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Development of Microfluidic Devices for Proteomics

Trust T. Razunguzwa

**Dissertation submitted to the
Eberly College of Arts and Sciences
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Doctor of Philosophy
in
Chemistry**

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2006**

Keywords: Microfluidic devices, Proteomics, Electroosmosis, Fabrication, Capillary electrophoresis, Electrospray, Mass spectrometry, Channel coatings

We have developed functional components for a comprehensive microfluidic system for proteomic analyses. This dissertation presents development of an electroosmotic pump for delivery of fluids to other microfluidic channels, a hydrodynamic flow restrictor for combining electrokinetic and hydrodynamic flow on a microfluidic chip-ESI-MS interface, and surface coatings to prevent analyte adsorption to microfluidic channels. A microfluidic chip ESI-MS interface with a novel voltage switching component is also developed for the separation of negative analytes at high pH, with positive ion mode ESI-MS detection of the analyte, converted to positive ions via a make-up solution induced pH change.

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There are many people I would like to thank who have contributed greatly to my years in graduate school. First, I would like to thank my advisor and mentor Dr. Timperman who has always challenged me to tackle difficult research problems, which normally are the source of greatness. When he assigned me my first microfluidic project, I did not realize that this research is multi-disciplinary that borrows from many areas of science including biology, physics, material science, surface chemistry engineering and would require a broad basic understanding of these areas to be able to communicate with collaborators. However, with his guidance, I managed to navigate through the problems of this research area and develop a good understanding of the problems in proteomics and how we can use microfluidics technology to solve some of these problems. Dr. Timperman also has had a profound effect on my skills of writing scientific communications and taught me how to write concise, clear and well structured literature. He always emphasized the need to keep abreast with recent advances in analytical technologies and I would like to thank him for sending me to conferences to present and keep up with cutting edge research.

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has grown since then. It was a great pleasure to be able to access and tap into Jim's broad technical expertise.

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Dedication

To my wife Tabitha Ruvarashe Razunguzwa and my daughter Zoe Razunguzwa

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Chapter 1

Introduction

1.1 Proteomics Research

The completion of sequencing of the human and other genomes has spurred an increase in the elucidation of the function, structure, and biochemical properties of gene products in a cell, such as RNA, transcribed from genomic DNA, and proteins, which act as a cell's machinery. Proteins have been studied to elucidate the mechanisms of cell function and signaling, since this information cannot be deduced directly from genomic data for four main reasons: i) the mRNA and protein levels do not correlate¹ ii) prediction of the genomic portion responsible for encoding of the protein, the open reading frame, is difficult,² iii) proteins are often activated and inactivated by post-translational modifications such as phosphorylations and glycosylations,³ and iv) protein-protein interactions and binding of other substrates is critical to their function.⁴ The need to study biochemical processes at the protein level gave rise research area of proteomics.

The term proteomics was coined by Marc Wilkins in 1994, and a goal proteomics is to identify, and characterize all proteins in a given biological system.⁵ Additionally, proteomics attempts to determine the interaction of proteins with each other, their functional roles, and expression levels under different conditions, such as healthy and diseased states. Valuable information and insights for many areas of biological research, such as drug discovery, therapeutics, diagnostics, biomedical research and molecular biology can be obtained.⁶⁻¹⁰

Proteomics entails large scale study of proteins, as opposed to traditional methods that have involved the study of one or a few proteins in great detail.¹¹ The techniques

used for proteomics therefore should be capable of dealing with complex protein mixtures exhibiting a wide dynamic range. The main technique for proteomics has been 2-dimensional gel electrophoresis (2DE) coupled with mass spectrometry.¹²⁻¹⁵ In 2DE, a complex protein sample is separated by isoelectric focusing in the first dimension and by SDS PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis) in the second dimension. The spots of the separated proteins are excised, digested and detected by tandem mass spectrometry. One of the main limitations of 2DE is difficulty in detecting low abundance proteins, due to its limited peak capacity.¹⁶ The proteins involved in cell signaling are usually in low abundance and are often masked by structural and housekeeping proteins on 2DE gels. Other limitations include: long analysis times, labor intensive and it is difficult to obtain good reproducible separations. Alternative technologies and approaches have been developed to improve detection of low abundance proteins and increase automation for faster analyses. Multidimensional Protein Identification technology (MudPit) was developed by Yates' group and involves initially digesting a complex protein mixture, detecting the digests by mass spectrometry and identifying the proteins from which the peptides were derived through database interrogation.¹⁷ This technique heavily relies on database searching, which is not always accurate due to inaccurate peptide-protein assignments and its dependence on proteins present in the database. MudPit also does not work very well with low abundance proteins due to the ultracomplex peptide mixture, although it performs as well or slightly better than gels for low abundance proteins. The main advantage for MudPit is that it is better for proteins of limited solubility on the gel, such as hydrophobic and basic proteins.

Microfluidic devices are well suited for proteomics demand for technologies that provide decrease in analysis time, smaller sample volumes, decreased contamination, parallel analyses and automation. With the development of new methods to fabricate microfluidic devices, investigation of fundamental behavior in miniature channels and invention of elements (units) from which complex microfluidic devices can be constructed; complex microfluidic platforms for comprehensive proteomic analysis can be assembled. Different microfluidic devices have been used for proof-of-principle experiments in proteomic processes such as protein purification, concentration, digestion and separation by a number of companies, government labs and academic research labs. These microfluidic devices can potentially be automated and integrated to process proteomic samples in various ways, and can be coupled on-line for mass spectrometry analysis. The subsequent sections will address device fabrication, the fundamentals of fluid dynamics in microfluidic channels and the detection schemes used.

1.2 Substrate Materials and Fabrication

The first microfluidic devices were fabricated on quartz and glass substrates using photolithography and wet etching techniques adopted from the micro-electronics industry. The advantages of using quartz or glass substrates are high electrical resistance, reproducible surface and optical transparency over a wide range of wavelengths compared to silicon and polymers. The etched glass surface supports high electroosmotic flow (EOF), and since glass surface chemistry is well understood, reproducible and contain many reactive silanol groups, many methods of modifying glass surfaces are available. Glass substrates, however, suffer from some limitations such as adsorption of

analytes to negatively charged wall, metal ion impurities, being fragile and slow fabrication. Due to amorphous nature of glass, there is isotropic etching, resulting in trapezoidal or semi-circular cross-sections. Glass bonding usually requires a cleanroom environment, high voltages, and elevated temperatures, which limits the use of temperature sensitive materials in the fabrication process. Glass microfluidic devices are therefore expensive, slow and difficult to fabricate, although they possess mostly good characteristics. In addition, their lack of flexibility (rigidity) makes sealing polymers and manufacturing valves difficult.

In order to overcome limitations of glass, the development of polymer microfluidic devices is an active and growing area of research. Polymer substrates are generally less expensive than glass substrates and there are a wider variety of fabrication methods that are faster and easier. Fabrication methods for polymers have included laser ablation for polystyrene, polycarbonate, cellulose acetate, poly(ethylene terephthalate), replica molding for PDMS, injection molding and hot embossing imprinting for polymethylmethacrylate (PMMA). However, polymers are not chemically inert and will dissolve in some organic solvents. Polymers also tend to be hydrophobic, exhibit poor wettability and need to be coated as well to minimize surface interactions with proteins.

1.2.1 Photolithography and Wet Chemical Etching

Photolithography is a process where a pattern is transferred to a substrate by optical means as depicted in Figure 1.1. For microfluidic devices, the pattern is a 2D layout of the channels and reservoirs. A chromium/gold film is sputtered on the surface of substrate. A positive photoresist is spin-coated on the chromium surface, and the

channel design is transferred to the substrate using a photomask and UV exposure of the photoresist. When ultraviolet light strikes the photoresist, it weakens the polymer in the portions exposed to the light. These portions are washed away during development and the image of the mask is transferred to the photoresist layer. After exposing and developing the photoresist the metal films are etched by using KI/I_2 for gold and $\text{K}_3\text{Fe(CN)}_6/\text{NaOH}$ for Cr. The channels are then etched into the glass substrate with a HF/HNO_3 solution. A summary of the process is outlined in Figure 1.1.

Figure 1.1: Photolithography and wet chemical etching process

1.2.2 Laser Ablation

Laser ablation refers to the removal of material using a laser beam, and has been used to write microfluidic channels on various substrate materials including glass¹⁸⁻²⁰ and polymeric substrates.^{21, 22} Unlike the multistep photolithography technique, laser micromachining offers a single step method of direct writing of channels of various geometries, depths and aspect ratios that cannot be achieved by traditional photolithographic techniques. The laser ablation process involves laser-substrate interactions that are dependent on the properties of the laser beam (wavelength, pulse duration, fluence, intensity) and the properties of the substrate.²³ The ablation process is not yet completely understood, but it involves a series of steps encompassing an initial absorption of the laser energy by the solid material, followed by local heating of the surface, and then evaporation of the substrate material resulting in an ablation plume (plasma) as shown in Figure 1.2. The plume contains molecular fragments, ions, free electrons, neutral particles and chemical products from reactions in the plume. The plume can also scatter and absorb the incident laser beam as well as re-depositing on the substrate or the laser beam delivery optics. A pressurized inert gas is usually used in the ablation process to blow away the plume as it is formed during the ablation process. The ablation depth is determined by the ability of the substrate material to absorb the laser energy and is a direct function of the beam fluence (energy per unit area), wavelength, repetition rate and pulse duration. The ablation wavelength is chosen from a region where the substrate material absorbs, thus allowing a high energy deposition in a small volume to ensure complete and fast ablation. A small pulse duration (ns-fs) is chosen to minimize thermal conduction to the surrounding substrate material and to maximize the

peak power.²⁴⁻²⁷ A high pulse repetition rate is chosen to prevent the loss of energy that is not used from a previous pulse and therefore making the ablation process more efficient.²⁸ The laser beam must be high in energy, homogenous, and easy to focus.

Figure 1.2: *Laser ablation process*

The work presented herein utilized a high repetition nanosecond UV (255nm) pulsed copper vapor laser (optical path shown in Figure 1.3) to micro-machine miniature channels ($>10\mu\text{m}$) on glass substrates. The laser head assembly consists of a ceramic plasma tube with copper pellets along its length. A fast-pulsed electrical discharge is used to vaporize the copper atoms from the pellets at temperatures of $1400\text{-}1500^{\circ}\text{C}$. The vaporized copper atoms exist as the minority species in a high speed longitudinal discharge in a buffer gas of high purity neon which removes impurities that could potentially build-up in the laser head and carries the discharge when the laser is operation at low temperatures and no copper atoms are present. The intense pulse of electrons from the electrical discharge collide and excite the copper atoms that produce light at 511 nm

and 578 nm when they relax back to ground state. The overall excitation efficiency is very low as only 1 % of the electrical power input is used to generate the 511 nm and 578 nm light, while 99 % is converted to heat. The 578 nm light is separated out and dumped by transmission through the beam turning mirror, while the 511 nm light is reflected by the beam turning mirror and spatially filtered in an off-axis mirror telescope. The beam is then focused using a cylindrical lens onto a BBO crystal where approximately 10 % of the power is converted to UV light at 255nm. The UV light beam is re-collimated by a cylindrical lens and the 511 nm and 578 nm radiation that passed through is filtered out using a pair of dielectric harmonic separators. The beam is directed to the substrate to be machined using 8 turning mirrors and finally passed through a focusing lens to the substrate surface.

The key for the components shown in the figure above is as follows:

RCM	Cavity Rear Mirror	HM	Holed Mirror
PBS	Polarising Beam Splitter cube	CFM	Cavity Front Mirror
BTM	Beam Turning Mirror	TM1	Telescope Mirror 1
TM2	Telescope Mirror 2	SF	Spatial Filter
AA	Alignment Aperture	CL1	Cylindrical Lens 1
CL2	Cylindrical Lens 2	XTAL	BBO Doubling Crystal
HS	Harmonic Separator	ICA	Intra Cavity Aperture

Figure 1.3: Optical path for a copper vapor laser used for micromachining of microfluidic channels

adapted from Oxford Lasers copper vapor laser manual.

The micromachining of microfluidic channels on glass is usually achieved using deep UV lasers since glass is optically transparent in the visible and near infrared regions.^{20, 29, 30} Alternatively, machining can be carried out 800 nm or 1660 nm by taking advantage of the non-linear absorption process that makes the material non-transparent to radiation at that wavelength.³¹ The laser is tightly focused beneath the surface of a glass slide and due the high intensity at the focal point, a multiphoton absorption occurs that results in the localized ablation of the glass sample.^{18, 25, 32, 33} Laser micromachining of glass usually suffers from poor edge definition, rough channel surfaces, microcracking and discoloration of the glass.^{34, 35} Recent studies have focused on better understanding of the mechanisms involved in the interactions of the different laser types with different glasses, such as borosilicate, borofloat and soda lime.³⁶

1.3 Transport in Microfluidic channels

Solutions are commonly transported through microfluidic channels by application of an electric potential (electrokinetically) or by applying a pressure (hydrodynamically). Electrokinetic transport can be used to steer fluids at channel junctions and for valving. This electrokinetic steering is required because no good large scale valves have been developed yet for microfluidic channels. The best valves developed are based on PDMS and these do not function well for hydrophobic and organic solvents.³⁷

1.3.1 Hydrodynamic Flow

Hydrodynamic flow is pressure driven flow and the driving force for the flow can be gravity, centrifugation, vacuum, a pressurized gas tank, or a pump to actuate flow

through the channels. Pressure driven flow obeys the no-slip boundary condition that states that the fluid flow at the channel wall must be zero, which produces a parabolic flow profile within the channel. The flow velocity of solution at the center is higher than near the channel walls and therefore a sample band will disperse in the axial direction as it migrates along the channel. This hydrodynamic dispersion is mathematically described by the Taylor-Aris theory is often termed Taylor dispersivity.^{38, 39} In electrokinetic manipulations on microfluidic platforms, hydrodynamic flow is typically an unwanted component of the flow. The hydrodynamic component arises from differences in the hydrostatic head pressure of the channel reservoirs, Laplace pressure,^{40, 41} variations in surface charges⁴² and solution conductivity.⁴³ Therefore, it is important to design of microfluidic devices that minimize Taylor dispersion by controlling the geometries and dimensions of the microfluidic channels, which has been a subject of research by various research groups.⁴⁴⁻⁴⁹

Hydrodynamic flow can be laminar or turbulent. Laminar flow is smooth and steady and flow paths do not change over time. However, in turbulent flow, convection of fluids is present where inertial and acceleration forces dominate flow, and flow paths do change over time. Reynold's number Re , defined by equation 1 is used to determine the boundary between turbulent and laminar flow,

$$Re = \frac{L V_{ave} \rho}{\eta} \quad (1)$$

where L is the most relevant length scale of the channel, ρ is the density of the liquid, V_{ave} is the average velocity of the fluid and η is the viscosity of the fluid. If Re is > 2000 then flow is turbulent and for $Re < 2000$ the flow is laminar. For microfluidic channels, $L = 4A/P$, where A is the area of the channel and P is the wetted perimeter of the channel.

Since the dimensions of the microchannels are very small, R_e is usually less than 100 and often less than 1.0. Therefore, the flow regime in microfluidic devices is laminar, and molecules can be hydrodynamically transported through microfluidic channels in a relatively predictable manner.

1.3.2 Electrokinetic Flow

Electrokinetic transport is mainly used for electrophoretic separations on microfluidic platforms. A sample plug is introduced at the inlet of a separation channel, and an electric field is applied across the separation channel, which is filled with electrolyte solution. The potential is applied via electrodes immersed in inlet and outlet electrolyte reservoirs. The sample migrates towards the outlet due to two electrokinetic phenomena: electroosmotic flow (EOF) and electrokinetic mobility. The migration velocity observed for each molecule (V_{obs}) is a sum of the the electroosmotic flow velocity (V_{EOF}) and the electrophoretic velocity V_E , as shown in equation 2

$$V_{obs} = V_{EOF} + V_E \quad (2)$$

Electroosmotic flow originates from the channel wall, which acquires a surface charge when it comes in contact with electrolyte solution. This phenomenon was first reported by Reuss, who demonstrated that water could migrate through porous clay by application of an electric field.⁵⁰ Most solid surfaces such as glass, quartz, silicon, fused silica and other polymeric materials acquire a surface charge when they come into contact with an electrolyte solution. In case of glass and silica, the surface charge density

is determined by the deprotonation of the surface silanol groups, which is a function of the local pH. In order to maintain overall charge neutrality, the surface charge attracts ions of opposite charge to form a layer of fixed immobile charges (Stern layer). This immobile charge layer repels ions of opposite charge and attracts free ions of opposite charge in solution, forming a thin layer of mobile charges called the Gouy-Chapman diffuse layer (Debye layer). The Debye and Stern layer form the electric double layer (EDL) which is separated by the shear plane as depicted in Figure 1.4.

Figure 1. 4: Gouy-Chapman electric double layer

The movement of the mobile ions of the Debye layer drags the rest of the electrolyte solution through viscous coupling, resulting in a bulk flow (EOF), and only works for small channels. The bulk electrolyte solution far from the wall is assumed to have net neutral charge. The electroosmotic flow velocity can be calculated from the Helmholtz-Smoluchowski equation (equation 3)

$$V_{\text{EOF}} = \frac{\epsilon \zeta E}{4\pi} \quad (3)$$

where E is the electric field, η is the viscosity of the electrolyte solution, ϵ is the dielectric constant of the electrolyte solution and ζ is the zeta potential (potential at the electrolyte/substrate interface). This formula is valid for a Debye layer thickness much smaller than the channel radius and assumes that the zeta potential is uniform over the length of the channel. The flow profile of EOF for practical purposes can be approximated as a flat, plug-like flow profile (Figure 1.5) under ideal situations, which means that the presence of electroosmotic flow does not lead to significant dispersion in capillary electrophoretic separations, assuming that the electric field, zeta potential and viscosity are constant. If any of these parameters vary, pressure gradients can be created which distort the plug-like flow profile.

Figure 1.5: Flow profiles of hydrodynamic and electroosmotic flow

Electrokinetic mobility is the movement of ions under the influence of an electric field through a solution or viscous medium. The electrophoretic velocity is a product of the electrophoretic mobility and the electric field as shown in equation 4.

$$V_E = \mu_E E \quad (4)$$

$$\mu_E = \frac{q}{6\pi \eta r} \quad (5)$$

The value of the electrokinetic mobility of each molecular species is function of the charge, size and shape of the molecule. The electrophoretic mobility dependence on the charge and size of a molecule is described by the Navier-Stokes equation (equation 5), where μ_E is the electrophoretic mobility, q is the charge of the molecule, and r is the radius of the molecule. Because the effects of shape are difficult to quantitate, the charged particle is assumed to be spherical. The electric force favors motion of the molecule and frictional force, which is determined by the size and shape, acts against motion.

There are several advantages associated with performing electrophoretic separations on microfluidic platforms than in fused silica capillaries. Rapid separation can be achieved by using very short injection plugs, short channels and high electric field strengths on microfluidic platforms. An example of such fast separation was reported Zhonghui et-al who demonstrated a separation of three amino acids in a 1.6 cm glass microfluidic channel in 4s using a 500 μm injection plug. Electric fields as high as 2500V/cm were utilized without experiencing joule heating effects due to the thermal mass and conductivity of the glass chip.⁵¹ The fast separations can be integrated with other sample processing procedures on the chip. High throughput can also be achieved by fabricating multiple separation channels on the same device.⁵²

1.3.3 Injection Schemes

One of the key elements in electrokinetic sample manipulation on microfluidic platforms is the sample injection system. The sample injection system is important because most sample handling processes on a microfluidic chip often include an electrophoretic separation step, which is directly dependent on the characteristics of the sample plug. Ideally, a short well defined sample plug is required to achieve high separation efficiencies as given by equation 6, where the injection plug length does not contribute significantly to the overall plug length.

$$\sigma_{inj}^2 = I_{inj}^2/12 \quad (6)$$

where σ_{inj}^2 is the variance due to injection plug length (I_{inj}^2).

There are several electrokinetic sample injection methods for microfluidic chips. Early devices used a Tee intersection shown in Figure 1.6A^{53, 54}, which has been mostly replaced by double Tee⁵⁵ and cross injectors⁵⁶⁻⁵⁹ shown in Figure 1.6B and C, respectively. These injectors are constant volume injectors although the cross can be used in gated mode⁶⁰⁻⁶³ (as opposed to the pinched mode⁶⁴⁻⁷⁰) for variable volume injections. Other configurations like the multi-Tee⁷¹ or the double cross have been used for variable volume injections. Also, different gated injection modes using a cross have been developed to achieve fast injections. It can take 10-150 s for a sample to migrate from the sample reservoir to the injection cross in the pinched injection mode, which can be longer than the separation time. In the gated modes, the sample is kept close to the cross during the separation, so that it takes very little to load it again at the cross.

Figure 1.6: Schematic diagrams of sample injection schemes on microfluidic devices. Shown in **A** is the Tee injector, in **B** is the double-Tee injector and **C** is the cross injector. The first schematic in **A**, **B**, and **C** depicts sample loading and the second schematic is sample injection.

1.4 Detection Schemes

A number of detection schemes have been employed in literature for detection of samples processed on microfluidic devices including fluorescence, mass spectrometry, electrochemical, backscatter, *UV/Vis*. These detectors are used as end or off-chip detectors. However fluorescence can be used for imaging flow through microfluidic channels and is the only real way to do so. Fluorescence imaging of the whole microfluidic device can be performed by dispersing light from a lamp to illuminate the whole chip to capture every part of the device. The major drawback of this major is poor light collection efficiency. Most of the fluorescence imaging is therefore performed using epi-fluorescence light microscopes which are very efficient in collecting fluorescent light. However, they can only be used to illuminate one part of the microfluidic device at a time and the microscope objective has to be translated to illuminate and collect light from different parts of the device. The detection systems

discussed here will be limited to fluorescence and mass spectrometry, which were the detection schemes used in this work.

1.4.1 Epifluorescence and Fluorescence microscopy

Laser-Induced fluorescence (LIF) is a popular on-chip detection technique due to its high sensitivity. However, this method requires fluorescent analytes, and so various labeling chemistries have to be employed to tag analytes such as proteins and peptides with fluorescent molecules (e.g. fluorescein, rhodamine or bodipy). For proteins, native fluorescence can be utilized where protein molecules are excited with UV radiation, and the fluorescence due to tryptophan and tyrosine residues detected. The optical set-up of an epi-fluorescence system is illustrated in Figure 1.7 and includes a light source, which can be a laser or a broadband source like as Hg or Xe lamp. If a lamp is used, the light must be passed through an excitation filter, which transmits a band of wavelengths to the dichroic mirror, where the light is reflected at 90° to the sample. The light is focused on the sample through a microscope objective, which is also used to collect the fluorescence from the sample. An emission filter is utilized to filter out the Rayleigh scattering before the fluorescence is transmitted to a detection device such as an imaging camera or photomultiplier tube PMT for measurement.

Greater sensitivity has been achieved by using confocal set-ups, where a laser excitation light is scanned on a fluorescent sample and the fluorescence is passed through a pin-hole placed before the emission filter to spatially reject fluorescent out-of-focus light before detection by a PMT⁷²⁻⁷⁵. One of the major drawbacks with confocal microscopes is photobleaching of the sample by the intense laser light. The use of the

pinhole results in a reduction in the light detected by the PMT which is often compensated for by increasing the intensity of the laser light, resulting in photobleaching fluorescent molecules. Another drawback is that, since they are scanning instruments, they are slow in capturing images and not very ideal for applications where flow is monitored in microfluidic channels in real time.

Figure 1.7: Schematic diagram of an Epifluorescence detection system

1.4.2 Electrospray Mass Spectrometry

There are a number of benefits in interfacing microfluidic devices with electrospray ionization mass spectrometry. One obvious advantage is the match in the flowrates (tens of nL/min to a few $\mu\text{L}/\text{min}$) used in microfluidic channels and those required for nanoESI-MS. More importantly, ESI-MS is concentration sensitive over a

wide range of flowrates, i.e, the signal response is a function of the concentration of the analyte in solution. For microfluidic devices, the highest sample concentration is achieved, due to the miniaturized sample introduction system, which translates into the lowest detection limits when ESI-MS is used. The mass spectrometer is a powerful analytical tool given its ability to provide a wealth of structural information from collision-induced experiments and its low detection limits. Moreover, microfabrication of the microfluidic devices allows the integration of a large number of electrospray emitters on the same device, resulting in high throughput analysis of the samples^{76, 77}

Various microfluidic chip-ESI-MS configurations have been used by different researchers to interface microfluidic chips with mass spectrometer. Early attempts to interface microchips to MS involved spraying fluid directly from an exposed channel on the microchip.^{78, 79} Although this design is attractive, due to simple fabrication, the large Taylor cones formed by the solution exiting out of the open end of the microchannel, causes sample dilution (lower sensitivities) and band broadening. Also, because the surface at the edge of the microchip was flat, these devices required an impractically high voltage to overcome the liquid surface tension and initiate electrospray. Other researchers attempted using hydrophobic coatings (to minimize surface tension and Taylor cone volume) at the edge of the microchip and on-chip nebulizers, but these methods have been met with limited success⁸⁰. More recently, a transfer capillary spray tip with a small orifice has been integrated with a microchip and has been found to be an effective means of sample delivery into a mass spectrometer.⁸¹⁻⁸³ For these devices, application of high voltage can be through a platinum electrode in contact with solution in a reservoir, a liquid junction or by a conductive coating at the outlet of the capillary. The most recent

advancements utilize microtechnology fabrication techniques to integrate nanospray tips directly onto the microfluidic device, which eliminates the large Taylor cone and the associated band broadening. An example of such a device was developed by the Henion group and Advion Biosciences, based on a silicon substrate with etched nozzles in the planar surface of the silicon wafer, used directly as microspray emitters perpendicular to the chip.⁸⁴ Smith's group recently reported a polycarbonate based device with an ESI tip constructed on the polycarbonate plates by laser micromachining, for IEF-ESI-MS.⁸⁵

1.4.2.1 The Electrospray Mechanism

For proper design and optimization of a microfluidic chip-ESI-MS interface, it is of paramount importance to understand the electrospray process. The electrospray process (depicted in Figure 1.8) has been theoretically and experimentally analyzed by several researchers, and can be divided into 3 steps which encompass droplet formation, droplet shrinkage and gaseous ion formation.^{86, 87}

Figure 1.8: A schematic diagram illustrating the electrospray process adapted from reference 72.

A high electric field is applied at the capillary tip (spray tip), while the analyte solution is passed through the capillary at a constant flowrate. The magnitude of the voltage required for the onset of electrospray can be calculated using the formula in equation 7,

$$V_o = A \left(\frac{2r\gamma \cos \theta}{\epsilon} \right)^{1/2} \ln \frac{4d}{r} \quad (7)$$

Where V_o is the electrospray onset voltage (V), A is a dimensionless constant, θ is the half-angle of the Taylor cone apex, γ is the surface tension (Nm^{-1}), ϵ is the permittivity of vacuum ($\text{N}^2\text{C}^{-1}\text{m}^{-2}$), d is the distance between the counter electrode and the tip (m), and r is the radius of the capillary (m). In the positive ion mode, the positive potential at the capillary tip results in the formation of a stream droplets carrying positive ions at the tip,

while the negative ions of the analyte solution migrate away from the tip. When the surface tension of the electrolyte solution exiting the spray tip is overcome by repulsive forces of the positive ions at the surface, and the attraction of the positive ions to the counter electrode, it expands into a ‘Taylor cone’, which is drawn out into a filament at its tip. Single droplets bud off from this filament and shrink via evaporation through the pressure gradient towards the counter electrode. When the droplet is sufficiently small such that the charge density of the droplet overcomes the surface tension holding the droplet together (Rayleigh Stability limit), coulombic explosion (fission) occurs where smaller daughter droplets are formed. However, the final process through which bare ions are produced from the small highly charged droplets has not been clarified. There are two proposed mechanisms are: the ion evaporation model (IEM) and the charge residue model (CRM). The formation of gas-phase ions by IEM is thought to occur by “evaporation” of ions from a high charge density droplet, with the driving force of the evaporation being the repulsion between the charged droplet and the other ions in the droplet.^{88, 89} Alternatively, gaseous ion formation in the charge residue model is thought to occur by shrinkage of a droplet until it contains a single charged ion which is released into gaseous phase by further solvent evaporation.^{90, 91} There is substantial evidence presented in literature that large ions such as non-denatured proteins are produced by CRM and small ions are produced by IEM.^{87, 92-94}

The parameters that affect electrospray generation at the outlet of the microchannel or capillary to obtain a functional interface are clear from an understanding of the electrospray process. The first requirement for a microfluidic chip-MS interface is maintaining stable flow of solution to the spray tip, which is needed to sustain droplet

formation in the electrospray source ⁷⁹. For an electrokinetically driven system, this means that electroosmotic flow (EOF) must be present in the microchannel, requiring charged sites on the channel surface. A basic pH is usually sufficient to meet this requirement, given that many microfluidic devices are fabricated on glass. Stable spray is dependent on the diameter of the tip and the spray capillary inner diameter (I.D), which generate more resistance to flow with decreasing sizes. At the low flowrates afforded by microfluidic devices, small spray tip sizes, and spray capillary I.Ds, small droplets with high surface-to-volume ratio form at the tip, allowing better ionization and desolvation efficiencies due to rapid droplet solvent evaporation. Mann and Wilm estimated the droplet size formed at a 1-2 μ m tip to be less than 200nm in diameter, corresponding to an average concentration of one analyte molecule per droplet for a 1pmol/ μ L solution.⁹⁵ By separating molecules into different droplets, cluster formation is minimized and the analysis of samples with high salt concentrations is improved. McLafferty and co-workers demonstrated this concept by further reducing the flowrate of 25nL/min used by Mann and Wilm to 1 nL/min by using a 5 μ m I.D capillary, 2 μ m spray tip and achieved attomole-sensitivity detection of large biomolecules.⁹⁶ It is therefore beneficial to use spray capillaries with as small capillary I.Ds and spray tip sizes as practically possible to achieve efficient electrospray and consequently high sensitivities. Although smaller spray tips and lower flowrates improve sensitivity, it is often difficult to operate the interface for a long time without clogging problems, which interfere with achieving stable electrospray. Therefore, practically, there is a trade-off between the smallest ESI orifice that can be used and the ruggedness of the interface. The interface must be able to operate for a long time with good stability and reproducible operation parameters.

There are other factors that need to be considered for the operation of a microchip-MS interface. The lowest detection limits in ESI-MS are usually realized when the ionization source is operated in the positive ion mode (*i.e.* a positive voltage is applied at the spray tip). This requirement dictates a low pH spray solution. In order to support sufficient EOF under acidic conditions, the native glass surface of the microchannel must be coated with the material that provides a high surface charge at acidic pH. A make-up or sheath flow solution may be necessary in some cases to modify the electrophoresis buffer to achieve the necessary conditions (pH, concentration or flow) for electrospray. Lastly, the chip substrate⁹⁷ must be compatible with organic and acids while producing minimal chemical background. Solvents must be volatile, of high purity and not contain ion-suppressing agents such trifluoroacetic acid (TFA).

1.5 Multiplexing

A future goal of microfluidic technologies applied to proteomics is the ability to concentrate, digest and separate real proteomic samples on a single device, with mass spectrometric identification. This goal requires integrating different microfluidic components (digestion micro-reactors, pre-concentrators, 2-D separation systems, MS interfaces) into a single automated device capable of high-speed analysis and high throughput sample processing. The challenge posed here, is that, the integration of components requires reproducibility in operation and fabrication. Moreover, the different components normally require different buffers and operating conditions, making it more challenging to integrate into one functional unit. Multiplexing of microfluidic components requires interdisciplinary collaborations, and there are attempts recorded in

literature to integrate different proteomic sample handling processes onto single devices. Some of the most promising integrated devices, including one by Li *et-al*⁹⁸, which is composed of a large channel with affinity media for binding of peptides or proteins, an electrophoretic separation channel and an ESI-MS interface. Gottschlich *et-al*⁹⁹ demonstrated digestion, separation and labeling of peptides and proteins for laser induced fluorescence (LIF) detection on single device. Also, Wang *et-al*¹⁰⁰ fabricated and operated a microfluidic device composed of protein digestion bed, an injector, a CE channel and an ESI-MS interface. However, these devices were used for proof-of-principle experiments and are still to be developed for analysis of real proteomic samples and commercialization. The use of these devices as viable miniaturized bioanalytical instruments has been hampered by lack good macro-world to chip interfaces as well good methods for pre-treating proteomic samples to minimize clogging of microchannels due to small particulates and sample precipitation. In addition, the use of minute samples volumes requires a very sensitive detection method, which imposes a substantial difficulty in detecting dilute samples.

1.6 Outline of Dissertation

This dissertation is organized into five major chapters. Chapter 2 describes the fabrication and characterization of a microfluidic electroosmotic flow pump that can be used to deliver fluids on microfluidic platforms. Most electrokinetic pumps designed and reported in literature are limited in the pH range in which they can operate. The electrokinetic pump designed in this work can operate over a wide pH range, which is important in proteomic analyses, since protein have widely varying pIs. The third

chapter details the fabrication and use of a hydrodynamic flow restrictor in a microfluidic chip CE-ESI-MS interface for the efficient introduction of make-up solution under low EOF conditions. Low EOF conditions are important because neutral, hydrophilic polymers such as polyvinyl alcohol (PVA) and polyethylene glycol (PEG), which are effective in reducing protein adsorption to the channel surfaces reduce EOF. Most microfluidic chip-ESI-MS interface described in literature utilize high electroosmotic flow to deliver samples for electrospray. Polyethylene glycol terminated silane self-assembled monolayers (SAMS) are developed to passivate glass microfluidic surfaces for efficient capillary electrophoresis separations, and their performance on fused silica capillaries and microfluidic channels are reported in the fourth chapter. The fifth chapter describes the development of a microfluidic chip ESI-MS interface that can be used for high pH, low EOF separation of proteins or peptides with positive ion mode ESI-MS detection. A novel voltage switching component is used for the application of electrospray voltage, and is integrated with the hydrodynamic flow restrictor interface to produce a chip-CE-ESI-MS system for the delivery of negatively charged samples at high pH with positive ion mode ESI-MS detection. The interface can potentially be used to separate on-chip tryptic digest typically performed at pH 8, followed positive ion mode MS detection by utilizing a low pH make-up solution to lower the pH of the digest and generate positive ions.

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Chapter 2

Fabrication and Characterization of a Fritless Microfabricated Electroosmotic Pump with Reduced pH Dependence.

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Abstract

A fritless electroosmotic pump with reduced pH dependence has been fabricated on a glass microchip and its performance characterized. The chip design consists of two 500 μm channels, one packed with anion exchange beads and the other packed with cation exchange beads, which produce convergent electroosmotic flow streams. The electroosmotically pumped solution flows away from the intersection of the two pumping channels through a field free channel. This simple design allows for the production of a fritless electroosmotic pump, and easy replacement of the ion exchange beads whose charged surfaces generate the flow. The pump was found to produce volumetric flowrates of up to 2 $\mu\text{L}/\text{min}$ for an applied voltage of 3kV at a pH of 6.8. Moreover, the electroosmotic pump can generate high flowrates over an extended pH range of at least 2-12, a significant advantage over previously fabricated electroosmotic pumps, which typically have a more limited range in which they can achieve high flowrates.

2.1 Introduction

Microfluidic devices hold much promise for the analysis of chemical and biological systems due to their ability to increase speed and sample analysis through massive parallelism, fast separations, the use of small volumes, and short diffusion lengths. Microfluidic systems have been developed for DNA analysis,¹ high throughput screening,² antigen detection,³ and point of care diagnostic medical devices.^{4, 5}

Fluid control involving mass transport and direction through the channels is crucial to the development of functional devices. Mass transport is achieved using either hydrodynamic or electrokinetic pumping. Control of flow direction in hydrodynamically driven systems requires on-chip active or passive valves for switching flow streams whereas electrokinetic systems utilize electrokinetic valving as originally demonstrated by Seiler et-al.⁶ Micropumps can be classified as either field-induced flow pumps or mechanical membrane-displacement pumps. Field-induced pumps include electroosmotic flow (EOF), electrohydrodynamic, centrifugal, and magneto-hydrodynamic pumps; while mechanical membrane-displacement pumps include electrostatic,⁷ electromagnetic,⁸ thermo-pneumatic,⁹ photo-thermal,¹⁰ and piezoelectric pumps.¹¹ A number of applications have been reported for EOF pumps including pumping mobile phases in chromatographic separations,¹² delivering reactants into on-chip reactors,¹³ flow injection analysis,¹⁴⁻¹⁶ and interfacing microchips to mass spectrometers.^{17, 18}

Electroosmotic pumps offer several advantages over membrane displacement pumps. Fabrication of field induced flow pumps is relatively simple, requires no moving parts, and they can be easily integrated into electrokinetically driven systems. These

pumps are also easy to operate and produce pulse free flow, which is important for microfluidic and flow injection analysis systems requiring a small and constant supply of solutions. Microfluidic EOF pumps are capable of generating flowrates from a few nL/min to hundreds of $\mu\text{L}/\text{min}$.¹⁹ However, EOF pumps generally have low stall pressures, and therefore are generally not used in high pressure systems. Also, electroosmotic pumping requires that the liquid is polarizable,²⁰ and does not work well for fluids that are non-conductive, highly conductive, and those at extreme pHs such as some strong acids and organic solvents, since the current levels are either excessive or inadequate to support any significant EOF. In contrast, mechanical pumps are able to pump almost any fluid with a typical flow range of 1-100 $\mu\text{L}/\text{min}$.

The use of electroosmosis for pressurized pumping emerged almost 40 years ago.²¹ Most surfaces develop an electric double layer (EDL) when brought into contact with electrolyte solutions produced by the zeta potential at the liquid/solid interface. In the case of glass surfaces, deprotonation of acidic silanol groups produces a negatively charged surface. Counter ions from solution are attracted to the wall and shield these charges, with dissolved counter-ions being repelled from the wall forming the EDL. When an electric field is applied, the mobile ions in solution move in response to the field, dragging the bulk of the solution with them, and thereby producing electroosmotic flow. Because EOF pumps typically have low stall or maximum operating pressures, much research has focused on increasing their useful pressure range. The primary approach to generating higher pressures with EOF pumps is to fabricate a device with many small channels in parallel. In such a design, smaller channels increase the stall pressure by increasing the pump's ability to resist hydrodynamic flow in the reverse

direction, which is produced by the backpressure. In hydrodynamically driven systems the linear flow rate is directly proportional to the square of the channel radius as shown by Equation (1):

$$\mathbf{V_{HD} = \Delta P r^2 / 8 \eta L} \quad (1)$$

where ΔP is the backpressure, r is the capillary radius, $\mathbf{V_{HD}}$ is the hydrodynamic linear flowrate, η is the viscosity and L the length of the capillary. In contrast, the linear flowrate developed by EOF is independent of the radius as shown by equation (2):

$$\mathbf{V_{EOF} = (\xi \epsilon / 4 \pi \eta) E} \quad (2)$$

where $\mathbf{V_{EOF}}$ is the electroosmotic linear flow rate, ξ is the zeta potential, ϵ is the dielectric constant, η is the viscosity and E is the electric field. The high surface area to volume ratio associated with this porous structure is also partially responsible for the generation of high pressures. Although smaller channels provide higher backpressures, the linear flow rate is constant and there is a reduction in the volumetric flowrate, which is proportional to the reduction in cross-sectional area at constant linear flowrate. This reduction in the volumetric flow rate can be compensated for by fabricating a device with small channels in parallel. The most simple and compact method for producing many small parallel channels is to pack a large channel with small beads, such as chromatography beads.²² The interstitial spaces between the particles in a packed column form multiple parallel channels with very small radii. Such packed EOF pumps are capable of generating higher pressures (in excess of 20atm) than EOF pumps made from open capillary columns.²³

Electroosmotic flow is strongly affected by a variation in pH, as the charge on the channel surface is influenced by pH.²⁴ Therefore, the EOF velocity generated by most EOF pumps is greatly influenced by the solution pH. Most microfluidic EOF pumps are made in glass or fused silica. For glass and fused silica devices, the surface charge or zeta potential is produced by deprotonation of silanol groups. A wide pH range is needed for optimizing separations, handling biological samples, and for reactions that are pH sensitive.

A number of EOF pumps have been described by various researchers. Zeng et-al recently designed and fabricated packed capillary column EOF pumps capable of generating flow rates of up to 3.6 μ L/min for 2kV applied potentials and pressures in excess of 20atm.²⁵ The EOF pumps were fabricated by packing 500-700 μ m I.D fused silica capillary columns with 3.5 μ m non-porous silica particles and using a silicate frit fabrication process²³ to hold the particles in place. Lazer and Karger developed an open channel micropump with hundreds of parallel small-diameter open microchannels that can generate flowrates of 10-400nL and pressures of up to 80 psi. EOF pumps of a similar design using coated open capillaries have been described but their performance was not characterized.²⁶ Various theoretical approaches such as full numerical simulations using computational fluid dynamics or the semi-analytical equivalent circuit theory, have been developed to predict pressures and flowrates associated with various pump designs and then correlating them to experimental results.²⁷

In this paper, an electroosmotic pump with both anion and cation exchange beads, packed in separate channels that pump towards an intersection is presented. Combining the two flow streams results in higher flowrates for the pump over a wide pH range. This

pump can be used to deliver solutions ranging from a pH of at least 2-12. The beads are immobilized in the channels using weirs and membranes, eliminating the need for frits. This configuration makes the removal and replacement of fouled beads easy. Since no frits are used, there is no difficulty of frit fabrication, no contaminants such as polymer leachates from frit fabrication, reduced outgassing, and reduced susceptibility to clogging. Therefore, these characteristics allow for easy fabrication and efficient operation of the EOF pump.

2.2 Experimental

2.2.1 Materials and reagents.

All solutions were prepared using ultrapure distilled deionised (DI) water from a Barnstead water purifier, 18M Ω , (Dubuque, Iowa, USA). The pH 2, 3.6, 4.2 and 5.6 acetate buffers were prepared using glacial acetic acid (99.9%) and sodium acetate trihydrate (98%, Fisher). The pH 6.0, 6.8 and 7.2. buffers were prepared using monosodium phosphate monohydrate and disodium phosphate heptahydrate (99.7%, Fisher). The pH 8.0, 9.1, 10 and 12 borate buffers were prepared using molecular biology grade boric acid (Promega) and sodium tetraborate decahydrate (99%, Acros Organics). All buffers were degassed before use with a sonicator (Aquasonics, VWR scientific products). Other reagents used were mesityl oxide (98%, Sigma), formamide (99.5%, Sigma) and Potassium silicate (Kasil no.1, PQ Corporation).

The poly(aspartic acid) cation exchange beads (5 μ m, 100 Å) and polyethyleneimine anion exchange beads (5 μ m, 100 Å) were purchased from Western Analytical Products, Inc (Murrieta, CA). The non-porous, 0.5 μ m cation and anion

exchange beads were purchased from Zirchrom Separation, Inc. Teflon filter membranes were obtained from Applied Biosystems (Foster city, CA). Fittings were obtained from Upchurch Scientific. Borosilicate D263 was obtained from Precision Glass and Optics (Santa Ana, CA) and 5 μ L disposable pipettes were purchased from Fisher.

2.2.2 Device design and fabrication.

The channels were patterned on the glass chips using standard photolithography and wet chemical etching methods on Borosilicate D263 glass.²⁸ The photomasks were designed using Freehand 10 program, and the negatives were printed on an Afga Accuset 1000 printer with a resolution of 3000 dpi. The weirs on the microchip were made by including a 50 μ m gap in the mask of the channel at weir locations and controlling the glass etching time. The glass substrates were etched 50% HF/ 70% HNO_3 / H_2O , 2/1/7, v/v/v at an approximate etching rate of 0.8 μ m/min for 25 min. *Hydrofluoric acid should be handled with extreme care as it is very hazardous. Exposure to vapour and skin contact is extremely dangerous and therefore accepted methods for handling and use should be followed.* Holes were drilled in the glass using a precision drill press (Tralmike's-Tool-A-Rama, Plainfield, N.J). After fabrication, the glass plates were cleaned with acetone and with a soap solution. The plates were placed in a 1:1:1 solution of $\text{NH}_4\text{OH}/\text{H}_2\text{O}_2/\text{H}_2\text{O}$ solution at 80°C, rinsed with DI water and finally in Piranha ($\text{H}_2\text{SO}_4/\text{H}_2\text{O}_2$, 3:1 v/v) solution at 100°C, after which the glass plates were washed in a wafer washer for 5 washing and drying cycles using deionized water.²⁹ A microscope (Leica GZ6 stereo-microscope) was used to aid with the alignment of the two glass chips. Permanent bonding was achieved by placing the glass plates in the furnace and applying the temperature program; 25-100°C at 400°C/min, 100°C for 15 min, 100°C-600°C at

10°C/min, 600°C for 15 min. The furnace was allowed to cool naturally to ambient temperature.

After bonding the microchip was rinsed first with 0.1 M sodium hydroxide for 5 min, followed by 25 mM phosphate buffer at pH 6.8. The same buffer was used to measure the resistance of each channel and obtain an Ohm plot. The current was determined indirectly by measuring the voltage drop across a 10 k Ω resistor using a Fluke 75 Multimeter. The channel dimensions were measured using a stylus instrument (Tencor Instruments). The beads were suspended in an acetonitrile slurry and packed by placing the cation exchange bead slurry in reservoir 1, the anion exchange beads in reservoir 2 and applying vacuum at reservoir 3, as shown in Figure 2.2.

Figure 2.1. Schematic diagram of the initial design of the microfluidic electroosmotic pump. With the polarity indicated, the device pumps solution from reservoirs 1 and 2 into the field free channel and reservoir 3. The pumping channels with packed beads are each 15 mm in length. The direction of flow in the field free channel can be reversed by reversing the polarity. The magnified area shows the cross-section of the weir used to contain the beads in the channel.

Changing to the aqueous buffer after packing caused the beads to agglomerate and become stable in the channel.^{30,31} Threaded, flat bottom, PEEK polymer nanopore reservoir (Upchurch Scientific, N-131) with nuts were attached to the top plate. A 3mm diameter teflon filter membrane (Applied Biosystems) was sealed against the cover plate with the nut and an O-ring. Alternatively, kasil frits were fabricated in glass reservoirs that were attached to the chip using epoxy. The design of the pump, shown in Figure 2.1, consists of a 500 μm wide channel intersecting a 50 μm wide channel to form a T-intersection. The 500 μm channel is 20 μm deep and 3.8 cm long with 2 weirs 5 mm from the center of the channel. The weirs are 12 μm high, producing an 8 μm gap

between the top of the weir and the cover plate. The 50 μm channel is 5 cm long and 20 μm deep.

A high voltage octochannel power supply (EMCO) connected to platinum wire electrodes was used to apply all electrophoretic voltages. *Caution should be exercised when applying the dangerous high voltages as they can be lethal without appropriate care.* Voltages were controlled using a program written in-house with Lab Windows, (National instruments, Austin TX).

2.3 Results and Discussion

2.3.1 Microfluidic EOF Pump Fabrication

The weirs were fabricated in a single step process by incorporating a 50 μm gap into the channel mask and carefully controlling the etching rate. An etching time of 25 min was found to reproducibly yield 20 μm deep channels with an 8 μm gap between the highest point on the weir and the cover glass. Longer etching times produced a deeper channel and larger gap between the weir and the cover glass. In a second design, the weir was eliminated and the pumping channels were narrowed from 500 μm to 50 μm before the intersection with the field-free channel. The 5 μm beads could be packed into the 500 μm channel and were retained by the 50 μm constriction. Incorporation of a weir onto the microchip at the beginning of 50 μm constriction produced current breakdown problems, so the weir was eliminated from the fabrication process for this design.

The inlet of the pumping channels was sealed with a Teflon membrane to prevent the beads from migrating into the buffer reservoirs. The Teflon membrane was held over the inlet with a threaded nanoport fitting that compressed an O-ring against the Teflon

membrane and the top of the glass substrate. This design was easy to fill with solution and the membrane did not cause buffer outgassing. The use of this membrane reduces clogging and provides an efficient method of sealing the beads in the device and allows for the removal of fouled beads. This method of bead entrapment also eliminates any impurities associated with frit fabrication processes that increase the chemical background.

2.3.2 Microfluidic EOF Pump Flowrates.

The maximum flow rate measured for the microfluidic EOF pump was 2 μ L/min, which was produced at an applied voltage of 3 kV using 50 mM phosphate buffer at pH 6.8. Volumetric flowrates obtained with the EOF pump on the microfluidic platform are shown in Figure 2.2, and as expected gave a linear relationship with respect to applied voltage. This observation is consistent with the literature as the maximum flow rate of the porous structure under conditions of no counter pressure is a direct function of the applied voltage and surface area.²⁵ The maximum flow rate is limited by the cross-sectional area of the porous media and the maximum voltage that can be applied.

Figure 2.2. Relationship between the volumetric flow rate and applied voltage for microchip EOF pump design in Figure 2.1. The EOF pump reaches a flowrate of 2 $\mu\text{L}/\text{min}$ at an applied voltage of 3kV at pH 6.8.

2.3.3 Flowrate dependence on pH.

An important characteristic of this pump is its ability to operate over an extended pH range. Most electroosmotic pumps are limited in the pH range in which they can operate because the zeta potential is dependent on the pH. For microfluidic devices based on a native glass surface, EOF is produced by the deprotonation of acidic surface silanol groups. Therefore, the EOF in glass devices is nearly zero below a pH of 4 and reaches a maximum above a pH of 8 where nearly all of the ionizable silanol groups are deprotonated.³² In this device, the pH dependence is reduced by the use of two pumping channels with oppositely charged surfaces. Conventional EOF pumps use either negatively or positively charged surfaces, but the pump reported here reduces the pH dependence by using both negatively and positively charged surfaces. The EOF pump shown in Figure 2.3 was used to determine the dependence of flowrate on pH for the

conventional single channel pump, which is packed with either the anion or cation exchange beads.

Figure 2.3. The design for equivalent conventional EOF pumps is shown. In one pump type, the pumping channel was packed with cation exchange beads, and for the other pump type, the pumping channel was packed with anion exchange beads. To be consistent with the conventional EOF pump design, some modifications were necessary compared with Figures 2.1 and 2.5. The electrode connection was completed at the end of the pumping channel by drilling a 200 μm access hole through the cover plate into the channel, at the end of the packed bed.

An access hole of 200 μm I.D is drilled at the end of the 500 μm channel to allow access of the palladium wire electrode of the same dimension. This EOF pump was designed to closely match the design of one pumping arm of the EOF pump with the two pumping channels, for good comparison. As shown in Figure 2.4, the cation exchange beads alone achieve a maximum flowrate of 0.4 $\mu\text{L}/\text{min}$ above a pH of 6, and the anion exchange beads alone achieve a maximum flowrate of 0.5 $\mu\text{L}/\text{min}$ below a pH of 6. The EOF pump presented here with both cation and anion exchange beads has a flowrate of 0.4 $\mu\text{L}/\text{min}$ or greater over the entire measured pH range of 2-12. Therefore, by combining

the flow streams from both the cation and anion exchange beads the dependence of the flowrate on the pH has been reduced, and the useful pH range has been increased. The channel with the cation exchange beads produces most of the flow at high pH because the poly(aspartic acid) functional groups are largely deprotonated providing a negatively charged surface at pHs above its pK_a . The pumping channel with the anion exchange beads produces flow at low pH because polyethyleneimine functional groups are predominantly positively charged at pHs below the pK_a of 9.1 of the conjugate acid. Thus, the opposite surface charges insure that one of the channels is pumping at high or low pH.

Figure 2.4. The flowrate as a function of pH for the conventional pumps. The pump utilizing anion exchange beads achieves a maximum flowrate below a pH of 6 and the cation exchange beads achieve a maximum flowrate above a pH of 6. It is important to note that for both pumps, the flowrate nearly goes to zero at high or low pH.

The design shown in Figure 2.1 was found to provide the highest maximum flowrate, but made only minimal improvements in extending the useful pH range. The pH range of

this design was limited because at low pH, the channel with the cation exchange beads did not pump and did not provide adequate resistance to hydrodynamic flow. Therefore most of the flow from the anion exchange beads would flow through the cation exchange bed, bypassing the field free channel. At high pH, the situation was reversed, and the solution pumped by the cation exchange bed would exit through the anion exchange bed. To reduce this leakage problem, an improved design was employed that restricts the flow between the two pumping channels by narrowing the pumping channel to 50 μm before its intersection with the field free channel, as shown in Figure 2.5.

Figure 2.5 The schematic diagram of an improved version of the microchip EOF pump shown in Figure 2.1. The design shown in Figure 2.1 did not exhibit improved flowrates over the entire pH range, and was modified to reduce the dependence of the flowrate on the pH. The 50 μm constriction of the pumping channels reduces backflow through the non-pumping section at pH extremes by increasing the resistance to hydrodynamic flow into the non-pumping section

The 50 μm channel section provides a much greater resistance to hydrodynamic flow back through the non-pumping bed, and forced most of the solution out of the field free channel. As shown in Figure 2.6, the pump produced flow rates ranging from 0.1 $\mu\text{L}/\text{min}$

– 1.0 $\mu\text{L}/\text{min}$ over a pH range of 2-12, with a maximum flow rate of 1 $\mu\text{L}/\text{min}$ obtained at a pH of 6.8. This maximum flow rate is less than the flow rate of 2 $\mu\text{L}/\text{min}$ obtained using the design in Figure 2.1 at pH 6.8.

Figure 2.6. The relationship between pH and flowrate at an applied voltage of 3kV for the microchip design in 2.5 packed with 5 μm anion and cation exchange beads.

The reduced maximum flow rate is caused by the narrowing of the ends of the pumping channels, which increases the frictional resistance to flow both into and out of the pumping channels. Thus, a trade-off is observed between the width of the pH range and the flow rate. The pump can potentially produce flow at extreme pH values (below 2 and above 12) since the graph in Figure 2.6 can be extrapolated to give flow rates at such pH values. A limitation in operating at extreme pH values is the degradation of the silica-based beads, but use of polymeric beads that are stable at extreme pH values can allow for the widening of the pH range in which the pump can operate. The useful pH range could be further extended by using acidic functional groups with a lower pK_a and basic functional groups with a higher pK_a for the conjugate acid.

The design described above still suffers from some degree of leakage of solution into the non-pumping channel. Flowrates at pH 2 and pH 12 are about 40% and 10% of the maximum flowrate respectively. This leakage is further minimized by using smaller beads to increase the resistance to the hydrodynamic back flow without inhibiting the EOF. The performance of the pump was improved considerably by incorporating smaller 0.5 μm non-porous beads into the pumping channels. Figure 2.7 shows a graph of the relationship between the pH and flowrate for the microfluidic pump packed with 5 μm anion and cation beads followed by a 1.5 mm length packing of 0.5 μm of non-porous anion and cation exchange beads respectively. The sum of flowrates at pH 2 and pH 12 in this case, is about 0.85 $\mu\text{L}/\text{min}$, which is the same as the maximum flowrate at pH 7.2. It is expected that at pH extremes the flowrate is equal to half the maximum flowrate. Therefore, with the 1mm section of the smaller 0.5 μm non-porous beads, the leakage through the non-pumping bed was minimal, as the flowrate at pH 2 and pH 12 was nearly half of the maximum flowrate.

Figure 2.7. The design shown in Figure 2.5 was further improved by the addition of a 1.5 mm section of 0.5 μm beads to further prevent leakage into the non-pumping channel, and the relationship between pH and flowrate at an applied voltage of 3kV is shown. The improved flowrates at both high and low pH produce the reduced pH dependence, compared to conventional designs shown in Figure 2.4.

2.4 Conclusions

A novel microchip based EOF pump capable of generating higher flowrates for a wider range of pH values of at least 2-12 has been presented. A design that improves the flowrates at extreme pH values has been described. Maximum flowrates of up to 2 $\mu\text{L}/\text{min}$ have been achieved using phosphate buffer at pH 6.8. The pump is fabricated using standard photolithography and wet chemical etching techniques allowing for easy and reproducible fabrication of microchips. Weirs have been fabricated within the microchip channels, eliminating the use of frits inside the channels to hold the beads and any frit synthesis that can introduce polymer leachates and contaminants. The pump has the capability of pumping non-conductive and highly conductive liquids to microreactors by the use of negative pressure applied to a reservoir containing the fluid. It is expected that the pump will be utilized in future microfluidic systems in applications such as reagent delivery, sample infusion for ESI-MS, flow injection analysis, and separations.

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Chapter 3

A Stable Electrokinetic/Hydrodynamic Flow Microfluidic CE/ESI-MS Interface utilizing a Hydrodynamic Flow Restrictor for Delivery Samples under Low EOF

The contents of this chapter were previously published by Razunguzwa, Lenke and Timperman and are reproduced here with minor modifications, (*Lab-on-a-chip* 2005, 5(8)).

Abstract

A hydrodynamic flow restrictor (HDR) has been fabricated in a microfluidic channel by laser micromachining that is used to combine electrokinetic and hydrodynamic flow streams. Combining electrokinetic and hydrodynamic flow streams is challenging in microfluidic devices, because the hydrodynamic flow often overpowers the electrokinetic flow, making it more difficult to use low electroosmotic flow in the electrokinetic portion of the system. The HDR has been incorporated into a capillary electrophoresis-mass spectrometry interface that provides continuous introduction of a make-up solution and negates the hydrodynamic backpressure in the capillary electrophoresis channel to the extent that low EOF can be utilized. Moreover, the hydrodynamic backpressure is sufficiently minimized to allow coatings that minimize EOF to be used in the electrokinetically driven channel. Such coatings are of great importance for the analysis of proteins and other biomolecules that adsorb to charged surfaces.

3.1 Introduction

Microfluidic analytical systems have been engineered for sample preparation, solid phase extraction, tryptic digestions and separations of biological samples.¹ Massive parallelism, shorter analysis time, increased separation efficiencies, higher sensitivities and reduced waste generation can be achieved with these devices.² Microfluidic devices are characterized by their zero dead volume intersections and small channels, having depths and widths ranging from a few microns to a few hundred microns. These intersections allow for fabrication of microfluidic networks that can be used to create microfluidic systems for integrating sample handling, analysis, and other processes.^{3, 4}

Mass spectrometry (MS) is one of the most widely used instrumental methods for molecular characterization, because of its ability to provide detailed structural information and its low limits of detection. Of the ion sources for MS, electrospray ionization (ESI) is best suited for the generation of molecular ions from the solution phase, and consequently ESI is the method of choice for combining on-line separations with the MS. ESI-MS is concentration sensitive,⁵ and consequently the best LODs are obtained for a given analyte mass by reducing the volume in which the sample is contained. Therefore, coupling microfluidic systems for sample handling and separation with ESI/MS can greatly improve performance for trace analyses.

However, development of a robust and flexible interface for microfluidic capillary electrophoresis and ESI is not as simple as it can appear initially. Complications arise because the flowrate at the ESI tip is largely determined by the orifice ID, and the orifice ID greatly influences the resistance to hydrodynamic flow. Increasing the spray tip orifice ID to reduce the resistance to flow also requires higher flowrates that can be

difficult to produce electroosmotically. As a result of the numerous competing processes in a CE/MS interface, many types of microfluidic-ESI-MS interfaces have been developed, and they can be generally classified in three different categories: sheathless, sheath flow, and liquid junction. The sheathless configuration utilizes an electrode immersed in solution of a reservoir on the chip, to apply both the CE and electrospray voltage.⁶ Alternatively, the electrospray voltage can be applied via a metal (gold or silver) coated spray tip.⁷ The sheath flow interface configuration utilizes a transfer capillary to transport the sample to a sheath flow interface where the electrospray voltage is applied and the make-up solution introduced.⁷ Lastly, a number of microfluidic/ESI-MS interfaces have used a liquid junction for the application of the electrospray voltage as well as make-up flow solution addition. Liquid junctions have been constructed on-chip^{8,9} and off-chip.^{10,11} A major limitation of these interfaces is that high EOF is required for these devices to function properly.

In general, microfluidic systems are generally driven by electrokinetic or hydrodynamic flow, because it is difficult to combine the two methods of mass transport in one system. However, electrokinetic and hydrodynamic mass transport can be combined advantageously for many applications, including the sample introduction into the MS using electrospray ionization sources. Hydrodynamic flow restrictors utilizing a series of small low aspect ratio channels were developed by Lazar et-al for electroosmotic pumping and as flow restrictors in microfluidic side channels.¹² The HDRs were used in a chip/ESI-MS interface that applied temporally alternating electrokinetic and hydrodynamic flow streams to load a sample into the sample introduction channel before infusion into the MS by electroosmotic pumping. Harrison

and co-workers described an interface that allows introduction of make-up solution via a side channel on the fluidic device and demonstrated its ability to stabilize electroosmotic flow through the separation channel.⁷ This interface requires the use of high EOF to prevent the electrokinetic transport through the separation channel from being overwhelmed by the hydrodynamic flow. The ability to use low EOF separations is critical in many applications because coatings that are required to minimize analyte adsorption to the capillary walls, such as polyethylene glycol coatings for protein separations, also minimize EOF. Additionally, the low EOF increases the separation time, which increases the resolution in capillary electrophoretic separations.¹³

The functional element of the system described here is a laser micromachined hydrodynamic flow restrictor in the separation channel. The HDR resists hydrodynamic flow because the linear velocity of the hydrodynamic flow is dependant on the width of the channel squared,¹⁴ while the electrokinetic flow is largely independent of the channel width. Laser micromaching is used to fabricate high aspect ratio channels for the HDR whose width is less than their depth. The channel profile and increased density of parallel HDR channels reduces Joule heating and improves resistance to hydrodynamic flow. These characteristics allow the HDR to be placed directly in the electrophoretic separation without excessive Joule heating.

In this report a laser micromachined hydrodynamic flow restrictor is used to combine electrokinetic and hydrodynamic flow streams continuously. The HDR is utilized in a CE/ESI-MS interface, making it possible to increase the flowrate at the spray tip with a make-up solution under low EOF conditions. The improved performance with low EOF permits the use of PEG coatings that minimize protein adsorption and EOF.

The addition of the make-up solution provides easy adjustment of the flow at the ESI tip, which is critical because the separation and ESI voltages are applied by the same electrode, preventing independent adjustment. For the ESI interface, the HDR is required to prevent back-flow of hydrodynamically introduced make-up solution into the separation channel, because the frictional resistance to flow through the ESI tip is significant. Additionally, introduction of the make-up solution can be used to adjust the solvent composition and pH, for independent pH optimization for the separation and electrospray. The performance of the ESI interface for introduction of analyte bands into the MS was characterized, and it was found to produce stable spray and contribute minimally to band broadening.

3.2 Experimental

3.2.1 Materials and Reagents

Bodipy FL succinimidyl ester and rhodamine B were purchased from Molecular Probes (Eugene, OR). Ammonium acetate was obtained from Sigma (St. Louis, MO). Distilled de-ionized water (18 M Ω) was produced with a Barnstead nanopure infinity system (Dubuque, IA). Methacryloxypropyltrimethoxysilane (MPTMS) was purchased from Gelest (Morrisville, PA). Irgacure 1800 (photoinitiator) was obtained from Ciba (Tarrytown NY). Fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ). Soda lime glass slides were purchased from Telic (Santa Monica, CA).

3.2.2 Microfluidic Device Fabrication

The microfluidic devices were fabricated using standard photolithography and wet chemical etching techniques as previously described.¹⁵ Briefly, 80 μ m wide (width

at half height) and 25 μm deep separation and side channels were patterned on soda lime glass using a photomask with patterns created with Freehand 10 software and etched using hydrofluoric acid. All microfluidic channel measurements were obtained using a stylus profiler with a 2 μm , 60⁰ angle tip, (Tencor Instruments, San Jose, CA). Access holes were created on the etched microchip using a microdrilling technique. A hydrodynamic flow restrictor of the make-up solution in the main channel was fabricated using an MP 100 UV laser micromachining system (Oxford Lasers, Oxfordshire, UK). Six parallel 10 μm channels were etched on the glass substrate, connecting the main channel and the intersection of the main channel and the make-up solution channel as shown in Figure 3.1. The cover glass was bonded by low temperature and high temperature bonding of the etched and drilled glass plate using a blank soda lime glass cover plate. The low dead volume connection of the spray tip to the microchip was achieved by drilling a 360 μm diameter hole through the junction between the cover plate and the etched glass plate at the edge of the bonded microchip and inserting a 2 cm long 50 μm I.D fused silica spray tip with a 208 μm tip capillary spray tip. Initial attempts to fabricate a hydrodynamic flow restrictor (HDR) involved the in situ polymerization of a small plug (<0.5cm in length) of polymer monolith. Methacryloxypropyltrimethoxysilane (MPTMS) was reacted with 1M HCl, toluene and Irgacure 1800 (photoinitiator).¹⁶ The channel was filled with the polymer solution and UV light at 365 nm was illuminated at the location of the flow restrictor for 10mins after the unpolymerized solution was rinsed out of the channel.¹⁷ The whole chip was masked leaving only a 3 mm portion of the separation channel exposed to the UV light.

Figure 3.1: Schematic diagram of the hydrodynamic flow restrictor (HDR) coupling hydrodynamic and electrokinetic flows, incorporated into microfluidic chip/ESI-MS interface.

The channel surfaces were derivatized with a PEG-silane self assembled monolayer (SAM). The channels were rinsed with sodium hydroxide, deionized water and methanol, with each solution delivered for 5 min. The PEG-silane was solution was then pumped through the channels for 6 hours, after which the coating was cured at 60⁰C in the oven for 2 hours.

3.2.3 Fluorescence microscopy

Fluorescence imaging experiments were performed using a Nikon inverted fluorescence microscope. A 4X microscope objective was used to focus the excitation beam and to collect the fluorescent light, which was passed through a 530±30 nm emission band pass filter. A Roper CCD camera (Roper Scientific, Tucson AZ) controlled by Metamorph software (Universal Imaging Corporation, Downingtown, PA) was used to capture images. A voltage of 4 kV was applied to reservoir 3 containing a solution of 60 µM bodipy FL, SE in 100 mM acetate buffer (pH 4) containing 50%

methanol. Rhodamine B in 0.1 M acetic acid was introduced hydrodynamically through the side channel at flowrates beginning at 50 nL/min and increased to 500 nL/min in 50 nL/min increments.

A microfluidic device with the channel design shown in Figure 3.2 was used to determine the band broadening due to the HDR. A sample plug of 1 μ M rhodamine B in 20 mM ammonium acetate at pH 8 was loaded by applying a voltage of 4.5 kV, 4.2 kV, 3.7 kV and ground to the sample, buffer, buffer waste, and sample waste reservoirs respectively. The sample was injected into the separation channel by applying a voltage of 4.2 kV to the buffer reservoir with the other electrodes at ground. The sample plug profiles of rhodamine B in 20 mM ammonium acetate at pH 8 were recorded before and after the restrictor to determine the extent to which band broadening increases due to the presence of the HDR.

Figure 3.2: Schematic diagram of the microfluidic device used for band broadening studies

3.2.4 Chip/ESI-MS Experiments

The microfluidic device was interfaced to a LCQ Deca XP ion trap mass spectrometer (Thermo Electron Corporation, Waltham, MA). The channels were prepared by rinsing with 0.1M sodium hydroxide solution for 10 min followed by a 5 min rinse with deionized water, after which it was rinsed with run buffer for 5 min. The channel was filled with the running buffer and the reservoirs loaded with the buffers

ensuring that there were no bubbles in the system. To determine the stability of the electrospray, reservoirs 1-3 were filled with the electrophoretic buffer, 100 mM acetate in water/methanol (80 /20% v/v), pH 4. The makeup solution, 0.1 M acetic acid in water/methanol (80/20% v/v) was hydrodynamically introduced via reservoir 4. The chip on a X,Y,Z translational stage was placed in front of the mass spectrometer with a distance of 3-5 mm between the mass spectrometer orifice and spray tip.

3.4 Results and discussion

A hydrodynamic flow restrictor was fabricated within the separation channel as shown in Figure 2.1, to prevent backflow in the separation channel. Initial attempts to achieve hydrodynamic flow restriction involved the in situ polymerization of a small plug of polymer solution. The resulting polymer monolith provided resistance to hydrodynamic flow because of its small pores that act like numerous small channels in parallel.¹⁴ However, the polymer monolith results in too large a backpressure in the separation channel, which makes it difficult to fill the channels. Moreover, the glass surface of the microchannel and the polymer monolith region exhibit different electroosmotic flow properties which complicates the flow through the electrophoretic channels of the device. In addition, because of the hydrophobicity of the polymer monolith, at least 50% organic was required in the electrophoretic buffer to elute protein adsorbed on the monolith and polymer leachates from the synthesis introduced more background noise in the MS signal. The use of the laser micromachined HDR prevents the generation of surfaces with chemistries that are substantially different from standard microfluidic channels.

For use in a CE/MS interface, a critical aspect with the interface is the band broadening introduced by the HDR. Several factors contribute to band broadening in electrophoretic separations on microfluidic platforms, including axial diffusion, Joule heating, channel geometry, injection plug length, and the detector path length.¹⁸ Adsorption of sample to the channel wall also contributes to band broadening especially for biological samples such as proteins and peptides. To determine the band broadening introduced by the HDR, a chip utilizing the flow restrictor and a second chip without the flow restrictor serving as a control (design shown in figure 3.2) were fabricated. Figure 3.3 shows the sample plug profiles of a sample of 1 μ M rhodamine B before and after the HDR. There is very minimal band broadening after the sample plug has passed through the restrictor, which was calculated to be a 2% increase in peak width of the sample band. Therefore, the HDR can be used to combine an electrokinetically driven system with a hydrodynamically driven system. In the case of upstream separations, the HDR contributes a negligible amount to band broadening.

Figure 3.3. Band profiles of a $1\mu\text{M}$ sample of rhodamine B, demonstrating that the contribution of the flow restrictor to band is minimal ($\sim 2\%$). The center of the sample bands obtained before and after the HDR are 27mm and 43mm from the injection cross respectively.

The make-up solution is used to adjust the flow rate at the ESI tip to optimize the stability of the electrospray. The minimal make-up solution flowrate found to stabilize electrospray is 100 nL/min. This flowrate is determined over a 100 min period by recording the spectra of the background buffer ions with the microfluidic chip spraying into the MS. The total ion chromatogram, shown in Figure 2.4, is representative of the background stability (RSD 14.1%) achieved with this device. With this sheathless configuration, the potential that generates the EOF and supplies the ESI voltage is provided by the electrode at the channel inlet. The required flowrate is primarily

determined by the orifice and ID of the spray tip and ESI voltage. Because the EOF and the ESI voltage are coupled and cannot be adjusted independently, the make-up flow provides a means to optimize the flowrate, increasing the stability of the electrospray.

Figure 3.4. The stability of the electrospray is shown with a total ion chromatogram of the solvent/buffer background. The electrophoretic buffer used was 100mM acetate in water/methanol (80 /20% v/v) and the make-up solution was 0.1M acetic acid in water/methanol (80/20% v/v) delivered at 90nL/min.

The performance of the HDR in resisting hydrodynamic flow is characterized by introducing the make-up solution at different flow rates, and observing the flowrate at which the make-up solution is forced into the narrow HDR channels. In order to image the flow profile, a bodipy solution is introduced electrokinetically from reservoir 1, and a rhodamine solution is introduced hydrodynamically through the make-up solution channel. The convergence of the bodipy solution carried by electroosmotic flow and the rhodamine B solution introduced hydrodynamically through the make-up solution channel is shown in Figure 3.5. As the make-up solution flowrate is increased from 50 nL/min, there is no backflow into the HDR channels up to 250 nL/min, but backflow of the make-up solution in HDR channels was evident at 300 nL/min. Therefore, the optimum make-up flow rate required by the ESI interface at 100 nL/min is well below the

maximum flowrate that the HDR can resist at 250 nL/min. The velocity of the electroosmotic flow in the separation channel using bodipy as the neutral marker is measured to be 0.5 ± 0.1 mm/s (60 nL/min volumetric flowrate) at a field strength of 470 V/cm. Chip-ESI-MS interfaces in literature employ much higher electroosmotic velocities in the separation channel. In one case, using a glass chip with an unmodified surface, the EOF velocity is measured to be 2 mm/s (240 nL/min) in 10 mM sodium borate (pH 9.6) and 0.5 mm/s in 10 mM ammonium acetate at a field strength of 550 V/cm.¹⁹ These velocities are at least an order of magnitude greater than the EOF velocity used in this work.

Figure 3.5. Convergence and mixing of the electrokinetically driven flow through the separation channel and the hydrodynamically driven make-up solution. The Bodipy FL, SE (green dye) is mobilized electrokinetically using a high voltage of 4kV at reservoir 2 and the Rhodamine B (red dye) is mobilized hydrodynamically using a syringe pump. This configuration allows the two dyes to be mixed without the Rhodamine B solution flowing upstream of the microfluidic channel due to the frictional resistance of the small parallel channels to hydrodynamic flow. The images correspond to the flow profiles observed at make-up flow rates of 100, 250 and 300 nL/min. At 300 nL/min the HDR does not provide adequate resistance to flow and the make-up solution prevents elution of the Bodipy.

In summary, the hydrodynamic flow restrictor (HDR) can be used to effectively introduce make-up flow solution on microfluidic platforms operating under low EOF conditions while maintaining stable electrospray. The make-up flow can be used to optimize the solution flowrate at the ESI interface, and to adjust the composition of the solution. The contribution of the HDR to band broadening in the separation channel has also been demonstrated to be minimal.

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Chapter 4

ESI-MS Compatible Permanent Coating of Glass Surfaces Using Polyethylene Glycol Terminated Alkoxysilanes for Capillary Zone Electrophoretic Protein Separations

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Abstract

Thin Polyethylene glycol silane (PEG-silane) coatings formed from N-(triethoxysilyl propyl)-O-polyethylene oxide urethane with different chain lengths polyethylene glycol (MW 750 and 4000-5000) are used to modify glass microfluidic channels and fused silica capillaries for electrophoretic separations of proteins. These coatings combine three important properties, which make them favorable for proteomic analyses including reduction of protein adsorption, compatibility with mass spectrometry due to their stability, and the ability to control the magnitude of electroosmotic flow (EOF). The coatings have been successfully used in microfluidic chips and fused silica capillaries for separation of protein sample mixtures under low EOF conditions. The long chain and mixed PEG-silane coatings suppress electroosmotic flow by more than 90%, whereas the short chain PEG silane suppresses EOF by 65-75% at pH values of 3-9. The long chain and mixed PEG-silane coatings are suitable for low EOF applications or for cases where negative effects of EOF are to be minimized. Efficient separations of unlabeled basic proteins at low pH and FITC-labeled proteins at high pH were achieved, as well as

excellent stability for at least 200 electrophoretic runs. Additionally, these covalent coatings produce no detectable background ions in ESI-MS, making them compatible with on-line mass spectrometry.

4.1 Introduction

Free zone electrophoresis in capillaries and microfluidic channels has emerged as an important separation technique, due to its highly efficient separations, small injection volumes, and short analysis times. These characteristics make channel and capillary electrophoresis (CE) an excellent tool for applications in numerous fields, including proteomics. Proteomics is an emerging field that seeks to understand the underlying molecular biology that controls cell function and dysfunction, through global analysis of protein expression. Therefore, it is anticipated that proteomics will contribute greatly to our understanding of nearly all diseases that affect human health. Full global analysis of protein expression implies identification, quantitation, and characterization of modifications for all the proteins in a cell, tissue, or organism. Currently, such complete analysis is not possible, and great improvements in analytical tools available for protein analysis are required for it to become a reality. Capillary electrophoresis and microfluidics have many characteristics necessary for their development as important tools in protein analysis. However, for protein applications, the development and implementation of microfluidic systems has been slow, with the lack of efficient and stable coatings to minimize protein adsorption being a great impediment to their development.

Controlling surface chemistries of microfluidic channels and capillary bores is particularly important due to their high surface area to volume ratios. For analysis of trace levels of analytes, it is particularly important to constrain samples to small volumes to maximize the concentration and achieve the best limits of detection (LODs), especially for electrospray mass spectrometry (ESI-MS) which is concentration sensitive.¹

Adsorption of proteins to the native glass surface is usually a problem in CE microchannels and microcapillaries² due in part to the highly concentrated, small volume samples in high surface area to volume ratio environments. Protein adsorption occurs via ionic interactions between silanol groups and cationic amine residues on the proteins, hydrogen bonding, hydrophobic and/or Van der Waals interactions.³ It is therefore necessary to modify the native glass surfaces for prevention of protein adsorption (especially for basic proteins) and to regulate the direction and magnitude of electroosmotic flow (EOF).

A variety of coatings have been used for passivation of glass surfaces and are described in a number of good reviews.⁴⁻⁹ The surface coatings can generally be classified into dynamic and permanent coatings. Dynamic coatings adsorb to the surface through secondary interactions such as hydrogen bonding and electrostatic interactions. They include adsorbed polymers such as polybrene,^{10, 11} polyvinyl alcohol (PVA),¹² polyethylene oxide (PEO),^{13, 14} hydroxyethylcellulose,¹⁵ PDAMAC,¹⁶ dextran sulphate,¹⁷ and polyarginine.¹⁸ These polymers, which can be neutral, positively or negatively charged, are physically adsorbed to the channel wall. Dynamic coatings also include small molecule additives such as surfactants (didodecyldimethylammonium bromide (DDAB),¹⁹ 1,2-dilauroyl sn-phosphatidylcholine (DLPC)²⁰), simple inorganic salts and

various types of amines.^{2, 21-23} The drawback of dynamic coatings is that most are incompatible with mass spectrometry, and they have to be continuously re-generated for effectiveness. In contrast, permanent coatings are covalently bound to the glass surface and have been regarded as the most effective way of modifying glass surfaces. This covalent modification of the surface results in the masking of residual silanols by the polymer, causing a change in the zeta potential. The increase in viscosity in the layer near the wall also contributes to the change in EOF.⁴ Self-assembled monolayers (SAMs) are a special class of permanent coatings, which can be differentiated from the traditional direct derivatization of surface active sites with polymer solutions. Through the self-assembly process, SAM molecules can form highly ordered and organized thin films. The main limitation, however, is that a well organized SAM requires a smooth surface for assembly. Tailoring SAMs to rough surfaces is subject of future research, but even on rough surfaces they still provide good coatings, compared to traditional methods.

SAM precursor or monomer molecules can be terminated with neutral and hydrophilic polymers such as polyacrylamide (PAA), polyvinyl alcohol (PVA) and polyethylene glycol (PEG) that are known to be the most effective for inhibiting protein adsorption to glass surfaces. These polymers have been used as dynamic and covalent coatings to demonstrate their effectiveness in preventing protein adsorption. In 1985, Hjerten was the first to demonstrate the covalent attachment of a hydrophilic polymer to a capillary by *in situ* polymerization of acrylamide to a capillary derivatized with γ -methacryloxypropyltrimethoxysilane (MPTS).²⁴ However, the *in situ* polymerization resulted in unattached polymer chains that need to be removed from the capillary under high pressure. The stability of the coating is also limited by formation of ester bonds

formed from the silanization process. Polyvinyl alcohol has been shown to achieve very efficient separations when used as a dynamic coating or attached to the capillary surface through silanization.²⁵ Polyethylene glycol has been demonstrated to have excellent resistance to protein adsorption on glass surfaces. Zhihao Yang, et-al attached polymer chains of PEG (MW 750 and 5000) to a glass surface through silanization with a trimethoxysilane.²⁶ Using atomic force microscopy (AFM), they determined that the smaller PEG forms an extended brushlike monolayer and that the larger PEG forms a highly entangled mushroom like structure. Both monolayers were found to reduce protein interactions with the surface. It has been suggested that PEG is effective in preventing protein adsorption by maximizing their entropic repulsion with the surface.²⁷ Exposure of water to a surface coated with polyethylene glycol causes the polymer to swell, and a protein that adsorbs to the polymer surface has to compress the swollen polymer, which is energetically and entropically unfavorable.²⁸ Other researchers have attributed this resistance to protein adsorption to the ability of the functional groups on the polymer to interact strongly with water.²⁹ SAM precursor molecules terminated with the neutral and hydrophilic polymers are therefore among the best for preventing protein adsorption.

There are several characteristics that are advantageous for the use of polyethylene glycol terminated silane coatings on glass surfaces for capillary electrophoretic separation of proteins. First, excellent resistance to protein adsorption is achieved by the PEG functionality that terminates the monomer molecules. Second, no buffer additives are required to maintain the coating. Buffer additives can interfere with CE-MS analyses by making the spray buffer less volatile and by also introducing more background into the

MS. The coating is very stable since the monomer molecules strongly interact with each other and are tethered to the surface by covalent linkages. Third, by varying the length of the polyethylene glycol molecule, EOF can be optimized. Meagher, et-al recently reported a tri(ethylene glycol)-terminated alkyltrichlorosilane that reduced EOF by 50% compared to the uncoated capillary, and was demonstrated to have remarkable stability, as well as good resistance to protein adsorption.³⁰ They tried to maintain high EOF by forming a thin layer of PEG-silane on the surface. However, it has been shown that the ability of PEG coated surfaces to resist protein adsorption increases with chain length of the grafted PEGs as well as with their grafting densities.³¹⁻³³

Our focus in this work is development of a coating compatible with CE-MS, since mass spectrometry is the instrumental method of choice for proteomic analyses. The coatings investigated here are formed from two silanes, one terminated by a low molecular weight PEG (MW 750), another with a high molecular weight PEG (MW 4000-5000), and a mixture of the two. We investigated coatings from mixed PEG-silanes because the longer chain PEGs potentially improve resistance to protein adsorption, and it is anticipated that the short chain PEGs increase the grafting density. Characterization of the mixed PEG-silane coating is achieved by electrophoretic separations of proteins in both fused silica capillaries and microfluidic channels. The long chain and the mixed PEG-silane coatings suppress EOF by more than 90% at pH values of 3-9, making the coatings good for low EOF applications. The short chain PEG-silane suppresses EOF to a lesser extent of 65-75% compared to the bare surface. Efficient separations of both unlabeled basic proteins at low pH and FITC-labeled proteins at high pH are achieved for the mixed PEG-silane coating. In addition, the coating exhibits excellent stability for at

least 200 electrophoretic separations of a FITC-labeled protein mixture. The coatings, therefore, combine three important properties that make them favorable for proteomic analyses: reduced protein adsorption, minimized EOF, and compatibility with mass spectrometry.

4.2 Materials and Methods

4.2.1 Materials and Reagents. N-(Triethoxysilylpropyl)-O-Polyethylene oxide urethane, (MW 750 and 4000-5000) was purchased from Gelest (Morrisville, PA). Anhydrous Toluene and triethyl amine were obtained from Aldrich (St. Louis, MO). Capillary tubes (363 μm OD X 75 μm ID) were purchased from Polymicro technologies (Phoenix, AR). Bodipy FL, SE, and Rhodamine B were purchased from Molecular Probes (Eugene, OR). Fluorescein isothiocyanate (FITC) labeled protein mixture, FITC labeled bovine serum albumin (FITC-BSA), cytochrome C, lysozyme, myoglobin, triethylamine and ammonium acetate were obtained from Sigma (St. Louis, MO). Distilled de-ionized water (18.2 M Ω resistance) was produced with a Barnstead nanopure infinity system (Dubuque, IA). Soda lime glass slides with chrome and photoresist coatings and cover plates were purchased from Telic (Santa Monica, CA).

4.4.2 Coating Procedure. The long PEG-silane solution (solution A) was prepared by adding 20mg of N- triethoxy silyl propyl polyethylene oxide urethane (MW 4000-5000) in 10 mL anhydrous toluene, and gently heating it to dissolve the polymer completely. The short chain PEG-silane solution (solution B) was prepared by adding 60 mg N-triethoxy silyl propyl polyethylene oxide urethane (MW 750) to 10mL of anhydrous

toluene and stirred thoroughly. Solution C was prepared by mixing 8 mg of triethyl amine in 10 mL toluene. The mixed PEG-silane solution was prepared by mixing solution B (1mL) and solution C (0.1 mL) and adding them to solution A and stirring for 10 minutes. A piece of fused silica capillary (75 μm I.D 360 μm O.D) was conditioned by passing 0.2 mL of 5 M sodium hydroxide solution through the capillary at 5 $\mu\text{L}/\text{min}$ using a syringe pump. The capillary was washed sequentially with deionized water and anhydrous toluene using the same conditions mentioned above for conditioning with sodium hydroxide. The coating solution was introduced through a glass syringe, and 7-8 drops were gently flushed through the capillary. The other end of the capillary was inserted through a septum into a vial containing the coating solution. A pressurized syringe was used to flush the coating solution through the capillary for 4-12 hours. The capillary was then flushed briefly with 7-8 drops of toluene, after which it was heated at 80 $^{\circ}\text{C}$ for at least 2 hours.

4.2.3 Microfluidic chip Fabrication. The microfluidic device with the design shown in Figure 1 was fabricated using standard photolithography and wet chemical etching techniques as previously described.³⁴

Figure 4.1: Schematic diagram of the microfluidic chip design used for electrophoretic separations

Briefly, 100 μm wide (width at half height) and 25 μm deep channels were patterned on soda lime glass using a photomask with patterns created with Freehand 10 software and etched using hydrofluoric acid. Access holes were created using manual microdrilling on the etched microchip. Upchurch nanoport fittings were used as reservoirs and facilitated the connection of capillaries to the chip. The uncoated chips were prepared by rinsing them with 1/1/5 v/v/v $\text{NH}_4\text{OH}/\text{H}_2\text{O}_2/\text{H}_2\text{O}$ solution at 80°C for 10 min followed by a 5 min rinse with deionized water, after which they were rinsed with run buffer for 5 min. The channels were coated using the same procedure as for the capillaries, but in this case, the coating solution was introduced through one of the access holes at a rate of 0.1-0.5 $\mu\text{L}/\text{min}$, which prevented the coating solution from filling the reservoir and prevented dissolution of the nanoport adhesive by the toluene.

4.2.4 Capillary Electrophoresis and EOF Measurements. Capillary Electrophoresis was carried out on a Beckman Coulter MDQ capillary electrophoresis system with a ± 30 kV power supply, capillary cooling system, laser induced fluorescence (LIF) and a photodiode array (PDA) detector (Fullerton, CA). The UV light source was a deuterium lamp, and a 488 nm Argon ion laser was used as the excitation source for LIF detection. EOF measurements were performed using a 1cm (48 nL) injection volume of 8.8 mM mesityl oxide (MO) as a neutral marker from pH 3 to pH 10, and the EOF mobility was calculated from the migration times of the neutral marker. A phosphate buffer was used at pH 3, acetate buffer at pH 4-5, phosphate buffer at pH 6-7, and borate buffer at pH 8-10. Each buffer was prepared at a concentration of 2 mM and the ionic strengths of all the buffers were matched by addition of 10 mM KCl and equalizing the conductivity of

the solutions. Electroosmotic flow measurements on the glass microfluidic device were performed using rhodamine B as the neutral marker and 50mM ammonium acetate at pH 8 as the electrophoretic buffer. Voltages of 2, 0, 1.5 and 1.7 kV were applied at the sample, sample waste, buffer and buffer waste reservoirs, respectively, for sample loading. For sample injection, voltages of 0.5, 0.5, 2 and 0 kV were applied to the sample, sample waste, buffer, and buffer waste reservoirs respectively. The detection point was 1.7 cm from the injection cross, where a 100 μm long injection plug was formed. A FITC-BSA sample plug on a PEG-silane coated microfluidic device was loaded at the cross by applying 0, 3, 0.7 and 1 kV to the sample, sample waste, buffer, and buffer waste reservoirs respectively. Sample injection and separation were performed by applying 0.5, 0.5, 0 and 2 kV to the sample, sample waste, buffer, and buffer waste reservoirs respectively. The loading and injection times were programmed to be 10 s and 30 s, respectively, for the FITC-BSA sample. All voltages were applied using the Labsmith HV448 high voltage sequencer (Labsmith, Livermore, CA).

4.2.5 Fluorescence Microscopy. Electropherograms and CCD camera images were acquired using a Nikon inverted epi-fluorescence microscope. The system utilized a broadband excitation light source (Exfo excite 120), whose beam was passed through a dichroic mirror and a 500 ± 10 nm, 570 ± 10 nm excitation band pass filter. The excitation beam was focused onto the surface of the chip through a 4X microscope objective, and the fluorescent light was collected through the same objective. The emitted light was passed through a 530 ± 30 nm emission band pass filter, after which it was collected by a Roper CCD camera (Roper Scientific, Tucson AZ). The acquired data

was processed using metamorph software (Downingtown, PA), with the exposure time set at 200ms for all experiments. All microfluidic channel measurements were obtained using a stylus profiler with a 2 μ m, 60⁰ angle tip, (Tencor Instruments, San Jose, CA).

4.2.6 Electrospray Mass Spectrometry. A coated capillary column 75 μ m I.D was connected to a spray tip and used for direct infusion of 0.1M acetic acid/50 % methanol spray solution and 0.5 pmol/ μ L angiotensin 1 in spray solution into an electrospray LCQ Deca XP ion trap mass spectrometer (Thermo Electron Corporation, Waltham, MA). The sample was delivered at a flowrate of 250 nL/min via a syringe with a voltage of 2.5 kV applied to the capillary to initiate electrospray. MS spectra of this solution were acquired from 150-2000 m/z for over three days for two hours a day to determine if any background due to the PEG-silane appeared in spectra due to degradation of the PEG-silane coating.

4.3 Results and Discussion

4.3.1 Electroosmotic Flow in Coated Capillaries. The coatings investigated in this work are formed by reacting N-(Triethoxysilylpropyl)-O-Polyethylene oxide urethane with silanol groups on the glass surface as shown in Figure 4.2.

Figure 4.2: Schematic diagram of *N*-(Triethoxysilylpropyl)-*O*-Polyethylene oxide linked to a glass surface

Two precursor molecules terminated with different length polyethylene glycol (PEG) tails are used. One of the precursors has a PEG tail with an average molecular weight of 750, which is referred to as the short PEG, and the other has a PEG tail with an average molecular weight of 4000-5000, which is referred to as the long PEG. Alkoxysilanes were chosen instead of trichlorosilanes, because alkoxysilanes have reduced water sensitivity and typically provide more reproducible layers than trichlorosilanes.³⁵ The coatings reduce the electroosmotic flow, and therefore measurement of the EOF is an important parameter in evaluation of the coating process. The reduction in electroosmotic flow for short, long and mixed PEG-silane coated capillaries compared to a bare capillary is shown in Table 4.1. For the long chain and mixed PEG-silane, the EOF marker does not elute within a 30 min electrophoretic run for pH values of 3-9, (corresponding to more than 90 % decrease in electroosmotic flow).

Table 4.1: Electroosmotic flow suppression by the short chain, long chain and mixed PEG-silane coatings.

	% Reduction in EOF compared to bare surface		
	PEG-silane (n=17)	PEG-silane (n=114)	Mixed PEGs (n=17 & n=114)
Fused Silica Capillary	65-75	>90	>90
Microfluidic chip	65-75	>90	>90

A coated capillary exhibits good protein separations when the EOF mobility is reduced by at least 90% compared to a native surface capillary for long and mixed PEG-silane, and 65-75% for short chain PEG-silane. The degree of EOF reduction is consistent with the study of electrokinetic effects of surface bound polymers by Burns et-al who reported a decrease in EOF with increase in PEG chain length and elimination of EOF by a PEG of molecular weight 35,000.³⁶ The observed maximum EOF reduction values are consistent, and values less than the maximum correlate with a decrease in the separation efficiency. Therefore, in routine tests to evaluate the coating, the EOF measurements are used as the indicator of the efficacy of the coating process. For the coating of a capillary or microfluidic channel to be deemed successful, the maximum EOF reduction must be obtained. More specifically, the coating process is considered successful if EOF is suppressed by at least 90% for the long and mixed PEG-silane coated capillaries, and 65% for the short PEG-silane coated capillaries. It is important to note that the best coating success rate of ~ 80% is obtained using the mixed PEG-silane coating. The coating success rate for the long chain and short chain PEG-silanes is ~60%. The success rate was based on electroosmotic flow measurements of 50 capillaries for each coating type.

4.3.2 Protein Adsorption in a Microfluidic channel. Protein adsorption to mixed PEG-silane coated and uncoated channel surfaces is measured by exposing the channels to FITC-BSA. The relative fluorescence intensities of adsorbed FITC-BSA are averaged over a channel length of 200 μm on coated and uncoated microfluidic channels. After 40 electrophoretic runs and a buffer rinse of the PEG-silane-coated channels, there is only a 2.5% increase in fluorescence intensity at the center of the channel. However, the uncoated channel exhibits a significant 35.7% increase in fluorescence intensity at the center of the channel after one electrophoretic injection and separation of the FITC-BSA, as shown in Figure 4.3.

Figure 4.3: Comparison of the background fluorescence from FITC-BSA on uncoated and N-(Triethoxysilylpropyl)-O-Polyethylene oxide urethane coated microfluidic channels. The relative fluorescence intensity is shown as a function of channel width for native surface and PEG-silane coated channels.

In the uncoated channel, the most protein adsorption is measured at the channel edges, but there is significant protein adsorption in the center of the channels as well. After exposure to the FITC-BSA, rinsing of the channels with running buffer solution does not decrease the fluorescence. The significant reduction in fluorescence after exposure to the FITC-BSA confirms that the PEG-silane coating minimizes protein adsorption to glass microfluidic channels. It also suggests that the greatest amount of protein adsorption occurs at channel edges where the substrates are bonded.

4.3.3 Band Broadening in a Microfluidic channel. The reduction of band broadening in a mixed PEG-silane coated microfluidic channel is characterized using rhodamine B and FITC-BSA. Sample band profiles at the detection point are compared with the profiles of the injected band at the injection cross. For rhodamine B, the sample width at half height increases 1.5-fold in a coated channel, whereas it increases 1.9-fold in an uncoated channel as shown in Table 4.2. In the case of FITC-BSA, the width at half height increases 2-fold for the coated channel and increases 6.5-fold for the uncoated channel. Result illustrate the decreased band broadening in the PEG-coated channel, which provides evidence that the PEG-silane coating provides a stable surface that minimizes the effects of the factors contributing to band broadening, particularly adsorption of the protein on the channel walls, and also minimization of non-uniform EOF.

Table 4.2: Increase in the sample band area of Rhodamine B and FITC-BSA samples.

The initial band profile was obtained immediately after injection and the final band profile was acquired 20 mm from the injection cross

Sample	Increase in band width at half height	
	PEG-silane coated channel	Uncoated Channel
Rhodamine B	1.5 fold	1.9 fold
FITC-BSA	2 fold	6.5 fold

4.3.4 Separations on Fused Silica Capillaries. The performance of the coatings in a CE separation is characterized by the separation of FITC-labeled proteins with molecular weights ranging from 20,100 – 66,000, which include trypsin inhibitor, carbonic anhydrase, bovine serum albumin, and alcohol dehydrogenase. These FITC labeled proteins are negatively charged at intermediate and high pH. The electropherograms of the protein separations on uncoated, long PEG-silane and mixed PEG-silane coated capillaries are shown in Figure 4.4.

Figure 4.4: Protein separations on native-surface and PEG-silane coated capillaries. Shown are electropherograms of a FITC-labeled protein mixture obtained using 50mM borate (pH 9) at an applied voltage of 10 kV and LIF detection. The fluorescently labeled proteins in the mixture are BSA (MW 66,000), alcohol dehydrogenase, equine liver (39,000), carbonic anhydrase, bovine erythrocyte (MW 29,000), and soybean trypsin inhibitor (MW 20,100), corresponding to peaks 1, 2, 3 and 4 respectively

The long-PEG silane shows comparable migration times to the mixed-PEG silane since the coatings exhibit the same amount of EOF suppression. The separation efficiencies of these two coatings are similar, but it is important to note that the intensities of two of the protein peaks from the mixed-PEG coated capillary are slightly higher than those of the

long PEG-silane coated capillary, signifying reduced proteins losses to capillary surface. All the proteins can be resolved in the coated capillaries, and the separation efficiencies of the protein peaks obtained as shown in Table 4.3. Theoretically, separation efficiencies of 2 million/m can be achieved in capillary electrophoresis, but protein separations on permanently coated surfaces are known to yield between 50 000–600 000 plate/m.^{20, 37, 38} Therefore, the mixed and long PEG coatings provide efficiencies comparable to those of other permanent coatings, such as polyacrylamide and poly (vinyl alcohol). The improved resolution and efficiencies compared to the native surface are primarily a result of the reduced protein adsorption to the channel wall. The proteins on the short PEG-silane coated capillary migrate slower since there is increased EOF opposing the electrokinetic mobility of the proteins. The separation efficiencies on short PEG-silane capillaries are reduced compared to those of the long and mixed PEG capillaries as shown in Table 4.3. Table 4.3 summarizes the performance characteristics of the long, short and mixed PEG-silane coated capillaries based on separations using the FITC labeled protein sample. The mixed and long PEG-silane coatings exhibit similar separation efficiencies, but the mixed PEG silane was deemed to be the best due to its superior success rate. The column to column reproducibility standard deviation values are calculated from the migration times of the separated components for 5 different capillary columns and show slightly better performance for the mixed PEG-silane capillaries.

Table 4.3: Performance parameters for the long, short and mixed PEG-silane coated capillaries

Coating	Separation efficiency / plates/m Peaks				Stability <i>no. of runs</i>	Column –Column reproducibility	Success Rate
	1	2	3	4		σ of migration times/min	
<i>Short PEG-Silane</i>	30 540	27 720	30210	19770	150	± 0.17	60%
Long PEG-Silane	111 760	99 630	343 570	146 360	>200	± 0.15	60%
Mixed PEG-Silane	115 110	82 480	78 720	117 800	>200	± 0.11	80%

Basic proteins are particularly challenging to separate by CE, since these positively charged proteins can interact electrostatically with the negatively charged silanols on the bare capillary surface. Thus, the separation of basic proteins at low pH is an excellent means to evaluate the coatings ability to mask the surface charge. A separation of a lysosyme, cytochrome C, and myoglobin mixture, performed at pH 3 using a 50 mM phosphate buffer, reveals separation efficiencies of 82 420, 42 026 and 9 984 plates/m for peaks 1, 2 and 3 respectively as shown in Figure 4.5. Although the three proteins are not resolved on the bare capillary, they are well resolved on the PEG-silane coated capillary.

Figure 4.5: Electropherograms of 0.1mg/mL of basic proteins (Lysozyme, cytochrome C and myoglobin) at pH 3 in 50 mM phosphate buffer. A photodiode array detector was used at 214nm and the electropherograms are offset by 0.02 absorbance units.

4.3.5 Coatings Performance and Stability. The stability of the coating on a mixed PEG-silane fused silica capillary is determined by performing 200 separations for the FITC labeled protein mixture over 3 days. As shown in Figure 4.6A, the reproducibility of the separations obtained with the protein mixture was excellent, with an RSD of 0.1% from run to run, as exemplified by runs 50, 52, 54, 56, 58 and 60. The stability is also very good over the entire 200 runs the column is tested over, represented by runs 10, 15, 25, 35, 45, 55, 65 and 200 as shown in Figure 4.6B. In the end, the EOF increased by 13 %, equivalent to a 90 % initial and 78 % final reduction compared to the bare capillary.

A

B

Figure 4.6: Electropherograms of the FITC-labeled protein sample for Panel **A** runs 50-60 and Panel **B** 10, 15, 25, 35, 45, 55, 65 and 200 on a *N*-(Triethoxysilylpropyl)-*O*-Polyethylene oxide urethane coated capillary. The separation was performed using a 50 mM borate buffer (pH 9.0) with an applied voltage of 10 kV and LIF detection. The electropherograms in **A** are offset by 0.1 RFU units and those in **B** by 0.2 RFU units

However, the stability of the mixed PEG-silane coating on a microfluidic device is reduced compared to the coated fused silica capillary. To test the stability of the coatings on the microfluidic channels, successive injections and separations of FITC-BSA are performed with a 10 s loading time period and 30 s injection and separation period. Table 4.4 summarizes the EOF results obtained before and after 40 successive injections of FITC-BSA on three different chips.

Table 4.4: *Electroosmotic flow suppression on three mixed PEG-silane coated channels before and after 40 successive injection and separation of a FITC-BSA sample.*

Microfluidic Channel	Initial % EOF suppression	Final % EOF suppression	% increase in EOF
1	93	80	14
2	92	81	12
3	94	84	11

In successfully coated channels, the EOF mobility is initially reduced by 94 %, which is comparable to the initial EOF reduction in the capillaries. However, the stability of the coatings is reduced in the microfluidic channels, and the EOF measurements as a function of run number exhibit a different behavior. As shown in Figure 4.7, the EOF measurements are very stable over the first ~30 runs, but after ~30 runs the EOF measurements increase rapidly to a level that is equivalent to an 84% reduction in EOF. This data is representative of the results obtained for 3 microchips. Clearly, the stability of the coatings is reduced in the microfluidic channels, and the stability behavior is not the same as observed with the coated capillaries. In the coated capillaries, the EOF is

stable for over 200 runs and during this period, the EOF reduction gradually decreased from 90% to 78%.

Figure 4.7: A graph of percent EOF suppression over 40 electrophoretic runs on a mixed PEG-silane coated microfluidic electrophoretic channel.

Although the causes of the observed stability differences can not be easily explained, it is important to note that both the surfaces and configuration of the fused silica capillaries and microfluidic devices differ, and may contribute to the differences in stability. First, the additives in the glass used for the microfluidic devices lower the surface density of silanol groups.³⁹ Additionally, the surface roughness is much greater in the acid etched microfluidic channels than in the capillaries, as observed by optical microscopy. A decrease in stability of the coating, could cause an increase in desorption of the coating, that would cause an increase in EOF, and increase in the elution time of the FITC-BSA. Previous reports in the literature support the idea that substrate morphology can effect SAM stability, albeit for a different substrate and SAM monomer system.⁴⁰ Second the channel layout configuration is different for the microfluidic device, in which the channels for the sample loading channels are integrated into the device. The EOF of the sample loading and waste channels can affect the flow through

the separation channel. An important difference in the sample loading and waste channels is that they are exposed to the BSA sample solution for much greater amounts of time and at higher concentration, because band broadening reduces the concentration in the separation capillary. Therefore, it is likely that the BSA will adsorb or precipitate within the PEG of the sample loading and waste channels at a greater rate than it will in the separation channel. Under the conditions used, the BSA is negatively charged and the additional BSA will increase the EOF (which is towards the sample inlet with the reversed polarity used here) and increase the elution time of the FITC-BSA as observed.

4.3.6 Compatibility with Mass Spectrometry. To evaluate the compatibility of the PEG-silane coating with ESI-MS, a spray solution (0.1M acetic acid in 20% acetonitrile) and a 0.5 pmol/ μ L solution of the peptide angiotensin 1 is directly infused into the MS through a coated capillary. As shown in Figure 4.7, no background peaks due to the PEG-silane coating appeared in the spectra of the spray solution. The ion counts for A, B, C and D are 2127, 2168, 2101 and 2102 respectively. The difference in the total ion counts between mass spectra **A** and **B**, **C** and **D** are therefore negligible. Most of the peaks in **A** and **B** are from solvent clusters of the buffer and are confined to low molecular weights. The background signal for the PEG-silane is shown in **E**. These results indicate that the coating is very stable once formed in the channel or capillary, and does not interfere with MS detection of the analyte of interest. This stability is a significant advantage over dynamic coatings, which must contain the coating in solution, and are therefore not compatible with ESI-MS. Finally, this coating is compatible with

CE/ESI-MS interfaces for protein separations, because they can be used to minimize adsorption and control the EOF during the separation.

Figure 4.8: Mass spectra of ESI spray solution (0.1 M acetic acid in 20% acetonitrile) directly infused through a bare capillary **A** and a mixed PEG-silane coated capillary **B**. A total of 180 scans are averaged for both mass spectra with ion counts of 2127 and 2168 for **A** and **B** respectively. The low molecular weight peaks at m/z 218.8, 259.8 and 278.7 are due to solvent clusters of the buffer ions. Mass spectra **C** and **D** are a result of direct infusion of 0.5 pmol/ μ L angiotensin 1 through a bare capillary **C** and another through a mixed PEG-silane coated capillary **D**. A total of 3586 scans were averaged, with ion counts of 2101 and 2102 for **C** and **D** respectively. The peaks at m/z 325.1, 433.1, 649.0 and 1296.6 correspond to the +4, +3, +2 and +1 charged states of the peptide angiotensin 1. Mass spectrum **E** is the background signal from 1 μ M of the PEG-silane spiked in a 1 pmol/ μ L angiotensin 1 solution.

4.4 Conclusions

The reported long and mixed chain PEG-silane coatings are excellent choices for surface modification of fused silica capillaries and glass microfluidic channels used in electrophoretic separations. The long and mixed PEG-silanes have comparable separation efficiencies, although the mixed PEG-silanes have a higher coating success rate. The PEG silane forms a thin layer on glass surfaces that resist protein adsorption and allow for efficient protein separations using a simple coating procedure. The coatings also minimize electroosmotic flow in the separation channel and the extent of EOF reduction is dependant on the PEG chain length. Since the coating is covalently bound to the glass surface, it exhibits good stability over 200 runs of protein separations in capillaries. The stability makes them attractive for use in CE-MS interfaces because they do not introduce detectable levels of unwanted background into the MS. However, the EOF stability was decreased in the glass microfluidic device compared to fused silica capillaries, which could be caused by lower coating stability from differences in surface chemistry and topography, or increased protein adsorption in the sample loading and sample waste channels. Finally, it is anticipated that PEG silane coatings will play an important role in the application and development of CE-MS methods for proteome analysis.

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Chapter 5

Toward a Microfluidic chip-ESI-MS Interface for High pH, low EOF Protein/peptide Separations and Positive Ion Mode MS Detection

Abstract

A microfluidic chip-CE-MS interface is developed that allows separation of negative analytes at an intermediate pH of 8, under low EOF conditions, followed by positive ion mode mass spectrometric detection, effected by addition of a low pH make-up solution. This format is well suited for the analysis of proteomic samples at intermediate pH with coatings that minimize protein adsorption and EOF. Incorporation of a down stream electrode is advantageous because it allows the electric field for the separation and the ESI voltage to be controlled separately. Typically, under low EOF conditions, negatively charged analytes will migrate toward positive ESI electrode, instead of migrating into the spray needle and the MS. This problem is solved by application of the ESI voltage through rapid switching at two opposing electrodes. A hydrodynamic flow restrictor is incorporated into the interface that provides a make-up flow to lower the pH and stabilize the electrospray at flowrates of 100 nL/min. The pH changes on the device are tracked by a fluorescent pH indicator (carboxy SNARF-1), as well as distributions of the charged states of a peptide. The performance of the interface is evaluated by imaging the flow properties at various intersections of the microfluidic channel network. Mass spectra are obtained by electrokinetically mobilizing standard protein and peptide samples through the main channel of the chip while adding make-up solution for stable electrospray.

5.1 Introduction

Microfluidic devices are excellent platforms for chemical analysis due to their potential for integration of many processes, such as separations, fractionation, digestion, and derivatization. Additionally, multiplexing, automation, high throughput sample processing and high speed analysis can be performed with these devices.¹⁻¹⁰ For these reasons, microfluidic systems provide an excellent technology for solving problems in many areas of research such as proteomics, genomics, cellomics and metabolomics. One of the main challenges of proteomics, in particular, remains the development of novel technologies that can handle the complexity, and wide dynamic range of proteins from cellular extracts. Proteomic investigations have primarily relied on high resolution two dimensional gel electrophoresis to map the protein composition of a sample and mass spectrometry to identify the separated protein sample components.^{11, 12} Alternatively, high pressure liquid chromatographic methods coupled to mass spectrometry have been used to separate and identify proteins from complex peptide mixtures.¹³⁻¹⁵ Capillary electrophoresis coupled to mass spectrometry is an attractive tool for proteomic analyses, and can be utilized as a complementary tool to established LC methods, especially for analytes that do not differ much in hydrophobicity but have different charge. Capillary electrophoresis is a highly efficient separation technique that separates analytes based on charge, size and shape, while electrospray mass spectrometry detection provides excellent limits of detection as well as structural information through collision induced dissociation (CID) or electron capture dissociation (ECD) experiments. When these two techniques are coupled together, complex protein samples can be separated using capillary

electrophoresis and identified using the high resolving power, mass accuracy, and good limits of detection (LODs) of mass spectrometers such as FT-MS.¹⁶⁻²⁰

There are significant benefits associated with the use of microfluidic devices as platforms for capillary electrophoresis. One of the main advantages is that it is possible to perform very fast CE separations using short separation channels on microfluidic platforms, afforded by the very short injection plugs that can be formed on a microfluidic device. Also, from a practical point of view, there are minimal length constraints when interfacing microfluidic devices to electrospray mass spectrometry, such as those practically experienced with fused silica capillary columns. Typically, at least a 60 cm long capillary is required to form a viable CE- ESI-MS interface using fused silica capillaries. In addition, automation and multiplexing of various sample preparation procedures prior to separation can potentially be accomplished on a single device as demonstrated by Wang et-al²¹ and Li et-al.²² For these reasons, a variety of microfluidic chip capillary electrophoresis/electrospray mass spectrometry (chipCE/ESI-MS) have been developed by different research groups based on liquid junctions,²⁴⁻²⁶ sheath flow junctions,²⁷ sheathless junctions,²⁸⁻³¹ and have been reviewed by different authors.^{18, 20, 32, 33} Detection limits for peptide samples separated on these devices have been shown to be similar, and in some cases better than conventional CE/ESI-MS.^{27, 34} These interfaces that have been developed are high EOF systems and do not function well when separations have to be performed under low EOF conditions, which is the case when neutral hydrophilic polymers effective in preventing protein adsorption are used to modify channel surfaces. The interface described in this chapter can be used with low

EOF systems, as well as to effecting a change in analyte charge before detection with the MS.

The microfluidic chip CE-ESI-MS interface developed in this work is designed to enable separation of protein/peptide samples at high pH, and at the same time allow positive ion mode ESI-MS detection. This goal is particularly important for integration of on-chip tryptic digestions, which optimally work at pH 8, followed by CE and positive ion mode ESI-MS. It is important to use positive ion mode ESI-MS whenever possible because it more sensitive than negative ion mode MS, when dealing with proteomic samples. Achieving this goal involves lowering the pH of the sample at some point along the separation channel of the device. In this particular device, a hydrodynamic flow restrictor (HDR) is fabricated at a specific point of the separation channel, and is utilized for hydrodynamic introduction of a low pH make-up solution that mixes with an electrokinetically mobilized sample through the separation channel.³⁵

The interface also utilizes rapid voltage switching between electrodes downstream of a microfluidic main channel for application of the electrospray voltage as well as an end electrode for the CE. The rapid voltage switching between two electrodes is used to provide the ESI voltage and prevent migration of the analytes to these electrodes, which are immersed in solution reservoirs terminating two side channels of the same resistance. This configuration also eliminates the need for coated tips, off-chip sheath flow and liquid junctions. To minimize protein adsorption, the channel walls are coated with a solution of a mixed polyethylene glycol terminated silane that suppresses EOF by 90% compared to uncoated channels. The performance of the device is characterized by fluorescence imaging experiments carboxy-SNARF-1 (fluorescent pH indicator),

rhodamine 6G and fluorescently labeled peptides. This fluorescent pH indicator has previously been used by other researchers for profiling pH changes in electrospray droplets,^{36, 37} for on column pH monitoring in capillary electrophoresis,³⁸ and to follow intracellular pH changes.^{39, 40}

5.2 Experimental

5.2.1 Materials and Reagents

Rhodamine B and 2 (or 4)-[10-(dimethylamino)-3-oxo-3H-benzo[c]xanthene-7-yl]-benzenedicarboxylic acid (Carboxy-SNARF-1) were purchased from Molecular Probes (Eugene, OR). Rhodamine 6G, Fluorescein isothiocyanate (FITC) labeled angiotensin 1, cytochrome C, myoglobin and ammonium acetate were obtained from Sigma (St. Louis, MO). A fluorescein labeled custom-made peptide (GCLEGQYEADK-fluorescein) was purchased from New England Peptide (Gadner, MA). Distilled de-ionized water (18 M Ω) was produced with a Barnstead nanopure infinity system (Dubuque, IA). Fused silica capillaries were purchased from Polymicro Technologies (Phoenix, AZ). Soda lime glass slides were purchased from Telic (Santa Monica, CA). N-(Triethoxysilylpropyl)-O-Polyethylene oxide urethanes, (MW 4000-5000 and 750), were purchased from Gelest (Morrisville, PA).

5.2.2 Fabrication of Microfluidic Devices

The microfluidic devices were fabricated using standard photolithography and wet chemical etching techniques as previously described.⁴¹ Briefly, 80 μ m wide (width at half height) and 25 μ m deep separation and side channels were patterned on soda lime glass using a photomask with patterns created with Freehand 10 software and etched

using hydrofluoric acid. All microfluidic channel measurements were obtained using a stylus profiler with a 2 μm , 60⁰ angle tip, (Tencor Instruments, San Jose, CA). Access holes were created on the etched microchip using a microdrilling technique. A hydrodynamic flow restrictor of the make-up solution in the main channel was fabricated using an MP 100 UV laser micromachining system (Oxford Lasers, Oxfordshire, UK). Six parallel 10 μm channels were etched on the glass substrate, connecting the main channel and the intersection of the main channel and the make-up solution channel as shown in Figure 5.1. The cover glass was initially attached through low temperature bonding, followed by high temperature bonding for permanent attachment. A low dead volume connection of the spray tip to the microchip was achieved by drilling a 360 μm diameter hole through the junction between the cover plate and the etched glass plate at the edge of the bonded microchip and inserting a 2 cm long 50 μm I.D fused silica spray tip with a 20 μm tip. The channel surfaces were modified with a mixed PEG-silane thin layer coating. A coating solution of variable chain length PEGs linked to a silane was prepared by weighing 20mg of N-(triethoxysilylpropyl) polyethylene oxide urethane, (Mwt 4000-5000) and 6mg N-(triethoxysilylpropyl)-o-polyethylene oxide urethane (Mwt 400-500), into a 20mL vial and adding 100 μL of a 8 mg/10mL triethylamine (TEA) catalyst in dry toluene. The channels were prepared by rinsing for 15 min with 0.1 M NaOH to activate the silanol groups on the glass surface and then rinsed for 5 min with de-ionized water followed by drying with nitrogen. The PEG-silane solution was delivered through the chip at a low flowrate of $\sim 0.5 \mu\text{L}/\text{min}$ for 4 hours, rinsed with toluene and blown dried with nitrogen. The coating was cured at 60⁰C in the oven for 2 hours.

A

B

Figure 5.1: Schematic of the chip CE-ESI-MS interface detailing the different components of the interface.

A make-up solution is introduced downstream to reduce the pH of the sample in the electrokinetic flow stream, and to aid migration of negatively charged sample past the voltage switching intersection towards the mass spectrometer. The electrospray voltage is rapidly switched between electrodes 4 and 5, directing the analyte through the electrophoretic channel toward the mass spectrometer. In **A**, the make-up flow is added after the voltage switching intersection and as opposed to **B**, where it is added before the voltage switching intersection.

5.2.3 Capillary Electrophoresis and Video microscopy

Fluorescence imaging experiments and microchip capillary electrophoresis were performed using a Nikon inverted epi-fluorescence microscope. A microscope objective was used to focus the excitation beam and to collect the fluorescent light, which was passed through an emission band pass filter via a dual view beam splitter. A Roper CCD camera (Roper Scientific, Tucson AZ) controlled by Metamorph software (Universal Imaging Corporation, Downingtown, PA) was used to capture images and for epi-fluorescence detection.

The voltage programs for experiments using carboxy-SNARF-1, rhodamine 6G, a protein mixture and rhodamine B for EOF measurements are shown in Table 5.1. All voltages were applied using a labsmith HVS448 6000D power supply (LabSmith, Livermore, CA). The ESI voltage was rapidly switched between 4 and 5 at 40 Hz. To enable voltage switching, the high voltage line from the power supply was connected to a relay switch (Kilovac, Santa Barbara, CA), which switched the high voltage between two electrodes. The relay switch was connected to a function generator (Wavetek, San Diego, CA) which provided a square-wave at 40 Hz.

Table 5.1: Voltage programs for loading and injecting samples of rhodamine B, rhodamine 6G, a protein mixture sample and carboxy-SNARF-1

EOF measurements using rhodamine B						
	1	2	3	4	5	7
Load	0	2000	1200	1500	1500	ground
Inject	500	500	3000	0	0	floating
Flow imaging using rhodamine 6G						
	1	2	3	4	5	7
Load	0	2000	1200	1500	1500	ground
Inject	500	500	3000	1500	1500	ground
Protein mixture separation						
	1	2	3	4	5	7
Load	0	2000	1200	1500	1500	ground
Inject	500	500	3000	1500	1500	ground
Imaging Carboxy SNARF-1						
	1	2	3	4	5	7
Load	500	2000	0	500	500	ground
Inject	0	700	700	3000	3000	ground

Reservoir and Electrode Key

- 1 = Buffer
- 2 = Sample
- 3 = Sample Waste
- 4 = Voltage switching
- 5 = Voltage switching
- 7 = Grounded copper plate

Initial assessment of the functioning of the microfluidic interface was performed by flow imaging experiments of rhodamine 6G in 20 mM acetate buffer (pH 5.0), while a make-up solution of 0.1 M acetic acid in 50 % methanol was delivered at a rate of 100 nL/min. The behavior of rhodamine 6G at the voltage switching, the hydrodynamic flow junctions, as well as the spray tip was imaged using an exposure time of 200ms and a 4X objective. The same experiments were performed using carboxy SNARF-1 and the pH drop after adding the low pH make-up flow determined. Carboxy SNARF-1 is a fluorescent pH indicator with a pK_a of 7.5 for its fluorescent forms shown in Figure 5.2.

Figure 5.2: The reaction and calibration curve for carboxy-SNARF-1.

The fluorescent protonated form has an emission maximum at 580 nm, whereas the unprotonated form has an emission maximum at 640 nm.⁴² Excitation of the protonated and unprotonated forms of the dyes was performed at 488nm and 530nm respectively. A calibration curve was generated using the ratio (R) of the fluorescence intensities obtained at $580 \pm 10\text{nm}$ and $640 \pm 10\text{nm}$ at different pH values (Figure 5.2).

5.2.4 Microfluidic Chip CE-ESI-MS

The microfluidic device was interfaced to a LCQ Deca XP ion trap mass spectrometer (Thermo Electron Corporation, Waltham, MA). The channels were prepared by rinsing with methanol for 1 min followed by a 1 min rinse with deionized water, after which it was rinsed with electrophoretic running buffer for 5 min. The channel was filled with the running buffer and the reservoirs loaded with the buffers

ensuring that there were no bubbles in the system. To characterize the functioning of the interface, all reservoirs were filled with the electrophoretic electrolyte, 20 mM ammonium acetate solution (pH 8). A make-up flow solution of 0.1 % acetic acid in 50% methanol was spiked with 1 pmol/ μ L angiotensin 1 sample and introduced hydrodynamically through channel 6 shown in Figure 5.1. In a second experiment, the sample reservoir was filled with a 10 pmol/ μ L mixture of myoglobin and cytochrome C in the running buffer. A make-up solution, 0.1 M acetic acid in water/methanol (80/20% v/v) was hydrodynamically introduced at 100 nL/min, via reservoir 6 (see Figure 5.1). The chip on a X, Y, Z translational stage was placed in front of the mass spectrometer with a distance of 5 mm between the mass spectrometer orifice and spray tip. The voltage program for loading the sample to the cross and injection is shown in Table 5.1.

5.3. Results and Discussion

5.3.1 Imaging Flow Through Microfluidic Device

The first part of the investigations into the operation of the interface involved fluorescence imaging of flow of a positively charged fluorescent dye (rhodamine 6G) through the microfluidic device, while configured to spray to a grounded copper plate as depicted in Figure 5.1A. In Figure 5.1A, the make-up flow is added after the voltage switching intersection, and so for delivery of positive analytes to the spray tip, a high positive voltage can be applied to electrode 1, and a less positive voltage switched between electrodes 4 and 5. This set-up will allow the sample to migrate from the double-tee injector toward 4 and 5, and focus through the voltage switching intersection towards the spray tip. In this case, there is sufficient electric field between the voltage

switching intersection and the counter electrode to drive a positive analyte through to the make-up solution intersection and to the spray tip. The scenario is different for a negative analyte. A negative voltage is applied to electrode 1, while a positive potential is switched between electrodes 4 and 5. In this case, there is insufficient electric field to drive the sample between the voltage switching intersection and make-up intersection, and this results in huge losses to the voltage switching channels. To solve this problem, a second design shown in Figure 5.1B is used, where the make-up flow is added before the voltage switching intersection. The hydrodynamically introduced make-up flow aids in transporting the sample past the voltage switching intersection and minimizes losses to the switching channels.

The use of a positive analyte is the simple case scenario, since the direction of the electrophoretic mobility of the positive ion in the same direction as EOF, unlike the case of a negative analyte, whose migration would oppose EOF under these low EOF conditions, achieved by modifying the surface using a polyethylene glycol terminated silane. A positive analyte will migrate with EOF, away from a positive electrode, while a negative analyte will move against EOF toward a positive electrode. A sample plug of rhodamine 6G is monitored at three positions of the microfluidic device: the voltage switching junction, the make-up flow solution intersection and at the spray capillary tip using a motorized stage to track the sample. The sample plug is injected (Panel A of Figure 5.3) from a double-tee injector (or in this case a cross injector) using the conditions described in experimental section and in Table 5.1 for rhodamine 6G. The behavior of the injected sample plug is monitored at the voltage switching intersection. Panels B-G of Figure 5.3 show successful transmission of the rhodamine 6G sample plug

through the voltage switching junction towards the grounded plate, with minimal losses to the side channels.

Figure 5.3: Images showing the concentration profiles of the passage of a rhodamine 6G sample plug through the voltage switching intersection. A voltage of 3 kV is applied at reservoir 1 to inject the sample plug into the CE channel, with 1.5 kV rapidly switched between electrodes 4 and 5 shown in Figure 5.1. (See table 1 for complete voltage program). Panels A-G show the flow behavior at the voltage switching intersection at successive time intervals of 0.5, 20, 21, 22, 23, 24 and 25 s after the injection of the sample plug respectively. Panel H shows the flow behavior at the make-up flow intersection. An image of the Taylor cone formed at the exit of the spray capillary attached to the microfluidic chip also is shown in panel I.

The images also show the concentration profiles of the sample plug as it migrates through the intersection with a calculated sample loss of 1 %. This value is determined from the average fluorescence intensities of the sample band at injection, the intensity as it passes through the voltage switching intersection, and the intensity of the sample that migrated toward the switching electrodes. In this device, hydrodynamic flow restrictors were fabricated at the entrance of the side channels to prevent the hydrodynamic pressure from forcing solution into the electrode channels to minimize sample losses.

The convergence point of the rhodamine 6G sample and the make-up solution (Panel H) is also monitored to ensure smooth and steady hydrodynamic flow as well as good mixing. Because low EOF conditions are used on the device, the make-up solution is necessary to achieve sufficient flowrate for stable electrospray. This method of adding make-up solution was reported in Chapter 4 and reference 35 as an effective means of combining hydrodynamic and electrokinetic flow streams, and is required to prevent the hydrodynamic pressure from overpowering the electrokinetic flow stream, which is particularly important with low EOF conditions. The rhodamine 6G is driven electrokinetically through the main channel, and is combined at an approximate 1:1 ratio with the make-up solution to provide a flowrate of 100 nL/min. An image of a stable Taylor cone obtained using these conditions is shown in Figure 5.3H. The spray does not appear in the image because it is out of the field of view.

5.3.2 pH sensing using carboxy SNARF-1

One the most interesting characteristics of the interface is the ability to effect a pH change on the device, particularly negative analytes at high pH that can be transformed to

positive analytes by shifting to a lower pH using the set-up in Figure 5.1B. Using a high pH electrophoretic buffer and a low pH make-up solution, the pH stability in the CE channel has to be ascertained. Also the mixing and pH change that occurs when the make-up flow meets the sample has to be determined. The magnitude of the pH change that occurs when make-up flow is added is characterized using the pH fluorescent indicator carboxy-SNARF-1. Carboxy-SNARF-1 is negative at pH 8 and is driven through the PEG-silane coated separation channel by electrophoresis. By monitoring the fluorescence at 580 ± 10 nm and 640 ± 10 nm before and after the hydrodynamic flow restrictor (HDR), the pH drop after adding the low pH make-up solution can be determined. Figure 5.4D₂ shows a CCD image of the pH shift occurring, as the high pH sample mixes with the low pH make-up flow. Panel D₁ of Figure 5.4 shows the fluorescence signal without the make-up flow (syringe pump turned off) and reveals the SNARF mainly in the unprotonated form (high intensity at 640 nm). However, when the low pH make-up solution is introduced, the fluorescence at 580 nm (protonated SNARF) increases and the pH is determined to be lower than 6 (Panel D₂) as indicated in Table 5.3. The exact pH value could not be ascertained because the effective working range of the SNARF is 6-9 as supported by the calibration curve (Figure 5.2) and as noted by other researchers.^{37, 42} The pH values at the double-tee (Panel A), before the hydrodynamic flow restrictor (Panel B) and in the HDR are determined to be 7.6 and 7.7 respectively, as indicated in Table 5.2. This result means the pH is effectively changed at the make-up flow intersection.

Figure 5.4: The figure shows the pH change occurring at the convergence of the electrokinetically mobilized carboxy SNARF-1 in 20 mM ammonium acetate CE electrolyte solution (pH 8) and a low pH make-up solution (0.1 M acetic acid in 50 % methanol at pH3). The images show carboxy-SNARF-1 at injection **A**, before the hydrodynamic flow restrictor, **B**, at the center of the HDR, **C**, at the make-up flow intersection, without delivery of make-up flow, **D₁** and with addition of make-up flow, **D₂**. The *R* values and corresponding pH values obtained from these images are shown in Table 5.2.

The effect of increasing EOF on the pH stability in the CE channel is investigated by using a lower concentration electrolyte solution. An increase in the EOF of the CE channel results in a greater mobility of protons toward the injection portion of the chip. The flow dynamics changes when the concentration of CE electrolyte solution is reduced by 50 %. In this case, the EOF, which is toward the sample inlet and opposes the electrophoresis migration of the SNARF, increases when 10 mM ammonium acetate is used instead of the 20 mM solution. As described by the Helmholtz-Smoluchowski equation, that EOF increases with decreasing electrolyte solution concentration. The

result of using this reduced electrolyte concentration is shown in Figure 5.5. The injection proceeds as normal (although the migration of the sample plug is slower than in the 20 mM solution case. However, half-way down the separation, there is a shift in pH to lower values as shown in panel C and all the way to the make-up intersection. There is a definite migration of protons upstream also supported by the fact that the pH does not change much after downstream of the make-up flow intersection. At the 10 mM electrolyte concentration, electroosmotic flow plus the electrokinetic mobility of the protons in the make-up solution is sufficient to overcome the hydrodynamic flow stream and migrate upstream of the main channel lowering the pH along the length of the of the main channel. Figure 5.5F shows that the protons eventually reach the double-tee resulting in regions of different pH's in the double-tee loading region. The high pH SNARF from the loading channel migrates toward the buffer reservoir, the same direction as the EOF and proton migration. In this case, with the anions migrating toward the outlet and the EOF toward the inlet, the magnitude of the EOF must be minimized.

Figure 5.5: The figure shows the pH change occurring at the convergence of the electrokinetically mobilized carboxy SNARF-1 in 10 mM ammonium acetate CE electrolyte solution (pH 8) and a low pH make-up solution (0.1 M acetic acid in 50 % methanol at pH3). The images show carboxy-SNARF-1 at injection **A**, 10 mm before the hydrodynamic flow restrictor, **B**, 25 mm before the hydrodynamic flow restrictor, **C**, at the center of the HDR, **D**, at the make-up flow intersection with delivery of make-up flow, **E** at and the double-Tee injector after the sample has exited the device **F**.

Table 5.2: Fluorescence intensity ratios (*R* values) and the corresponding pH values for different sections of the microfluidic CE-MS chip for 10 mM and 20 mM ammonium acetate CE electrolyte solutions.

Electrophoretic Electrolyte Solution	Background Subtracted Fluorescence Intensities		R Value	pH
20mM Acetate pH 8				
	580 nm	640 nm		
At Double Tee	968.70	107.13	9.04	7.6
Upstream HDR	1067.70	90.13	11.85	7.7
Make-up flow junction	556.70	857.13	0.65	<6
Voltage Switching junction	455.70	655.13	0.70	<6
10mM Acetate pH 8				
At Double tee (before injection)	1051.40	160.28	6.56	7.4
10 mm after double Tee	1568.70	234.35	6.69	7.4
25 mm after double tee(before HDR)	969.42	1630.82	0.59	<6
In the center HDR	181.77	302.82	0.60	<6
At make-up junction	215.53	92.77	2.32	7
At double tee (Final)				
<i>main channel</i>	462.07	700.35	0.66	<6
<i>entrance channel</i>	2100.08	263.19	7.98	7.5
<i>exit channel</i>	457.99	380.86	1.20	~6

5.3.3 Microfluidic ChipCE-ESI-MS

To demonstrate the device can provide stable spray, the microfluidic chip (Figure 5.1B) was interfaced to a mass spectrometer. The stability of the electrospray signal was monitored by setting-up the microfluidic device to spray a hydrodynamically driven solution of 1 pmol/ μ L angiotensin 1 from the reservoir (6) into a mass spectrometer instead of a grounded copper plate, while electrokinetically mobilizing a 20 mM ammonium acetate solution (pH 8) in the main channel. The distribution of the intensities of angiotensin 1 charged state peaks (m/z = 433.1 (+3), 649 (+2), 1297 (+1))

are monitored over a period of 20 minutes. The total ion electropherogram (TIE) signal and the corresponding mass spectra averaged over the entire 20 minutes are directly compared to the signal and spectra from hydrodynamic direct infusion of the same angiotensin 1 solution electrosprayed at pH 3, 4 and 5. The distributions of these charged states of the peptide shown in Figure 5.6 and Table 5.3 suggest a pH value between 3 and 4. It is also noted that the distributions of the charged states fluctuated significantly with each spectrum acquired, more so than the hydrodynamically infused samples. This trend can be explained by an uneven change in pH for portions of solutions exiting the spray tip. There are a significant number of scans in the averaged spectrum in which the $m/z = 649$ dominated corresponding to a pH between 4 and 5. The stability of the electrospray signal is very comparable to that of the direct infusion using the same conditions (MS tuning, solutions, flowrate, tip size, and voltages), although there was much more background in the angiotensin spectrum from the hydrodynamic infusion. The TIE also shows stable electrospray signal during the 20 min operational period of the device.

Figure 5.6: TIC and mass spectrum of angiotensin I delivered through the chip-CE-ESI-MS interface, with the 20 mM ammonium acetate CE electrolyte solution (pH 8) in reservoirs 1-5 and 0.1 pmol/ μ L angiotensin I in 0.1 M acetic acid in 50 % methanol make-up solution hydrodynamically delivered through reservoir 6.

Table 5.3: The intensity ratios of Angiotensin I charged states $m/z = 649$ and $m/z = 433$ at pH 3-5, and for chip-CE-MS interface.

	Intensity		Intensity Ratio
pH	$m/z = 433$	$m/z=649$	$(m/z\ 649)/(m/z\ 433)$
3	1064379387	39810708	0.04
4	3481736	9305327	2.67
5	549491	15293615	27.83
ChipCE-ESI-MS	159805925	45546063	0.29

An initial attempt to separate two small proteins with the interface is performed using the configuration in Figure 5.1A. Figure 5.7 shows a total ion electropherogram (TIE) of a mixture of myoglobin and cytochrome C. Although the two peaks are barely

resolved, this data demonstrates that the microfluidic chipCE-ESI-MS configuration can be used to deliver protein samples into the mass spectrometer while adding make-up flow. This electropherogram however highlights the several practical challenges involved with operating a microfluidic chipCE-ESI-MS interface due to the complicated coupling between the chipCE and MS. The migration time of the sample in this electropherogram is much longer than expected from the fluorescence imaging experiments, suggesting a perturbation in the electrokinetic flow regime.

Figure 5.7: Total ion electropherogram of a mixture of cytochrome C and myoglobin injected and separated on the microfluidic chip. The sample contained 10 pmol/ μ L of each protein in the electrophoretic electrolyte (20 mM ammonium acetate). The voltage program used is shown in Table 5.1.

One of the major challenges is the deposition of minute particulates on the channel surface during operation of the microfluidic device, particularly at channel junctions.⁴³ A small particle deposited in the channel at a junction usually grows in size as the device is operated, due to adsorption on the particle surface, thereby creating local pressure gradients. These pressure gradients eventually perturb the flow through the device, which

affects the balance between the CE and ESI voltages required for optimum performance of the device, as well as increasing band broadening.⁴⁴⁻⁴⁶ Real time adjustments of the CE and ESI voltages are therefore often required to maintain stable electrospray and/or flow in the CE channel to compensate for these pressure gradients.

Maintaining stable spray for an extended operational period of the device is also a challenge. The spray stability is dependent on a variety of factors which include, the flowrate, the inner and outer diameters of the capillary, the applied voltage, the distance between the capillary tip and counter electrode and spray solution properties such as conductivity, surface tension, viscosity and dielectric constant, which all determine the electrospray mode of operation. All these parameters have to be kept constant during the operation of the device, which is practically challenging due to the operational complexity and small channel sizes of the device.

5.4 Conclusions

The microfluidic chip CE- ESI-MS interface designed and developed in this work has the unique ability to separate anionic proteins and peptides under low EOF conditions with positive ion mode MS detection. Effective pH reduction has been demonstrated using SNARF-1 pH indicator as well as the distribution of charged states of a peptide. Although it is not demonstrated in this work, this chipCE-ESI-MS interface can be potentially be used for integrating on-chip tryptic digestions followed by high pH CE separation, pH reduction and positive ion mode detection of the sample. The voltage switching component of the interface also provide an alternative method of applying the

electrospray voltage and as an end electrode to the CE providing dual control points for both the CE and ESI voltages.

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‘This is not the end. It is not even the beginning of the end. But it is, perhaps, the end of the beginning.’ *Sir Winston Churchill.*