused by their pI values in 1 cm by 100 μm by 20 μm PDMS microfluidic channel.

4.4.2 Factors that affect Separation – Viscosity and EOF

Therefore, to have a relatively high resolution dynamic gradient by using carrier ampholyte, we have to control the viscosity, electrical field, ampholyte concentration, as well as the channel passivation very carefully. By controlling the viscosity using different methyl cellulose concentration, we can focus those high pH makers before being flushed out by EOF. However, the uneven buffering capacity (conductivity) and EOF makes the pH gradient in the channel nonlinear, especially in the high pH side. One of the major reasons having uneven buffer capacity is the commercial available ampholyte mixture we used was optimized for coated fussed silica capillary. It is believed that, by balancing the concentration distribution for each ampholyte component to mach the local EOF condition, and doing better channel passivation, one can have more linear gradient. In these experiment, we had tried mixing 3.8-5.2, 4.8-7.2, and 8.1 to 9.8 ampholytes in different ratio with observable difference in pH gradient distribution. These experiments show that changing the composition of each component in ampholyte mixture does affect the linearity of the pH gradient. However, no satisfying results have yet been obtained, and further study and characterization could be done to make the IEF more linear.

Unlike IEF in IPG gel, the capillary IEF (CIEF) is establishing a dynamic pH gradient. Typically, capillary IEF uses 27 cm fused silica CIEF with 50 μm i.d. and on-line UV detection. To perform a CIEF, one can either do a two-step CIEF including focusing and mobilization or one-step CIEF that simultaneous focusing and mobilization proteins. The first two-step CIEF was first developed by Hjerten and Zhu(Hjerten and Zhu 1985) where a coated 27 cm capillary were used to perform IEF in 1.5 % ampholyte and 0.4%

Methylcellose. After the focusing, the peaks were driven through the UV detector by low pressure or chemical salt. The sharp peak can be maintained because the voltage was applied constantly during the flushing. In the contrast, one-step CIEF takes advantage of the strong EOF in uncoated capillary to focus and mobilize proteins simultaneously. To prevent samples being flush out before focused, 90 cm long untreated capillary must be used, which in turn required much longer separation time and higher potential. Compare to short fused silica CIEF done by Pawliszyn et al., which take 280 to 360 seconds to reach equilibrium. The presented ultra short IEF on microfluidic PDMS chip has better S/N and separation properties than the experiment by Pawliszyn et al. (Wu and Pawliszyn 2001)

Known from these operations, the control of EOF is crucial to have a reliable isoelectric focusing. (EOF in untreated fused silica in pH 10 solution is about 7×10^{-4} cm^2/Vs)(Kutter, Jacobson et al. 1998) Compare to CIEF where 25 cm or longer fused silica capillary is being used, where the acrylamide coating can decrease the EOF from 10^{-6} to 10^{-8} cm^2/Vs (Munro, huhmer et al. 2001) In a 25 cm channel, it takes two to three hours for a focused peak to move out from the middle to the exit. However, because of the extremely short channel (1 to 5 cm) used in chip separation, no mater how we decrease the EOF, samples will be flushed out in about one minute. However, this problem can be managed as long as the operation of peak selection and isolation is performed before the complete flushing of samples out of the device.

Among the control factors, the control over EOF seems to be a dominating one. In our experiment, to prevent high pH proteins being flushed out by EOF before being focused,

channel no longer that 2 cm can be used, because proteins in a short IEF channel doesn't have to move as much they did in capillary IEF. Likewise, by having an ultra short channel, we can cut down the equilibrium time (from 40 mins to 30 seconds) and overall separation voltage required (from 30 kV to 500V). Although, ultra short channel makes the IEF much more sensitive to the EOF and harder to control compare to CIEF, the short focusing time enable proteins overcome strong EOF in high pH region that makes 3-10 focusing possible.

To overcome the catholic EOF in the channel, the electrical field and viscosity of the solution should be carefully calibrated. In general case, protein molecules moving from the anode side would get focused much more quickly, while molecules coming from the cathode side of the channel get focused slowly because they have to overcome EOF fluid flow during the focusing process. (Figure 8) As a result, IEF looks like started from the anode toward the cathode. However, by coating the channel and tuning the viscosity, a stable IEF that focuses from both cathode and anode can be done.

However, the coating did not solve the entire problem. The biggest challenge in establishing 3-10 pH gradient in chip format is to compete the pressure developed across the channel. After established the pH gradient for IEF, it is unavoidable having uneven charge densities in different regions of the chip. Therefore, the essential pH gradient built in IEF certainly leads to the pressure developed across the channel. To avoid this, we can possible coat the channel with pH inert material, or adjust local EOF by tuning ampholyte composition.

4.4.3 IEF coupled with Second dimension capillary gel electrophoresis

In this experiment, one fluorescent GFP (aprox. pI 5.6, MW 26k) and three fluorescein or Alexa Fluor 488 labeled proteins were used as pI and MW markers, including ovalbumin (aprox. pI 5.1, MW 45k), low density lipoprotein (aprox. pI 5.11, MW 179k), and trypsin inhibitor (aprox. pI 4.6, MW 20k). The liquid gel buffer used here is SDS 14-200 Gel Buffer (Serial No. 477420, Beckman Coulter) containing 3.0% Tris(hydroxymethyl)aminomethane and 2.0% Ethylene Glycol.

Shown in Figure 23, four steps separation and transfer process described before were used to perform 2D separation of these protein samples. In the first step, 500 V/cm electric field was applied on the 1 cm IEF channel, which is isolated from the second dimension CGE channel by closing the hydro-valve. After 130 seconds into the first dimension separation, target proteins were mobilized into (by EOF) and trapped in the peak transfer region by closing the left valve set. Then the valves to CGE channel were opened for 30 seconds without applying electrical field for CGE, to prevent abrupt concentration change between distinct separation media. However, no significant differences were observed without doing so.

At last, 200 V/cm electrical fields were applied to mobilized proteins for CGE separation. In these labeled proteins, the fluorescein and Alexa Fluor dyes are designed to replace the -Lys groups on them. Thus, depending on the uniformity of labeling, proteins could

acquire different numbers of dyes with slightly negative charged and therefore could have multiple pI values(Richards, Stathakis et al. 1999). Shown clearly in figure 23 (a), the broadened IEF bands were mostly coming from the labeling process, while the local non-uniformity of EOF could also be a possible reason for it. In Figure 23(c), the stacking mechanism was shown while low conductivity ampholyte solution move into 0.4 M high conductivity Tris-Cl gel. This stacking can be a pre-concentration process before second dimension CE. Because higher-conductivity environment (lower resistance) has lower field strength, proteins will encounter a sudden decrease in electrophoretic velocity at the buffer boundary (electrokinetic focusing). Therefore, samples will stack together until buffers get thoroughly mixed together. Also, the high viscous gel media for CGE has a sieving effect that contributes to this stacking mechanism too. if we compare CE and CGE result, CGE separation has more peak broadening The broadened peaks result from the sieving interaction between gel polymer and protein molecules.

The existence of ampholyte in the CGE channel does not affect the resolution of CGE separation. Most likely, the ampholytes in IEF mixture will be flushed out by electrophoresis in very short time, due to their smaller MW. Just after closing the valves, proteins are surrounded by ampholyte molecules with the same pI values, which makes the protein neutral. However, if one waits a short time these different ampholyte buffer molecules inter-diffuse, and render protein molecules charged again. This waiting period does not affect the separation resolution since the protein peaks are physically isolated from other proteins.

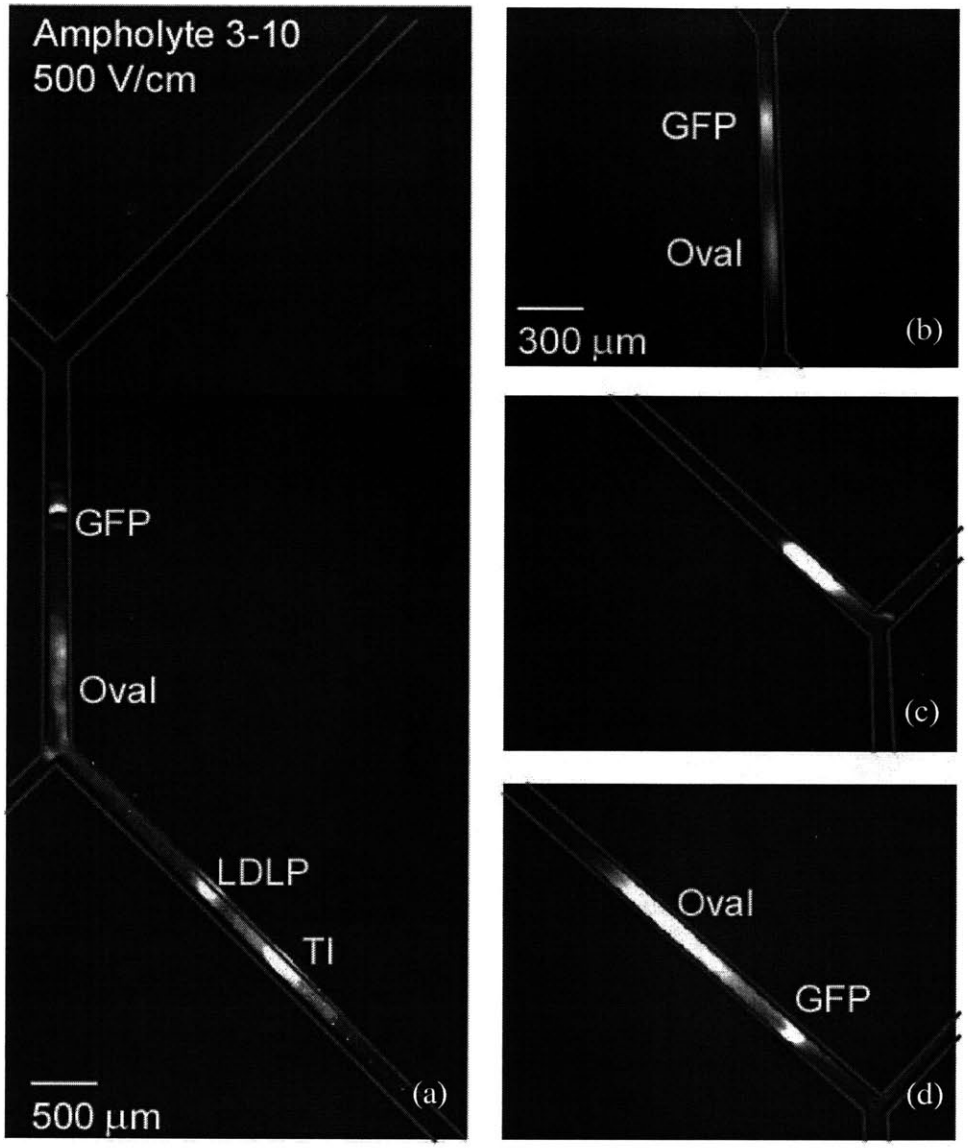

Figure 23: 2D protein separation CCD camera images:
(a) IEF done 130 secs after applying 500 V/cm to 1cm long, 12 μm deep, 150 μm wide channel; (b) 30 seconds after trapping. Peaks gradually diffuse and reacquire charges; (c) Stacking effect; (d) Proteins further separated by different charge to mass ratio.

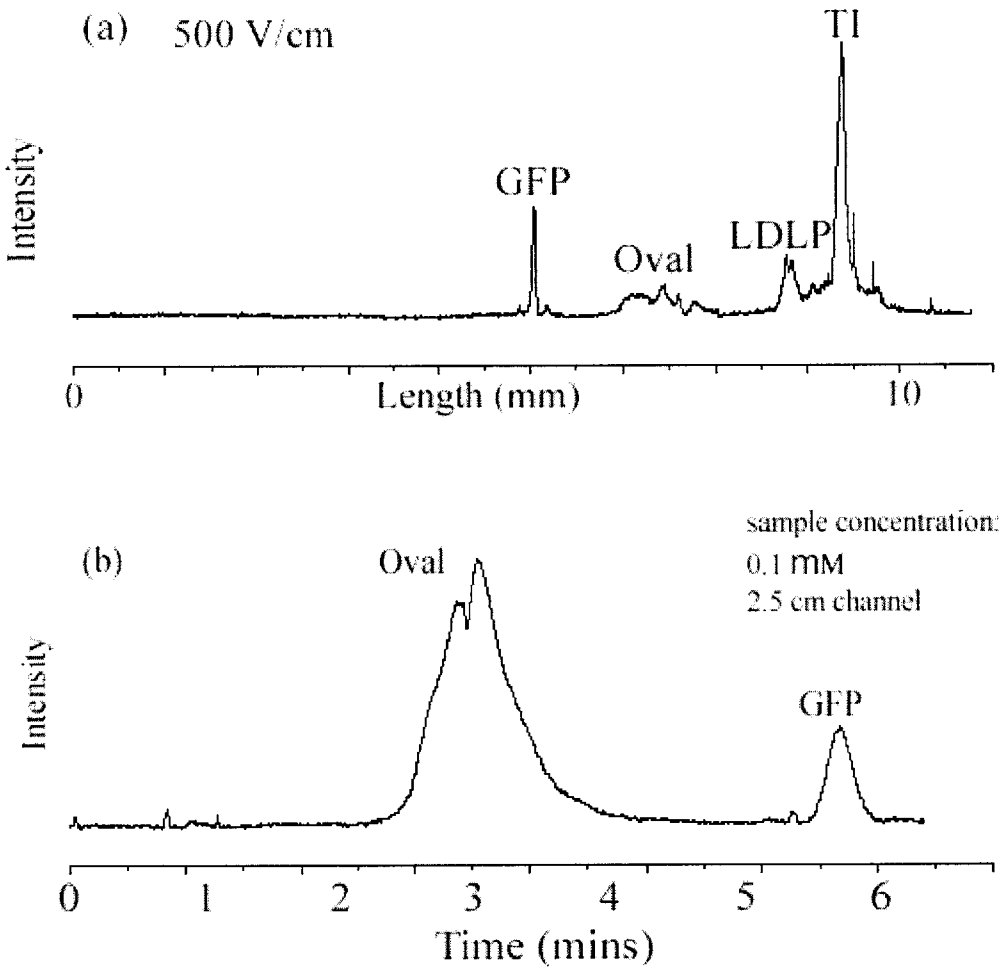

Figure 24: 2D separation intensity charts:
(a) whole column intensity chart after IEF, 3-10 ampholyte; (b) Second dimension CGE (fixed point detection).

The whole separation was done in 10 minutes including the valve operation, IEF, concentration balancing, and CE. As for capillary electrophoresis, the control and understanding over electrophoretic mobility is the key toward a nice separation. Factors including channel wall passivation, ion strength in the solution, buffer additives all affect the final result. Tuning buffer additives and ionic strength of the solution increases the

peak resolution. With the wall passivation, the unwanted protein adsorption and EOF can be eliminated. The intensity charts shown in Figure 24 were taken by fix the objective at the end of 25 mm CE channel.

4.4.4 IEF-CE coupling

For IEF-CE coupling, instead of Beckman gel buffer, 3% wt., 8.4 pH Tris-Cl solution with 0.1% methylcellose, was used as the second dimension free solution CE separation media. The most important difference between CGE and CE is the free solution CE has higher resolution and electrophoretic mobility under the same level buffer strength. The CGE experiment is performed mainly to demonstrate the ability coupling separation media with distinct viscosity. Moreover, CGE can separate proteins based on protein length, if denatured by SDS first. The same stacking effect happens at the boundary even without the presenting gel matrix, because protein are entering high conductivity buffer region from low-conductivity ampholyte mixture. However, the liquid-liquid interface mixing occurs much faster than the liquid-gel case, mainly due to lower viscosity of the CE buffers.

To test the independence of the couping between CE and IEF, either sending proteins showed the same second dimension separation results in cathode-anode sequence or anode-cathode sequence. In the cathode-anode case (figure 25 (b)), GFP moves into second dimension earlier than the ovalbumin while in anode-cathode case, ovalbumin heads GFP. In both case, proteins first affected by stacking effect and then run thought the CE channel with distinct mobility. By having the same second dimension separation intensity chart, one can prove the second dimension CE is driven by the charges reacquired in Tris-Cl or gel buffer, therefore, has better resolution and control compare to unfocused ampholytes CE(Herr, Molho et al. 2003). In the second dimension separation with 500 V/cm electrical fields, ovalbumin (45K) has higher electrophoretic mobility

than GFP (26K). The mobility of biomolecules in CE separation depend on the their complex titration behaviors. Detail discussion is listed in 2.2.1 (Figure 9). Shown in the figure 25 (c), only GFP being trapped in the peak transfer region and then sent into second dimension CE with 200 mM, pH 8.0 Tris-Cl and 0.1 % methyl cellulose by valve operation. It demonstrates our ability of controlling target proteins. A clear GFP pattern with the same mobility is shown in the intensity chart. These two experiments prove the reliability and isolation power of the second dimension CE.

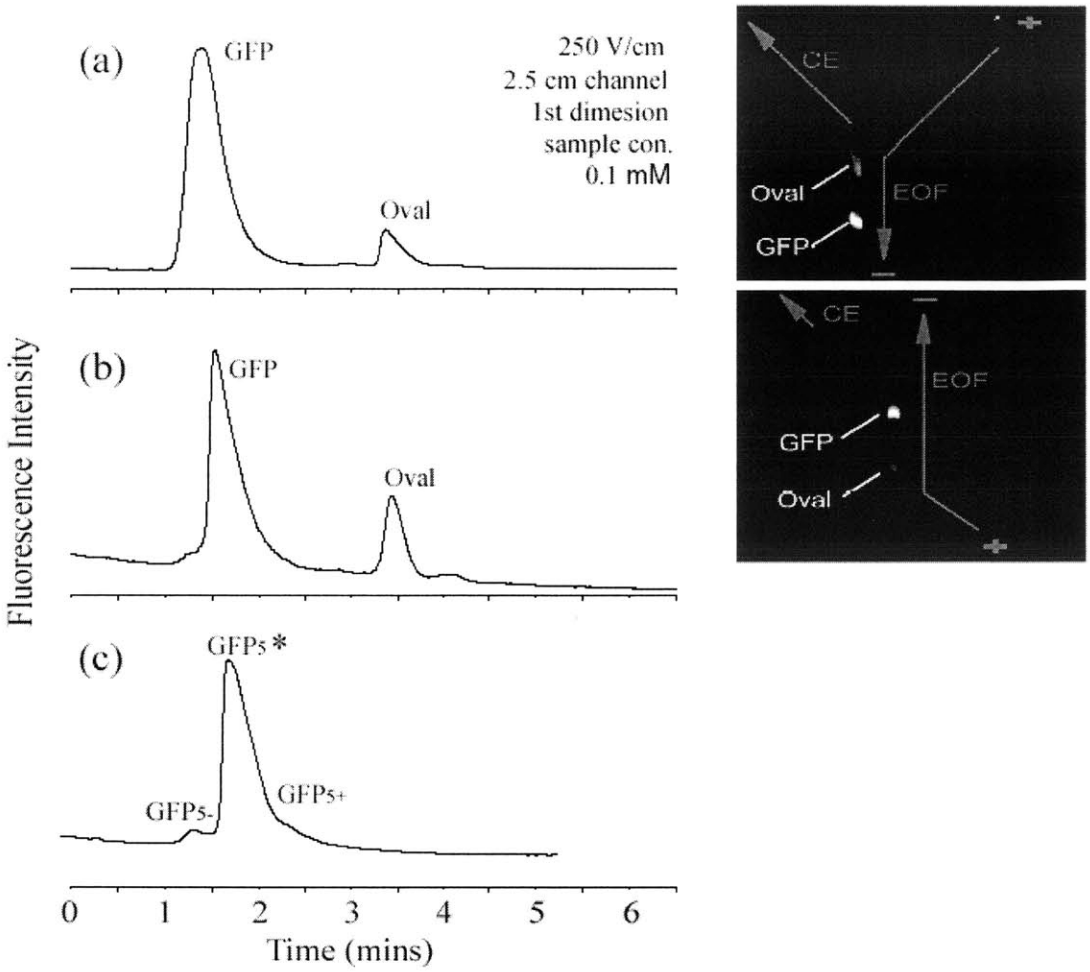

Figure 25: The further analysis of 2D on chip separation:

(a) Send proteins into second dimension CE with ovalbumin-GFP sequence, (b)Send proteins into second dimension with GFP-ovalbumin sequence, (c)Isolate and send only the GFP into second dimension CE by valve operation

4.4.5 Characterization of IEF-CE/ CGE Coupling Issues

Known from the isolation experiment described above, different electrophoresis mobility between proteins in second dimension CE comes from different mass to charge ratio. In typical CE, the mass to charge ratio in the same species is related to the buffer condition (including ion-strength and pH value) only. It is known that proteins tend to crystallize when it reaches high concentration and precipitate when sits in its pI values(Voet and Voet 1995). Therefore, if we perform IEF separation too long, crystallization and precipitation will occur, and resulting crystal will have slower mobility. On the other hand, if IEF was cut down and capture protein peaks before reaching the equilibrium, proteins with different pI values that are not quite yet focused can get trapped with the target proteins, causing backgrounds. In either case, the descending second dimension separation will be affected because they have different mass to charge ratio compare to correct focused one.

The intensity chart in figure 26(a) presents a second dimension CE that was isolated from IEF before reaching equilibrium. Under this circumstance, proteins obtain various charges and therefore show up all over the second dimension channel. The intensity chart in figure 26(b) shows the other case where IEF was cut off too late. Therefore, most of the proteins precipitated together and became larger molecule with slower electrophoresis mobility.

To suppress the precipitation, one can use lower concentration sample to prevent saturation. In typical proteomics research, proteins are handling in sub mM concentration

range, which is 1000 times lower than the operation condition we have. Therefore, no serious precipitates are expected. However, the concentration here is limited by the detection limit of the system. As a result, in order to have a repeatable high resolution CE, the timing of IEF has to be well controlled. Also, it is believed by decreasing the electrical field and channel passivation, we should be able to get IEF done without serious dispersions and precipitations.

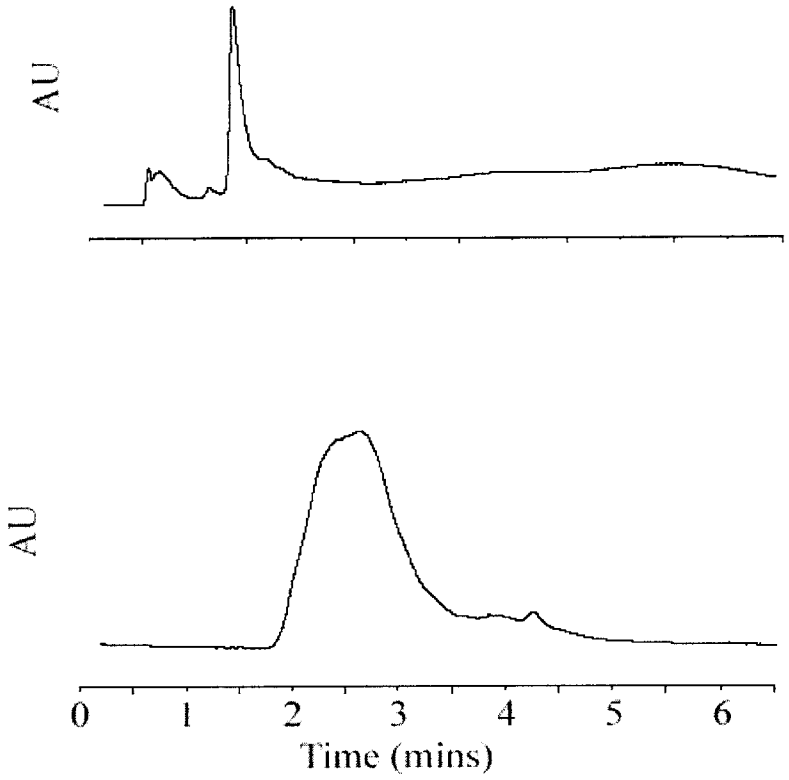

Figure 26: The discussion about IEF-CE relation.
(a) Proteins were isolated before reaching their pI points; therefore, each of them acquired different total charge. (b)Proteins were isolated long after reaching their pI points; therefore, larger molecular mass conjugates were formed.

Chapter 5 Summary

5.1 Conclusion

The goals of this thesis are 1) describing the importance of separating biomolecule by multi-dimensional separation in chip format, 2) presenting an important strategy for surface passivation and gel solidification by free radical polymerization, and 3) demonstrating our ability to couple chip based capillary IEF and CE or CGE together.

To achieve reliable microfluidic multi-dimensional protein separation, we developed an on-chip protein peak manipulation strategy to integrate two different separation techniques together. A three-step process was described in detail. First, a high-resolution pH gradient from pH 3 to 10 for IEF was established within a short channel. Under this pH gradient, proteins were focused into sharp regions and immobilized in their corresponding pI within a short amount of time. As target proteins move into the peak transfer region, microfluidic valves were closed for protein trapping. Second, the buffer exchange region was connected to second dimension CE channel by opening one set of valves. These microfluidic valves were made by the multilayer Polydimethylsiloxane (PDMS) stacking process(Jo, Lerberghe et al. 2000; Unger, Chou et al. 2000). Finally, CE in liquid gel or aqueous solution was applied as the way to further resolve selected samples. The advantages of better isolation between the two separation techniques were

demonstrated.

For the purpose of controlling protein adsorption and EOF, channel passivation by UV-initiated polymerization was illustrated. Such polymerization processes will be useful in microfluidic systems. Besides, to ensure analytical second dimension separation results, the control and calibration of first dimension IEF was carefully examined.

The presented multi-dimensional biomolecule separation strategy is a useful and quick solution toward integration between different heterogeneous separation/ analysis techniques such as IEF, PAGE, CE, reversed-phase liquid chromatography (RPLC), and mass spectrometry (MS). Microfluidic multi-dimensional separation devices, integrating various separation techniques in series or in parallel, will play an important role in the analysis of clinically-important biomolecule samples.

5.2 Directions for Future Research

5.2.1 Improving the Performance of Current System

There are many different promising ways to improve the performance of our current system. For example, refinements in channel wall passivation, detection instrument, injection scheme, and labeling techniques.

The minimization of protein binding was presented in this thesis by using high ionic strength CE buffer, high electrical field strength rapid separation, and channel passivation. It is believed that we can further improve the performance by developing a permanent (covalently-bonded) passivation coating.

In current detection system, the sensitivity is limited by the excitation light intensity a mercury burner can provide (mercury burner has trough in 488 nm, the excitation wavelength of FITC). By using laser-induced fluorescence with at least 10 mW/cm^2 in required wavelength, the detection limit can be brought down to 10^{-13} M range. (Chen, Aldelhelm et al. 1994; Timperman, Khatib et al. 1995; Landers 1997)

Non-covalent post column or on-column labeling or proteins with commercially available quantitative dyes are superior ways to label and detect proteins(Jacobson, Koutny et al. 1994; Bousse, Mouradian et al. 2001; Giordano and Landers 2001; Harvey, Bablekis et al. 2001; Sze, Huang et al. 2002). It is expected, by post or on-column labeling, we can achieve protein separation and detection on chip without the peak broadening issue caused by pre-labeling(Richards, Stathakis et al. 1999).

Besides, a better IEF ampholyte injection techniques by high fluid resistant ultra-thin capillaries (Hosokawa, Fujii et al. 1999) can help control the ampholyte location which is the key to a reproducible second dimension separation.

5.2.2 Future Areas of Research Interest

Coupling microfluidic separation (pre-concentration) device with the MS always be a significant study in this field(Zhang, Foret et al. 2001; Mohan, Pasa-Tolic et al. 2003). With the peak control ability, we can use our system as an injector performing IEF as a pre-concentration step before MS analysis. More advanced couplings like IEF-IEF/MS and IEF-RPLC/MS are also possible. Easy automation and low-dead volume sample processing are the one of the key advantages one can get from such microfluidic sample preconcentration/ preseparation device.

The other ultimate goal in this project is having a portable proteome analysis device similar to the integrated DNA chip announced by ACLARA BioSciences (including heater for PCR, microfluidic valves, injection channels, and CE channels) (Fan, Ricco et al. 2003). With the large-scale integration microfluidic fabrication technique presented by Thorsen et al.(Thorsen, Maerkl et al. 2002), we could integrate hydrophobic microcapillary vent as the injector, IEF and CE as the separation media, post column labeling as the label scheme, and/ or built-in conductivity sensors as the detector (Dasgupta and Bao 1993; Kar, Dasgupta et al. 1994). Such kind of micro total analysis devices will dramatically improve the speed of proteomics analysis and make on-line tracking of human health possible.

Appendix I IEF troubleshooting guide

Problem	Cause	Solution
Missing or faint bands	A. Wrong CCD camera setting	A. Check exposure time and enhancement mode
	B. Not enough sample	B. Increase protein sample concentration
	C. Ampholyte mixture flush out by reservoir buffer	C. Balance cathode and anode solution carefully
Fuzzy bands	A. Not focused long enough	A. Increase focusing time or increase voltage at end of run for a few minutes
	B. Focusing too long	B. Decrease focusing time by incrementally increasing voltage gradient
	C. Wall contamination	C. Flush solvents like methanol though the channel
Skewed bands	A. Electrode not clean	A. Clean electrode
	B. Uneven electrode contact	B. Level instrument and add weight to maintain good electrode contact.
	C. Uneven EOF caused by additives	C. Change ampholyte mixture composition
Isoelectric precipitation	A. Focusing too long	A. Decrease focusing time
	B. Samples reach threshold concentration	B. Decrease protein sample concentration 10 to 100 fold
Wavy bands	A. Salt in sample	A. Desalt sample
	B. Improper anolyte	B. Replace anolyte
	C. Old electrolyte solution	C. Make fresh
	D. Improper voltage	D. Initially reduce to 100 V/cm
	E. Dirty electrodes	E. Clean thoroughly

References

Auroux, P.-A., D. Iossifidis, et al. (2002). "Micro Total Analysis Systems. 2. Analytical Standard Operations and Applications." Analytical Chemistry **74**: 2637-2652.

Beebe, D. J., J. S. Moore, et al. (2000). "Functional hydrogel structures for autonomous flow control inside microfluidic channels." Nature **404**(6): 588-590.

Berg, J. M., J. L. Tymoczko, et al. (2002). Biochemistry. N. Y., W. H. Freeman.

Bjellqvist, B., K. Ek, et al. (1982). "Isoelectric focusing in immobilized pH gradients: Principle, methodology and some applications*1." Journal of Biochemical and Biophysical Methods **6**(4): 317-339.

Bongers, J., T. Lambros, et al. (1992). J. Liq. Chromatogr. **15**: 1115.

Bousse, L., S. Mouradian, et al. (2001). "Protein Sizing on a Microchip." Anal. Chem. **73**(6): 1207-1212.

Bousse, L., S. Mouradian, et al. (2001). "Protein Sizing on a Microchip." Analytical Chemistry **73**: 1207-1212.

Brahmasandra, S. N. and V. M. Ugaz (2001). "Electrophoresis in microfabricated devices using photopolymerized polyacrylamide gels and electrode-defined sample injection." Electrophoresis(22): 300-311.

Burns, M. A., B. N. Johnson, et al. (1998). "An Integrated Nanoliter DNA Analysis Device." Science **282**(5388): 484-487.

Camilleri, P. (1998). Capillary electrophoresis : theory and practice, CRC Press.

Catsimpoolas, N. (1976). Isoelectric Focusing. N. Y., Academic Press.

Chen, D. Y., K. Aldelhelm, et al. (1994). Analyst **119**: 349-352.

Dasgupta, P. K. and L. Bao (1993). Anal. Chem. **65**: 1003-1011.

Duffy, D. C., J. C. McDonald, et al. (1998). "Rapid Prototyping of Microfluidic Systems in Poly(dimethylsiloxane)." Analytical Chemistry **70**: 4974-4984.

Fan, Z. H., A. J. Ricco, et al. (2003). "Integrating Multiplexed PCR with CE for Detecting Microorganisms." Proceedings of Micro Total Analysis Systems 2003 **1**: 849-852.

Giordano, L. J. J. B. C. and J. P. Landers (2001). "Dynamic Labeling during Capillary or Micrchip Electrophoresis for Laser-Induced Fluorescence Detection of Protein-SDS Complexes without Pre-or Postcolumn Labeling." Anal. Chem. **73**(20): 4994-4999.

Greenway, G. M., S. J. Haswell, et al. (1999). Anal. Chem. **387**: 1-10.

Grodzinsky, A. J. (2003). Fields, Forces and Flows in Biological Systems. Massachusetts, MIT.

Guenat, O. T., D. Ghiglione, et al. (2001). Sens. Actuators, B **72**: 273-282.

Han, J. and A. K. Singh (2002). Miniaturization of Protein Separation: Isoelectric Focusing and SDS-PAGE. Micro Total Analysis Systems, Nara, Japan, Kleuwer Academic Publishers.

Han, J. and A. K. Singh (2002). Miniaturization of Protein Separation: Isoelectric Focusing and SDS-PAGE. Proceedings of the microTAS 2002 Symposium. A. van den Berg. Nara, Japan, Kleuwer Academic Publishers. **1:** 596-598.

Harvey, M. D., V. Bablekis, et al. (2001). "Utilization of the non-covalent fluorescent dye, NanoOrange, as a potential clinical diagnostic tool Nanomolar human serum albumin quantitation." Journal of Chromatography B. **754**: 345-356.

Herr, A. E., J. I. Molho, et al. (2003). "On-Chip Coupling of Isoelectric Focusing and Free Solution Electrophoresis for Multidimensional Separations." Anal. Chem. **75**(5): 1180-1187.

Herr, A. E., J. I. Molho, et al. (2003). "On-Chip Coupling of Isoelectric Focusing and Free Solution Electrophoresis for Multidimensional Separations." Analytical Chemistry **75**: 1180-1187.

Hjerten, S. (1985). "High-Performance Electrophoresis Elimination of Electroendosmosis and Solute Adsorption." Journal of Chromatography **347**: 191-198.

Hjerten, S. and M.-d. Zhu (1985). "Adaptation of the equipment for high-performance electrophoresis to isoelectric focusing." Journal of Chromatography A **346**: 265-270.

Hosokawa, K., T. Fujii, et al. (1999). "Handling of Picoliter Liquid Samples in a Poly(dimethylsiloxane)-Based Microfluidic Device." Anal. Chem. **71**(20): 4781-4785.

Ikuta, K., K. Ikeda, et al. (2001). Micro Total Analysis Systems, Netherlands, Kluwer Academic Publishers.

Jacobson, S. c., L. B. Koutny, et al. (1994). "Microchip Capillary Electrophoresis with an Integrated Postcolumn Reactor." Anal. Chem. **66**(20): 3472-3476.

Jain, M. K. (1972). The Biomolecular Lipid Membrane. N. Y., Van Nostrand Reinhold.

Jo, B.-H., L. M. V. Lerberghe, et al. (2000). "Three-Dimensional Micro-Channel Fabrication in Polydimethylsiloxane (PDMS) Elastomer." Journal of Microelectromechanical System **9**(1): 76-81.

Kar, S., P. K. Dasgupta, et al. (1994). Anal. Chem.(66): 2537-2543.

Kutter, J. P., S. C. Jacobson, et al. (1998). "Solvent-Programmed Microchip Open-Channel Electrochromatography." Anal. Chem. **70**(15): 3291-3297.

Landers, J. P. (1997). Handbook of Capillary Electrophoresis. N. Y., CRC Press.

Li, Y., J. S. Bush, et al. (2004). "Intergration of Isoelectric Focusing with Parallel Sodium Dodecyl Sulfate Gel Electrophoresis for Multidimensional Protein separations in a Plastic Microfludic Network." Anal. Chem. **ASAP web Edition**.

Liu, Y., J. C. Fanguy, et al. "Dynamic Coating Using Polyelectrolyte Multilayers for Chemical Control of Electroosmotic Flow in Capillary Electrophoresis Microchips." Anal. Chem.

Lu, H., M. a. Schmidt, et al. (2001). Lab Chip **1**: 22-28.

Mao, Q. and J. Pawliszyn (1999). "Capillary isoelectric focusing with whole column imaging detection for analysis of proteins and peptides." Journal of Biochemical and Biophysical Methods **39**(1-2): 93-110.

Mao, Q. L. and J. Pawliszyn (1999). "Demonstration of isoelectric focusing on an etched quartz chip with UV absorption imaging detection." Analyst **124**(5): 637-641.

Mohan, D., L. Pasa-Tolic, et al. (2003). "Integration of Electrokinetic-Based Multidimensional Separation/Concentration Platform with Electrospray Ionization-Fourier Transform Ion Cyclotron Resonance-Mass Spectrometry for Proteome Analysis of Shewanella oneidensis." Anal. Chem. **75**(17): 4432-4440.

Munro, N. J., A. F. R. huhmer, et al. (2001). "Robust Polymeric Microchannel Coatings for Microchip-Based Analysis of Neat PCR Products." Anal. Chem. **73**(8): 1784-1794.

Ocvirk, G., M. Munroe, et al. (2000). "Electrokinetic control of fluid flow in native poly(dimethylsiloxane) capillary electrophoresis devices." Electrophoresis **21**: 107-115.

Odom, T. W., J. C. Love, et al. (2002). "Improved Pattern Transfer in Soft Lithography Using
Composite Stamps." Langmuir(18): 5314-5320.

Offord, R. E. (1966). Nature **211**: 591.

Raisi, F., P. Belgrader, et al. (2001). "Microchip isoelectric focusing using a miniature scanning detection system." Electrophoresis **22**: 2291-2295.

Reyes, D. R., D. Iossifidis, et al. (2002). "Micro Total Analysis Systems. 1. Introduction, Theory, and Technology." Analytical Chemistry **74**: 2623-2636.

Richards, D. P., C. Stathakis, et al. (1999). "Labeling effects on the isoelectric point of green fluorescent protein." Journal of Chromatography A. **853**: 21-25.

Rocklin, R. D., R. S. Ramsey, et al. (2000). "A Microfabricated Fluidic Device for Performing Two-Dimensional liquid-Phase Separations." Anal. Chem. **72**(21): 5244-5249.

Schasfoort, R. B. n. M., S. Schlautmann, et al. (1999). "Field-Effect Flow Control for Microfabricated Fluidic Networks." Science **286**(5441): 942-945.

Schmalzing, D., L. Koutny, et al. (1997). "Ultrafast STR Analysis by Microchip Gel Electrophoresis." Eighth International Symposium on Human Identification.

Schwartz, H. and T. Pritchett (1995). Separation of Proteins and Peptides by Capillary Electrophoresis: Application to Analytical Biotechnology, Beckman Instruments, Inc.

Seong, G. H., w. Zhan, et al. (2002). "Fabrication of Microchambers Defined by Photopolymerized Hydrogels and Weirs within Microfluidic Systems: Application to DNA Hybridization." Anal. Chem. **74**(14): 3372-3377.

Service, R. F. (2001). "PROTEOMICS: High-Speed Biologists Search for Gold in Proteins." Science **294**(5549): 2074-2077.

Sze, N. S.-K., T. Huang, et al. (2002). "Laser-induced fluorescence detection of noncovalently labeled protein in capillary isoelectric focusing." J. Sep. Sci. **25**: 1119-1122.

Tanaka, T. (1981). "Gels." Scientific American **244**(1): 124-138.

Thorsen, T., S. J. Maerkl, et al. (2002). "Microfluidic Large-Scale Integration." Science **298**(5593): 580-584.

Timperman, A. T., K. Khatib, et al. (1995). Anal. Chem. **67**: 139-144.

Ugaz, V. M., D. T. Burke, et al. (2002). "Microdevice-based measurements of diffusion and dispersion in cross-linked and linear polyacrylamide DNA sequencing gels." Electrophoresis **23**: 2777-2787.

Unger, M. A., H.-P. Chou, et al. (2000). "Monolithic Microfabricated Valves and Pumps by Multilayer Soft Lithography." Science **288**(7): 113-116.

Voet, D. and J. G. Voet (1995). Biochemistry, Wiley.

Wen, J., Y. Lin, et al. (2000). "Microfabricated isoelectric focusing device for direct electrospray ionization-mass spectrometry." Electrophoresis **21**: 191-197.

Wu, X.-Z. and J. Pawliszyn (2001). "Application of Whole Column Imaging Capillary Isoelectric Focusing in Study of Protein-Protein Interaction." Analytical Sciences **17**: 189-192.

Wu, X.-Z. and J. Pawliszyn (2002). "Whole-column imaging capillary electrophoresis of proteins with a short capillary." Electrophoresis **23**: 542-549.

Wu, X.-Z., N. S.-K. Sze, et al. (2001). "Miniaturization of capillary isoelectric focusing." Electrophoresis **22**: 3968-3971.

Xie, J., X. Yang, et al. (2001). "Surface micromachined leakage proof parylene check valve." Proc IEEE Micro Electro Mech Syst MEMS: 539-542.

Yamamoto, T., T. Nojima, et al. (2001). Micro Total Analysis Systems, Netherlands, Kluwer Academic Publishers.

Yao, S., D. S. Anex, et al. (1999). "SDS capillary gel electrophoresis of proteins in microfabricated channels." Proceedings of the National Academy of Sciences of the United States of America **96**: 5372-5377.

Yu, Q., J. M. Bauer, et al. (2001). "Responsive biomimetic hydrogel valve for microfluidics." Applied Physics Letters **78**: 2589-2591.

Zhan, W., G. H. Seong, et al. (2002). "Hydrogel-Based Microreactors as a Functional Component of Microfluidic Systems." Anal. Chem. **74**(18): 4647-4652.

Zhang, B., F. Foret, et al. (2001). "High-Throughput Microfabricated CE/ESI-MS: Automated Sampling from a Microwell Plate." Anal. Chem. **73**(11): 2675-2681.

Zheng, J., C. Mastrangelo, et al. (2002). "Single Stranded DNA Separations in Oxide-Passivated Thin-Walled Plastic Microchannels." Proc. 2nd Intl. IEEE-EMBS Conference on Microtechnologies in Medicine & Biology: 442-446.