# PROTEIN STAMPING FOR MALDI MASS SPECTROMETRY USING AN ELECTROWETTING-BASED MICROFLUIDIC PLATFORM

Vijay Srinivasan[1], Vamsee Pamula, Phil Paik, and Richard Fair
Department of Electrical and Computer Engineering, Duke University,
Durham, NC 27708-0291.

## ABSTRACT

MALDI-MS (matrix-assisted laser desorption/ionization mass spectrometry) is one of the most commonly used techniques for protein analysis. In conventional systems sample preparation is typically done in well-plates and transferred onto a MALDI target by robotic systems, which are complex, huge, expensive and slow. In this paper, we present a droplet-based microfluidic interface to transfer protein samples from a well-plate format onto a MALDI target for MS analysis. The droplets are actuated using the electrowetting phenomenon, and are immersed in silicone oil which prevents non-specific adsorption and enables the manipulation of high concentrations of proteins. Droplet transport and droplet formation were evaluated as a function of protein concentration using bovine serum albumin (BSA) as a test system. Droplet transport was possible for BSA concentrations up to 10mg/mL which is three orders of magnitude higher than previously reported results on handling proteins by electrowetting. Droplet formation from on-chip reservoirs, using only electrowetting forces and no external pressure assistance, was possible up to concentrations of 0.01mg/mL. An interface between a well-plate format and the electrowetting chip, and a scheme to passively stamp droplets onto a target substrate was then designed and tested by stamping BSA solutions. In two separate experiments 3.6fmoles and 16fmoles of BSA were stamped onto a glass slide using 0.001mg/mL and 0.01mg/mL samples respectively. A protein mixture with known constituents (ABI 4700 proteomics analyzer calibration solution) was stamped onto a MALDI plate and the individual proteins were correctly identified in the mass spectrum obtained using MALDI-TOF MS. The preliminary results establish the feasibility of using an electrowetting-based microfluidic system to handle proteins especially for protein stamping applications. The proposed system has a small footprint, is easy to control, and is very fast compared to conventional robotic systems. In addition, there are no moving parts and the associated mechanical reliability issues. Future work involves scaling to a larger number of samples and integration of sample preparation steps on-chip.

**Keywords** – electrowetting, droplet, lab-on-a-chip, microfluidics, MALDI, protein stamping.

## 1. INTRODUCTION

Mass spectroscopy (MS) is increasingly becoming the method of choice for protein analysis in biological samples [1]. Among the various MS methods, MALDI-TOF (Matrix Assisted Laser Desorption-Ionization Time of Flight) is the most commonly used due to its simplicity, high sensitivity and resolution. A typical MALDI-MS protocol for protein identification involves sample preparation, stamping onto a MALDI target and analysis on a MALDI-TOF mass spectrometer. Sample preparation steps (such as digestion and concentration) are usually done in the well-plate format and last for several hours at the least. The stamping is done using complex robotic systems which are huge, expensive and immobile. Sample volumes required are also very high, which is a concern for proteins available in very small quantities.

The emerging paradigm of microfluidic lab-on-a-chip systems can alleviate these drawbacks by reducing volume requirements to nanoliters, decreasing sample preparation time (due to more efficient kinetics on the microscale) and by providing a simpler interface to MALDI-MS instrumentation without moving parts. Several microfluidic platforms have been proposed for protein analysis using MALDI-MS. Ekström et al [2] integrated a microchannel immobilized enzyme reactor (for protein digestion of 1µL samples within 3 minutes) with a piezoelectric microdispenser for stamping in a

---

[1] vijay@ee.duke.edu, Phone: 919-660-5229, FAX: 919-660-5293

high-density format. The microfluidic device used a syringe pump for inducing fluid flow. Brivio et al [3] developed a continuous flow lab-on-a-chip consisting of an on-chip microfluidic device with reaction microchannels and integrating the microdevice with a MALDI-TOF MS standard sample plate. A pressure driven pumping mechanism using the vacuum of the instrument was used as a driving force in the reaction microchannel. Gyros AB (Uppsala, Sweden) [4][5] has developed a system to integrate sample concentration, elution and crystallization on a CD platform for MALDI analysis.

All the microfluidic devices discussed above are based on continuous flow in fixed microchannels, offering very little flexibility in terms of scalability and reconfigurability. An alternative approach is to manipulate the liquid as discrete microdroplets. This approach, referred to as digital microfluidics, has several advantages over continuous-flow systems, the most important being the ease of fabrication, and reconfigurability and the scalability of architecture.

Electrowetting is one of several techniques that have been proposed to actuate microdroplets. Electrowetting refers to the modulation of the interfacial tension between a conducting liquid phase and an insulated solid electrode, by the application of an electric potential between the two. Kim et al [6] have proposed an electrowetting-based system for analysis of peptides and proteins by MALDI using the electrowetting chip itself as the MALDI target. However since the droplets are manipulated in air, non-specific protein adsorption problems arise and only sufficiently hydrophilic proteins or ultra low concentrations of other proteins ($<<$ μg/mL) would work. The system proposed in this paper uses silicone oil to encapsulate the droplet and minimize adsorption, enabling the manipulation of very high concentrations of protein solutions. Using this technique we have previously demonstrated the manipulation of real physiological samples containing proteins for a clinical diagnostic application [7]. In this paper we extend our system to format protein samples for MALDI-MS analysis and present preliminary results to establish the proof of concept. Droplet transport and formation from an on-chip reservoir is first evaluated as a function of the protein concentration using bovine serum albumin (BSA) as a test system. An interface between a well-plate format and an electrowetting chip, and a scheme to passively stamp droplets onto a target substrate is then designed and tested using water and BSA solutions. Finally droplets of a known protein mixture are stamped on to a standard MALDI target plate and analyzed on an ABI 4700 MALDI-MS TOF system.

## 2. MATERIALS AND METHODS

### 2.1 Chip fabrication

The electrowetting system comprises of a photolithographically patterned metal electrode array (chrome or indium tin oxide) on a glass substrate and a continuous ground plane (indium tin oxide on glass or polycarbonate) parallel to it. A spacer of known thickness (H) separates the electrode array and the ground plane and the droplets are sandwiched between the two. The spacer material is also used to physically define the on-chip reservoir. 1cSt silicone oil (DMS-T01, Gelest, Morrisville, Pennsylvania, USA) is used as the filler medium surrounding the droplets to prevent evaporation and facilitate transport. The electrode array is insulated from the droplet by layer of Parylene C (~800nm) and both the surfaces are hydrophobized by a thin layer of Teflon AF 1600 (~50nm). Figure 1 shows the vertical cross-section of a typical electrowetting device. The fabrication and operation of the electrowetting system are described in detail [8].
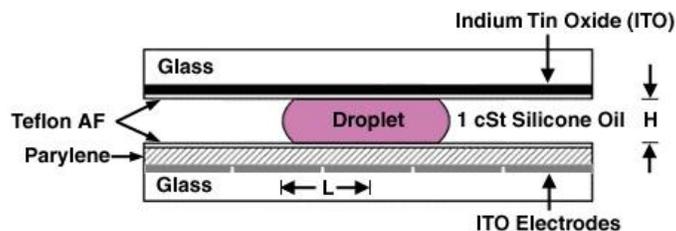


**Figure 1 – Vertical cross-section of the electrowetting chip showing the material layers**

### 2.2 Chemicals

Bovine Serum Albumin (BSA, A-4378) was obtained from Sigma Chemicals (St Louis, Missouri, USA) and stock solutions of 0.1mg/mL, 0.01mg/mL and 0.001mg/mL were prepared in de-ionized water. BSA (10mg/mL) was also provided by GlaxoSmithKline (Durham, North Carolina, USA) and diluted to 1mg/mL, 0.1mg/mL, 0.01mg/mL and 0.001mg/mL in deionized water. ABI 4700 proteomics analyzer calibration solution was provided by the Duke Univer-

sity Proteomics Center (Durham, North Carolina, USA) and consisted of 1.0µM des-Arg[1]-Bradykinin, v Angiotensin I, 1.3µM Glu[1]-Fibrinopeptide B, 2.0µM ACTH (1-17 clip), 1.5µM ACTH (18-39 clip), and 3.0µM ACTH (7-38 clip) in α-cyano-4-hydroxycinnamic acid/acetonitrile/TFA matrix.

## 3. MANIPULATION OF PROTEIN SAMPLES BY ELECTROWETTING

The manipulation (droplet formation, transport etc) of non-biological electrolytes using electrowetting has been demonstrated both in air [9] and in other immiscible media such as silicone oil [8]. However dealing with fluids containing proteins is not as straightforward, since they tend to irreversibly adsorb to hydrophobic surfaces such as the Teflon AF surface in the electrowetting setup. Any contact between the protein sample and the Teflon AF surface will therefore contaminate the surface and also render the surface permanently hydrophilic [10]. This is detrimental to electrowetting since electrowetting works on the principle of reversible modification of the wettability of a hydrophobic surface. Therefore any contact between a liquid containing proteins and the Teflon surface should be avoided to prevent contamination and not inhibit transport. As a consequence, air is not a suitable filler medium for applications involving proteins, since the droplet is always in contact with the Teflon surface. Silicone oil with its low surface tension (~20dynes/cm) and spreading property is an ideal alternative. From visual observations we have inferred the presence of a thin film of oil, encapsulating the droplet. This oil film isolates the droplet from the Teflon surfaces, minimizing adsorption and facilitating reversible electrowetting. However, we need to evaluate if lipophilic proteins in droplets would partition into the surrounding oil.

The stability of the oil film, though yet to be extensively characterized, appears to decrease with increasing protein content which causes lowering of the interfacial tension between the liquid (droplet) and oil. A less stable oil film implies more adsorption of proteins on to the Teflon surface and consequently liquids having high protein concentrations are more difficult to manipulate and require higher actuation voltages. The droplet transport and droplet formation processes are therefore studied as a function of protein concentration using bovine serum albumin (BSA) as a test protein. We have previously shown the transport of a variety of biological fluids including whole blood, serum, plasma and urine on our system using silicone oil as the filler fluid [7].

### 3.1 Protein Droplet Transport

Due to the discrete nature of operation of a digital microfluidic system, the maximum switching frequency (defined as the highest rate at which a droplet can be completely moved across two adjacent electrodes) is the measure of the transport-performance of the system. Higher the switching frequency, more the number of discrete fluidic operations that can be performed per second and higher the throughput of the system. The maximum switching frequency is evaluated for droplets containing different concentrations of BSA as a function of the applied voltage. Droplets are manually dispensed using a pipette in order to nullify the effect of the concentration dependency of automated droplet formation.

### 3.2 Protein Droplet Formation

Unit droplet generation from a larger volume liquid of is a very important component of the world-to-chip interface of any droplet-based microfluidic device. In an electrowetting system, droplets can be generated from on-chip reservoirs using purely internal electrowetting forces, from external reservoirs using active pressure sources, or a combination of the two. We have previously demonstrated droplet generation with and without external pressure assistance for aqueous electrolytes [7][8]. However, as in the case of transport, droplet formation also needs to be evaluated for protein solutions to completely establish the compatibility of the liquid with our platform. The formation process is likely to be more severely affected by protein adsorption than transport, since the liquid in the reservoir has a much larger surface area to adsorb to than a unit droplet. Concentrations for which formation would work are therefore expected to be smaller than the maximum concentration for which simple transport would work. Droplet formation using only electrowetting forces is tested for BSA solutions with concentrations ranging from 0.001-0.1mg/mL.

## 4. PASSIVE PROTEIN STAMPING

Stamping refers to the process of transferring a protein droplet on to a MALDI substrate which can be analyzed on a MALDI MS system. Figure 2 shows a high level schematic of an electrowetting-based stamping system which interfaces a standard well-plate format and a MALDI target substrate. The electrowetting chip consists of an on-chip reser-
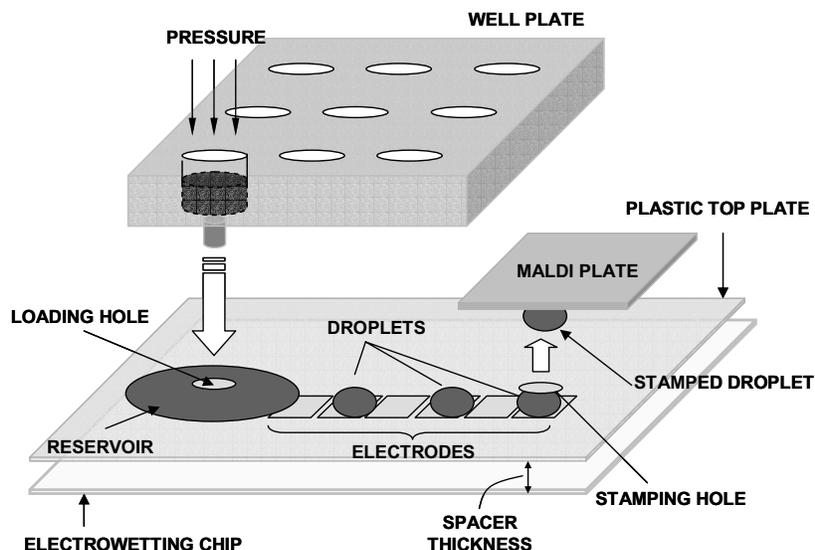
**Figure 2 - High level schematic of electrowetting-based protein droplet stamping system.**

voir (physically defined by the spacer material) and a set of contiguous electrodes connecting it to the stamping site. A polycarbonate sheet (coated with conductive indium tin oxide to act as the ground plane) is clamped to the electrowetting chip, and has holes for injecting liquid into the reservoir from a well-plate and for extraction (stamping) onto the MALDI target. By designing the diameter of the stamping hole to be much larger than the spacer thickness (which is also the droplet height), the liquid is passively pushed out of the stamping hole. The larger diameter creates a lower pressure at the stamping hole as compared to the transport layer on the chip, which forces the droplet out spontaneously without active forces [11]. However some liquid is still expected to be left behind in the hole as dead volume.

Preliminary stamping experiments are done with water or protein solutions containing 0.01mg/mL or 0.001mg/mL bovine serum albumin. Sample is injected into the on-chip reservoirs either from a well-plate (using a syringe pump to apply pressure) or a pipette. Droplets are then automatically generated from the reservoir, configured on the chip by electrowetting, and vertically stamped onto a MALDI target. To complete the feasibility study, a known protein mixture (AB1 4700 proteomics analyzer calibration solution) is stamped on to a standard MALDI target plate and analyzed on an ABI 4700 MALDI-MS TOF system to determine if any proteins are lost on the electrowetting platform or the stamping hole. We would like to note that though the eventual objective is to do sample preparation on-chip, the well-plate format still represents a standardized high-throughput input format. Hence the emphasis on interfacing the electrowetting chip to the well-plate format.

## 5. RESULTS AND DISCUSSION

### 5.1 Transport of BSA droplets

The maximum switching frequency of droplets containing different concentrations of BSA (0.001-10mg/mL) was evaluated as a function of voltage. The electrode pitch was L=1mm, the gap was H=500μm (refer to Figure 1 for definition of L and H), and the droplet volume was 0.75μL. Silicone oil (1cSt) was used as the filler fluid. Figure 3 plots the maximum switching frequency as a function of the voltage, for various concentrations of BSA. As expected, droplets with higher protein content are more difficult to transport and require higher voltages for operation. This is the first time such high concentrations of protein have been demonstrated to transport on an electrowetting system. Using air as a filler medium, static electrowetting (i.e. static change in contact angle by application of a potential) of 4μg/mL BSA droplets has been previously demonstrated in a limited sense [8]. However dynamic transport has not been shown in air and will indeed be a big challenge due to the reasons mentioned in section 3. Even in the static case the reversibility of the contact angle change reduces over time due to continuous non-specific and electric field driven adsorption.

Proc. of SPIE Vol. 5591    29

**Transportability of BSA**
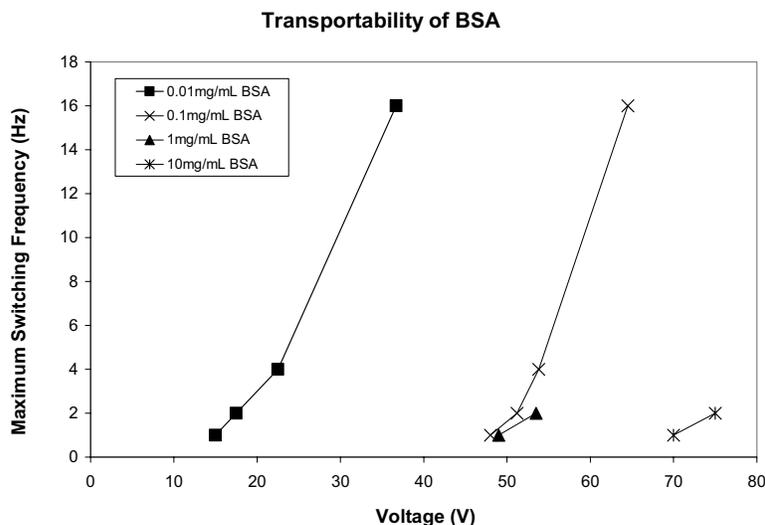


Figure 3 – Maximum switching frequency of BSA droplets as a function of voltage for various concentrations.

## 5.2 BSA Droplet Formation

Droplet formation from BSA solutions of various concentrations (0.001-0.1mg/mL) using only electrowetting forces was evaluated. The electrode pitch was L=750µm and the spacer thickness was H~75µm. For concentrations up to 0.01mg/mL reliable on-chip dispensing was possible. For solutions containing higher concentrations of BSA (>= 0.1mg/mL) it was not possible to generate droplets using electrowetting alone, despite the fact that individual droplets containing up to 10mg/mL BSA have been transported previously. This is likely due to protein adsorption, since the liquid in the reservoir has a much larger surface area (~20 times more than a unit droplet) and therefore a higher probability of adsorbing to the surfaces of the reservoir.
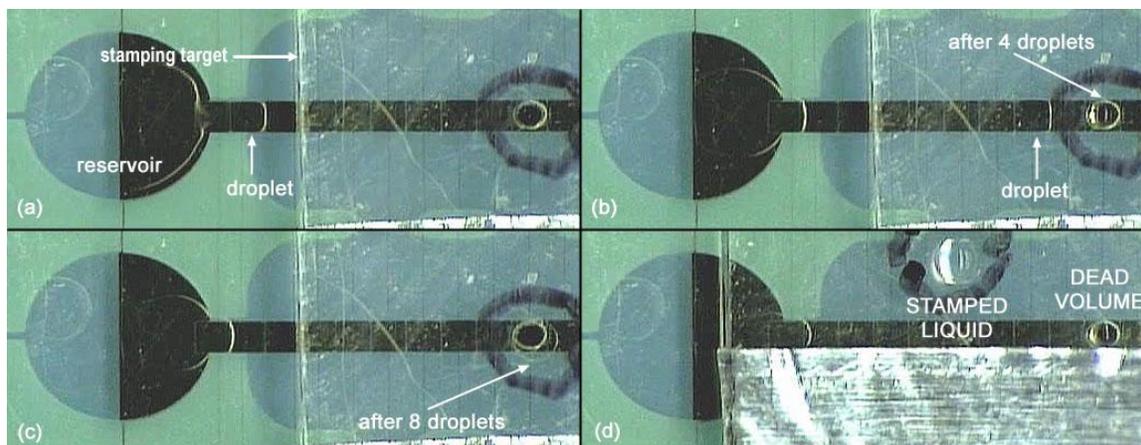
## 5.3 Protein Stamping

Preliminary experiments were conducted with water, and protein solutions containing 0.01mg/mL or 0.001mg/mL bovine serum albumin. The electrode pitch was L=750µm and the spacer thickness was H~75µm, resulting in a unit droplet volume of ~40nL. The thickness of the top plate of polycarbonate sheet was 500µm, and the diameter of the stamping hole was 600µm. A hydrophobic glass slide with a hydrophilic patch (aligned to the stamping hole) was used as the stamping target. The spacing between the stamping target and the polycarbonate top plate was ~90µm. Liquid was loaded from a well-plate (for water samples) or using a pipette (for protein solutions). Droplets were then automatically generated from the reservoir, routed to the stamping hole, and vertically stamped onto the stamping target.

Figure 4 shows the time lapsed images of an experiment where 10 droplets (~40nl each) of 0.001mg/mL were dispensed from the reservoir, of which 6 droplets (~240nL corresponding to 3.6fmoles) was stamped onto the glass slide. The remaining 4 droplets (~160nL) were left behind in the stamping hole as dead volume. In a similar experiment, an estimated 16fmoles of BSA was stamped using 0.01mg/mL BSA solution. The number of moles stamped can be further increased by increasing the concentration of the protein solution or the volume of liquid i.e. the number of droplets stamped.

## 5.4 MALDI-MS on protein calibration solution

To complete the feasibility study, a protein stamping experiment was performed using ABI 4700 calibration solution as a test protein sample. The electrode pitch was L=1.5mm and the spacer height was H=300µm. The calibration solution contained des-Arg-Bradykinin (mol. Wt. = 904.468 Daltons), Angiotensin I (1296.685 Da), Glu-Fibrinopeptide B (1570.677 Da), ACTH (1-17) (2093.087 Da), ACTH (18-39) (2465.199 Da), and ACTH (7-38) (3657.929 Da). Two 1µL droplets of the calibration solution were dispensed manually onto chip, transported by electrowetting, and passively stamped through the polycarbonate top plate on to a MALDI plate. The MALDI plate was then analyzed on an

**Figure 4 - Time lapsed images of a protein stamping experiment.**

ABI4700 MALDI TOF/TOF system and the spectrum obtained is shown in Figure 5. The figure shows that all the proteins in the calibration solution are detectable in the spectrum even after on-chip operations of transport and passive stamping were performed. ACTH (7-38) does not appear on the graph since the mass spectroscopy was done only till a molecular weight of 3400 Daltons as suggested by the manufacturer protocol.

The preliminary results establish the feasibility of using an electrowetting-based microfluidic system for protein stamping applications. Further work is required to integrate sample preparation on-chip which would reduce the total analysis time significantly. Mechanical design issues also need to be considered at the injection and ejection orifices, such as air bubbles and dead volume. Ways to increase the total amount of protein stamped also need to be investigated. This could be done by spotting more number of droplets at the same location, by increasing the concentration of the protein in solution or by scaling the system up in dimensions. Eventually the system also needs to be scaled to accommodate an actual 96-well plate or 384-well plate.

**MALDI-MS spectrum for Protein Calibration Solution**
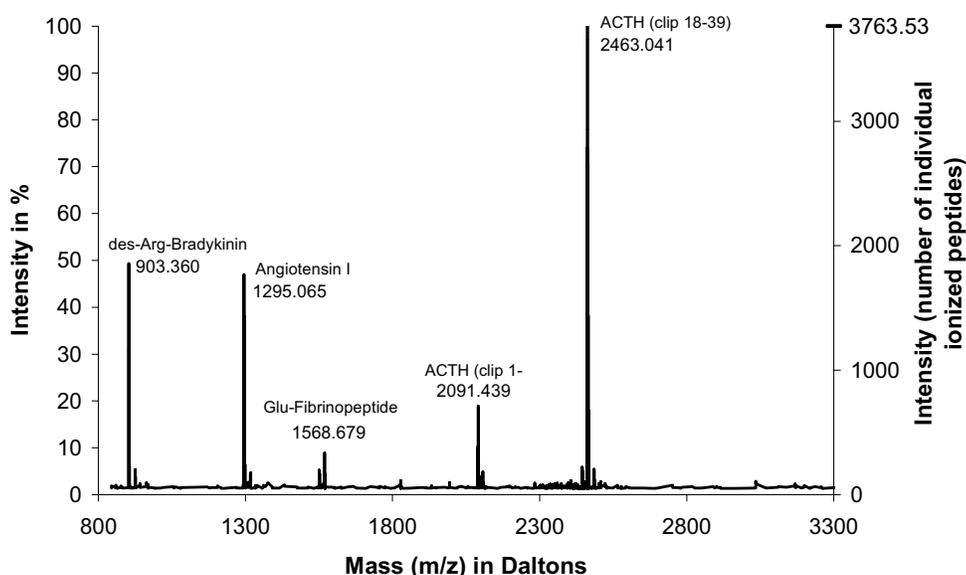


**Figure 5 – Mass spectrum of the protein calibration solution done on the ABI 4700 Proteomics Analyzer.**

# 6. CONCLUSIONS

An electrowetting-based microfluidic platform for protein stamping applications was described in this paper. Protein droplet transport and formation from an on-chip reservoir was initially evaluated to establish the compatibility of the protein with the electrowetting system. Bovine serum albumin (BSA) was used as a test protein, and concentrations up to 10mg/mL were transportable, which is more than three orders of magnitude higher than previously reported results. Droplets formation was possible up to concentrations of 0.01mg/mL using only electrowetting forces. We were able to manipulate droplets containing such high concentrations of proteins due to the presence of an oil film encapsulating the droplet and minimizing non-specific adsorption to Teflon AF. A passive stamping scheme was designed to vertically actuate droplets onto a MALDI target and tested using BSA and water. Liquid was loaded from a well-plate (for water samples) or using a pipette (for protein solutions), and droplets were automatically generated from the reservoir and configured on the chip by electrowetting to be vertically stamped onto the stamping target. By designing the diameter of the stamping hole to be much larger than the spacer thickness, a lower pressure is created at the stamping hole as compared to the transport layer on the chip, which forces the droplet out spontaneously without active forces. In two separate experiments 3.6fmoles and 16fmoles of BSA were stamped onto a glass slide using 0.001mg/mL and 0.01mg/mL samples respectively. MALDI-MS was also performed on an ABI 4700 proteomics analyzer calibration solution and from the mass spectra the proteins in the calibration solution were correctly identified. The preliminary results establish the feasibility of using an electrowetting-based microfluidic system for protein stamping applications. The proposed system has a small footprint, is easy to control, and is very fast compared to conventional robotic systems. In addition, there are no moving parts and the associated mechanical reliability issues. Future work mainly involves integration of sample preparation on-chip and scaling the system to a larger array.

# 7. ACKNOWLEDGEMENTS

# 8. REFERENCES

1. R. Aebersold and M. Mann, *Mass spectrometry-based proteomics*, Nature, v.422, pp.198-207, 2003.
2. S. Ekström, P. Önnerfjord, J. Nilsson, M. Bengtsson, T. Laurell, and G. M.-Varga, *Integrated Microanalytical Technology Enabling Rapid and Automated Protein Identification*, Analytical Chemistry, v.72, pp.286-293, 2000.
3. M. Brivio, R. H. Fokkens, W. Verboom, D. N. Reinhoudt, N. R. Tas, M. Goedbloed, and A. van den Berg, *Integrated Microfluidic System Enabling (Bio)chemical Reactions with On-Line MALDI-TOF Mass Spectrometry*, Analytical Chemistry, v.74, pp.3972-3976, 2002.
4. M. Gustafsson, D. Hirschberg, C. Palmberg, H. Jörnvall, and T. Bergman, *Integrated Sample Preparation and MALDI Mass Spectrometry on a Microfluidic Compact Disk*, Analytical Chemistry, v.76, pp.345-350, 2004.
5. Gyros AB, Uppsala, Sweden, *http://www.gyros.com.*
6. A. R. Wheeler, H. Moon, C-J. Kim, J. A. Loo, and R. L. Garrell, *Electrowetting-Based Microfluidics for Analysis of Peptides and Proteins by Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry*, Analytical Chemistry, v.76, pp.4833-4838, 2004.
7. V. Srinivasan, V. K. Pamula, and R. B. Fair, *An Integrated Digital Microfluidic Lab-on-a-chip for Clinical Diagnostics on Human Physiological Fluids*, Lab on a Chip, v.4, pp.310-315, 2004.
8. M. G. Pollack, A. D. Shendorov, and R. B. Fair, *Electrowetting–based actuation of droplets for integrated microfluidics*, Lab on a Chip, v.2, pp.96-101, 2002.
9. S-K. Cho, H. Moon, and C-J. Kim, *Creating, Transporting, Cutting, and Merging Liquid Droplets by Electrowetting-Based Actuation for Digital Microfluidic Circuits,* J of Microelectromechanical Systems, v.12, pp.70-80, 2003.
10. J-Y. Yoon and R. L. Garrell, *Preventing Biomolecular Adsorption in Electrowetting-Based Biofluidic Chips*, Anal Chemistry, v.75, pp.5097-5102, 2003.
11. U-C. Yi and C-J. Kim, *Soft Printing of Droplets Digitized by Electrowetting*, Transducers 2003, pp.1804-1808, 2003.