

RAPID PROTEIN SEPARATIONS IN MICROFLUIDIC DEVICES

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ABSTRACT

This paper describes fabrication of glass and plastic microfluidic devices for protein separations. Although the long-term goal is to develop a microfluidic device for two-dimensional gel electrophoresis, this paper focuses on the first dimension–isoelectric focusing (IEF). A laser-induced fluorescence (LIF) imaging system has been built for imaging an entire channel in an IEF device. The whole-channel imaging eliminates the need to migrate focused protein bands, which is required if a single-point detector is used. Using the devices and the imaging system, we are able to perform IEF separations of proteins within minutes rather than hours in traditional bench-top instruments.

1. INTRODUCTION

Microfluidics technology has been used to construct miniaturized analytical instruments called “lab-on-a-chip” devices. The principles of microfabrication and microfluidics, as well as their current and potential applications, have been reviewed in literature (Reyes, et al. 2002; Boone, et al. 2002; Soper, et al. 2000). Common analytical assays, such as polymerase chain reaction (PCR), have been reduced in size and fabricated in a centimeter-scale chip (Liu, et al. 2004; Koh, et al. 2003; Krishnan, et al. 2002; Legally, et al. 2001; Belgrader, et al. 2001). The size reduction of an analytical instrument has many advantages including high speed of analysis, minimization of required sample and reagents, and portability.

Proteomics is emerging as an important tool in biodefense, modern drug discoveries, and medical diagnostics. As a novel countermeasure to biological warfare, proteomics has recently been developed as a method for microorganism detection, identification, and classification (Jia, et al. 2004; McBride, et al. 2003; Warscheid, et al. 2003). This method is based on protein or peptide biomarkers in a microorganism;

identification is carried out by protein separations and/or mass spectrometry. Recent demonstrations include microorganism identification based on peptide biomarkers in proteolytic digests generated from spore mixtures of *Bacillus*.

Among many approaches being developed for proteomics, two-dimensional gel electrophoresis (2DGE) is an essential tool (Service, 2001; Chen, et al. 2002; Li, et al. 2004). 2DGE consists of first dimensional separation–isoelectric focusing (IEF)—and second dimensional separation–polyacrylamide gel electrophoresis (PAGE). One major advantage of 2DGE is its enormous separation resolution. However, its poor reproducibility and time-consuming process make it cumbersome in large-scale proteomics studies and biodefense environments. To address these challenges, Chen, et al. (2002) and Li, et al. (2004) recently described their effortson 2D electrophoresis device. For the same objective, we are developing an integrated, miniaturized device for rapid, reproducible 2DGE that is capable of mapping proteomes and searching for biomarkers of biological warfare agents. This paper will focus on device fabrication and the first dimensional separation, IEF.

2. EXPERIMENTAL

2.1 Device Fabrication

Plastic devices were fabricated generally following our published procedures (Boone, et al. 2002). Briefly, the layout was first designed using AutoCAD and fabricated in either silicon or a glass substrate using conventional photolithography and chemical etching. The pattern made in the substrate was then transferred onto a metal master using electroplating. The metal master was exploited as a molding tool to make plastic devices through compression molding.

Report Documentation Page				Form Approved OMB No. 0704-0188	
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1. REPORT DATE 00 DEC 2004		2. REPORT TYPE N/A		3. DATES COVERED -	
4. TITLE AND SUBTITLE Rapid Protein Separations In Microfluidic Devices				5a. CONTRACT NUMBER g	
				5b. GRANT NUMBER	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S)				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Department of Mechanical and Aerospace Engineering Department of Biomedical Engineering University of Florida Gainesville, FL 32611				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION/AVAILABILITY STATEMENT Approved for public release, distribution unlimited					
13. SUPPLEMENTARY NOTES See also ADM001736, Proceedings for the Army Science Conference (24th) Held on 29 November - 2 December 2005 in Orlando, Florida., The original document contains color images.					
14. ABSTRACT					
15. SUBJECT TERMS					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 5	19a. NAME OF RESPONSIBLE PERSON
a. REPORT unclassified	b. ABSTRACT unclassified	c. THIS PAGE unclassified			

For glass devices, a glass substrate was first etched with a desired pattern using photolithography. The substrate was then bonded thermally with a cover plate, which possesses holes in alignment with the etched pattern in the substrate. These holes function as reservoirs for reagents and provide accesses to the channels. The holes in glass were drilled ultrasonically by Bullen Ultrasonics (Cincinnati, OH). The bonding protocol has been described previously (Fan and Harrison, 1994).

2.2 Materials and Chemicals

Glass substrates (25 mm x 75 mm) were purchased from Fisher Scientific (Atlanta, GA), as were acrylamide:bis-acrylamide (electrophoretic grade, 5%C), tetramethylethylenediamine (TEMED), ammonium persulfate, and acetic acid. Ampholytes (pH 3-10) were from Amersham Biosciences (Piscataway, NJ) while ethanolamine was from Sigma (St. Louis, MO). Recombinant green fluorescent protein (GFP) was obtained from BD Biosciences Clontech (Palo Alto, CA). GFP was supplied in a concentration of 1 mg/mL and it is diluted in a ratio of 1:1000 in CA of desired concentration (usually 1-2%).

For IEF, acrylamide monomer solution (5%T) and the sample were mixed before polymerization, or alternatively the sample was loaded electrokinetically by adding the sample in the anode compartment. Solutions of 15 mM acetic acid and 15 mM ethanolamine served as anolyte and catholyte, respectively.

3. RESULTS AND DISCUSSION

3.1 Device Fabrication.

Most microfluidic devices are made from silicon, glass, or plastics, as reviewed by Reyes et al. (2002). When electrokinetic pumping or electrophoresis is carried out, silicon wafer is too conductive and the current will flow through silicon bulk rather than the fluid in channels. As a result, glass is advantageous than silicon in this regard. In addition, glass has very good transparency so that it is amenable to optical detections.

Figure 1 shows a picture of a glass device, which consists of 6 channels with different length. The different length was for studying the effects of channel length on IEF separation resolution. The side channel is for possible introduction of labeling reagents if needed. All channels are about 50 μ m deep and 120 μ m wide.

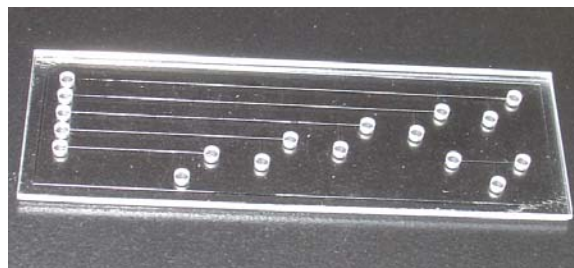


Figure 1. Picture of a device consisting of 6 IEF channels with different separation lengths.

Plastics offer advantages compared to silicon and glass (Boone, et al. 2002, Soper, et al. 2000). They are compatible to chemical and biological reagents, evidenced by many plastic lab wares. There are vast experience and development in manufacturing plastic parts such as compact discs (CD). The well-developed manufacturing process, as well as low cost of plastic materials, makes microfluidic devices inexpensive; thus we can afford to dispose a device after each use. Disposability is very important for diagnostic applications when cross-contamination among samples is not tolerated.

Figure 2 shows a scanning electron micrograph (SEM) of a plastic device made from poly(cyclic olefin). Also shown in the inset of the figure is the silicon wafer, which was used as a master to fabricate plastic parts. The result indicates that there is a good fidelity between the master and device.

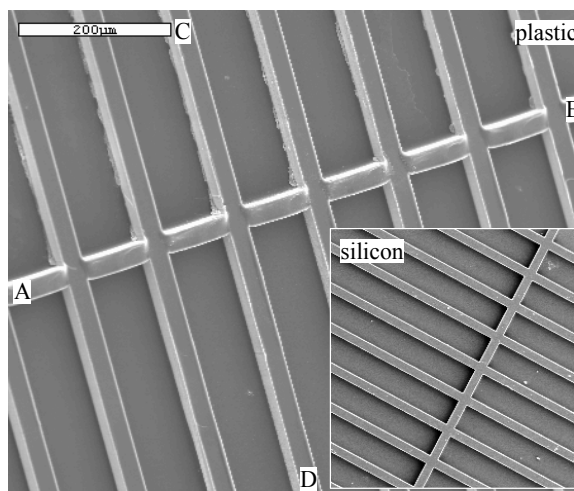


Figure 2. Part of a microfluidic device made from poly(cyclic olefin). The length of the bar in the figure is 200 μ m. (inset) A picture of silicon master used to mold plastic parts. The same area for both plastics and silicon is viewed.

The device in Figure 2 is designed for developing a microfluidic device for two-dimensional separations. The layout consists of one channel (AB) for IEF and parallel CD channels for PAGE. The channels for two dimensions are perpendicular to each other. The size of the device is 25 x 75 mm; 86 parallel CD channels were fabricated in this design. All channels are 30 μm wide and the space between channels is 90 μm .

3.2 Laser-Induced Fluorescence Imaging System

Among several detection methods used in microfluidic devices, laser-induced fluorescence (LIF) detection is the preferred approach due to superior sensitivity (Verpoorte, 2003). Most LIF systems are built for single-point detection, in which a laser beam continuously illuminates a fixed point along the separation channel to detect the signal when fluorescent molecules pass by. While single-point detection is sufficient for many applications, it is troublesome for other operations such as isoelectric focusing.

IEF is an electrophoresis technique for protein separations (Wu and Pawliszyn, 1995; Rodriguez-Diaz, et al., 1997; Tan, et al., 2002). Under an electrical field, a protein migrates and is then focused at a spot where its pH value is equal to the protein's isoelectric point (pI). Focusing takes place because the net charge of the protein is zero at pI and thus the protein will not move in the electric field. Since each protein has a unique pI, proteins can be separated along a pH gradient in a capillary or channel. If a single-point detector is used, the focused proteins must be transported to pass the detection point. Thus an addition step called mobilization (e.g., by hydrostatic flow) must be implemented, causing adverse effects on IEF performance (Rodriguez-Diaz, et al., 1997).

To eliminate mobilization step, we assembled an LIF imaging system to detect simultaneously all focused proteins by imaging the entire channel. Mobilizing focused proteins is then not needed, thereby reducing proteins' de-focusing and improving separation resolution. Wu and Pawliszyn (1995) developed a similar imaging system using a UV lamp. The advantage of UV imaging is that there is no need to label proteins, but only ~10% of amino acids in proteins have UV absorbance at ~280 nm. The benefit of LIF imaging is its sensitivity; LIF is typically several orders of magnitude more sensitive than UV absorbance.

The setup of our LIF imaging system is shown in **Figure 3**. An Ar⁺ laser beam (488 nm, 30 mW) is directed by two mirrors into a 20x beam expander, and subsequently through a cylindrical lens. The expander increases the laser beam diameter, and the cylindrical lens then converts it from a column of light to a line beam with minimum beam divergence. The resultant laser line is focused onto an IEF channel in a microfluidic device. A cooled, scientific grade, 14-bit charge-coupled device (CCD) camera collects the fluorescence emission after it passes through a bandpass filter (535 nm/50 nm or 585/40 nm). The pixel size of the CCD is 6.8 x 6.8 μm while the imaging area is 14.9 x 10 mm.

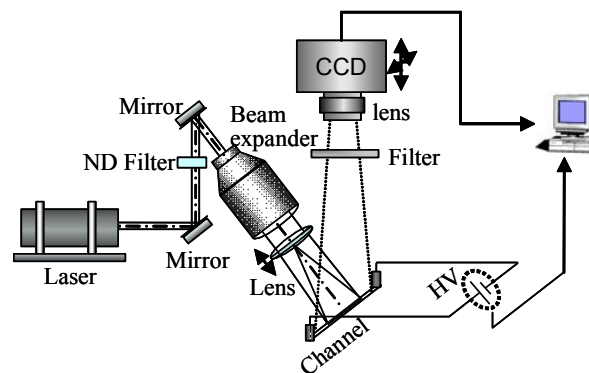


Figure 3. LIF imaging system for IEF in a microfluidic device.

We completed the characterization of the imaging system and established the guideline for operation. For example, we found that the effect of photobleaching is negligible when the laser power is less than 3 mW and the exposure time is less than 60 seconds for a fluorescein solution at a concentration of 1 μM or less. Using the guideline, we studied the detection limit of the imaging system using a series of concentrations of fluorescein solutions. The calibration curve between fluorescence intensity and fluorescein concentration is shown in **Figure 4**. The detection limit of the imaging system is determined to be ~1 nM fluorescein. The dynamic range of the linear relationship is over 5 orders of magnitude. This result was obtained by using the laser at 3 mW with an exposure time of 50 s. For concentrations higher than 1 μM when photobleaching cannot be neglected, a shorter exposure time (e.g., 5 s) must be used.

3.3 Effects of Separation Distance on Separation Resolution

Both theory and experimental results (Tan, et al., 2002) indicate that IEF resolution is independent of

the separation distance under certain conditions (e.g., no Joule heating). **Figure 5** shows the effects of the focusing length on IEF resolution in glass capillaries (Tan, et al., 2002). Capillaries with various lengths were filled with IEF gel containing 4% acrylamide, 9.2 M urea, 20% Triton X-100, 1.6% Bio-Lyte 5/7 ampholyte, 0.4% Bio-Lyte 3/10 ampholyte, 0.01% ammonium persulfate, and 0.1% TEMED. Protein pI standards consisting of 9 proteins and 14 pI bands were used. Gel was stained and imaged after focusing.

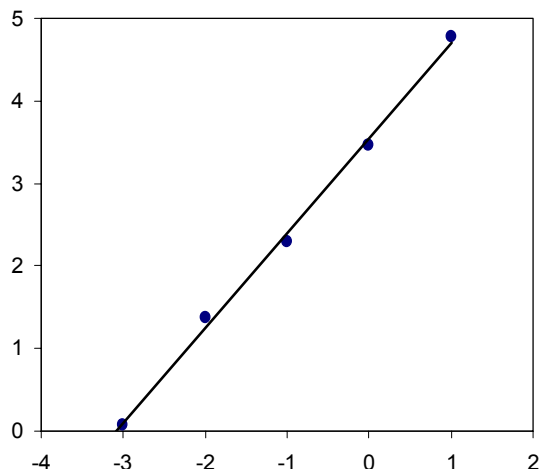


Figure 4. Calibration curve for fluorescein using the imaging system in Figure 3. Both axes are in log scale to show large dynamic range.



Figure 5. Effect of focusing length on separation resolution in glass capillaries (Tan, et al., 2002). The two arrows in the top and bottom electropherograms indicate two closely spaced proteins that are separated at all focusing lengths utilized. Gel was stained and imaged after focusing.

Two of the most closely spaced protein bands, indicated by arrows at the top and bottom of the figure, have a difference of 0.1 pH units in their isoelectric points (pI). These two proteins (hemoglobin A and myoglobin) were well separated in all cases, suggesting that there is no significant

change in the minimum pI difference required to resolve them. As a result, a short focusing length is advantageous, especially for a microfabricated device, since it should provide more rapid analysis without sacrificing the resolving power. We are in the process to verify this conclusion using the device in Figure 1.

3.4. Rapid Protein IEF in a Microfluidic Device

Using the device in Figure 1 and the imaging system in Figure 3, we demonstrated isoelectric focusing of proteins, including myoglobin, green fluorescent protein (GFP), and bovine serum albumin (BSA), and phycoerythrin. **Figure 6** shows an image of separated B-phycoerythrin (BPE), R-phycoerythrin (RPE). IEF was carried out using polyacrylamide linear polymer as a separation medium containing carrier ampholyte (pH 3-10). The length of the channel used is 42 mm long while the electric voltage was 1000 V. BPE and RPE can be excited by the Ar⁺ laser and they have the maximum emission at 575 nm. The concentration of each protein was 2 ng/ μ L.

The typical separation time is between 7 to 40 minutes, depending on the voltage and the length of channel used. The separation time for Figure 6 is 11 minutes. The analysis time is one order of magnitude faster than the typical time of conventional IEF experiments using bench-top slab gel electrophoresis apparatus. It is also faster than IEF in a typical capillary electrophoresis instrument, mainly due to elimination of the mobilization step.

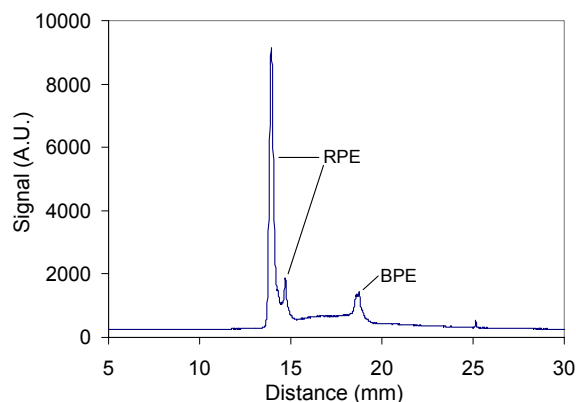


Figure 6. IEF electropherogram of 2 proteins in a microfabricated device in Figure 1. The IEF pattern was obtained by imaging the channel directly using the setup in Figure 3. The distance in X axis is calculated from the location of the pixels of CCD camera. Two peaks for RPE are likely due to protein's heterogeneity.

4. CONCLUSIONS

In summary, both glass and plastic microfluidic devices have been fabricated for protein separations. An LIF imaging system has been built to image an entire channel in an IEF device. The whole-channel imaging is advantageous over a single-point detector due to elimination of mobilization step. The detection limit of the imaging system was determined to be about 1 nM fluorescein. Using the device and the imaging system, we are able to perform IEF separations of proteins within minutes, compared to hours needed in traditional bench-top instruments.

ACKNOWLEDGEMENT

We acknowledge the financial support from McKnight Brain Institute, the startup fund from the University of Florida, and the grants from National Aeronautics and Space Administration (NASA) via UF Space Biotechnology and Commercial Applications Program, and UCF-UF Space Research Initiative.

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