SANDIA REPORT

SAND2006-5873
Unlimited Release
Printed October 2006

Development of a High-Throughput Microfluidic Integrated Microarray for the Detection of Chimeric Bioweapons

Jay A.A. West, Kyle W. Hukari, Brent Satterfield, Timothy Sheppod, and Gary Hux

Prepared by
Sandia National Laboratories
Albuquerque, New Mexico 87185 and Livermore, California 94550

Sandia is a multiprogram laboratory operated by Sandia Corporation,
a Lockheed Martin Company, for the United States Department of Energy's
National Nuclear Security Administration under Contract DE-AC04-94-AL85000.

Sandia National Laboratories

Issued by Sandia National Laboratories, operated for the United States Department of Energy by Sandia Corporation.

Available to DOE and DOE contractors from
U.S. Department of Energy
Office of Scientific and Technical Information
P.O. Box 62
Oak Ridge, TN 37831

Telephone: (865) 576-8401
Facsimile: (865) 576-5728
E-Mail: reports@adonis.osti.gov
Online ordering: http://www.doe.gov/bridge

Available to the public from
U.S. Department of Commerce
National Technical Information Service
5285 Port Royal Rd
Springfield, VA 22161

Telephone: (800) 553-6847
Facsimile: (703) 605-6900
E-Mail: orders@ntis.fedworld.gov
Online order: http://www.ntis.gov/help/ordermethods.asp?loc=7-4-0#online

SAND2006-5873
Unlimited Release
Printed October 2006

Development of a High-Throughput Microfluidic Integrated Microarray for the Detection of Chimeric Bioweapons

Jay A.A. West, Kyle W. Hukari, Brent Satterfield, Timothy Sheppod, and Gary Hux

Microfluidics Department
Sandia National Laboratories
P.O. Box 969
Livermore, California 94550-MS9292

Abstract

The advancement of DNA cloning has significantly augmented the potential threat of a focused bioweapon assault, such as a terrorist attack. With current DNA cloning techniques, toxin genes from the most dangerous (but environmentally labile) bacterial or viral organism can now be selected and inserted into robust organism to produce an infinite number of deadly chimeric bioweapons. In order to neutralize such a threat, accurate detection of the expressed toxin genes, rather than classification on strain or genealogical decent of these organisms, is critical. The development of a high-throughput microarray approach will enable the detection of unknowns chimeric bioweapons. The development of a high-throughput microarray approach will enable the detection of unknown bioweapons. We have developed a unique microfluidic approach to capture and concentrate these threat genes (mRNA's) upto a 30 fold concentration. These captured oligonucleotides can then be used to synthesize in situ oligonucleotide copies (cDNA probes) of the captured genes. An integrated microfluidic architecture will enable us to control flows of reagents, perform clean-up steps and finally elute nanoliter volumes of synthesized oligonucleotides probes. The integrated approach has enabled a process where chimeric or conventional bioweapons can rapidly be identified based on their toxic function, rather than being restricted to information that may not identify the critical nature of the threat.

This page intentionally left blank.

Contents

This page intentionally left blank.

Figures

This page left blank intentionally

Introduction

In the last fifteen years, technologies surrounding DNA cloning have truly accelerated. In the present day, genes from almost any organism (human, bacterial or 'Viral) can be copied and inserted into another organism. This is a common practice when researchers are attempting to identify whether the function of an unknown gene is related to a disease (i.g. cancer, Alzheimer's, heart disease). The bioengineered organisms produced from these experiments are often termed "chimeras", named for the mythical Greek monster with the lion's head, goat's body, and serpents' tail. This technology, applied with malevolent intentions, poses a significant risk to national security. The production of such organisms as bioweapons has been reported [I]. The production of the organisms can be achieved with relative ease and, in the coming future, may not require Ph.D. level training. Even today, commercialized kits complete with directions can be purchased for production of such organisms.

In the last five years, the development of DNA gene array or "microarray" technology capable of detecting thousands of genes in single experiment has rapidly advanced. This technology allows for the detection of chimeric bioweapons; however, few if any, microarrays with capability to detect biotoxin genes exist. Complicating the use of microarray for biotoxin detection is the synthesis of cDNA probes employed to detect the expressed genes of an organism. In its current format the synthesis process is long and tedious. Samples isolated for analysis are exquisitely sensitive to sample degradation, and the cDNA probe synthesis takes a minimum of two days.

Microarray analysis of gene expression hinges on the isolation of those expressed genes or "mRNA". The current process by which mRNA is isolated is the rate limiting step to performing a successful microarray analysis. The system has two major problems for use as a detector for bioweapons. First the process to capture the mRNA is labor intensive and requires a minimum of four hours to complete. Second, these isolated mRNA are extremely sensitive to degradation from a class of enzymes known as RNases. In order to overcome these issues, a microfluidic channel will be filled with a polymer based material that has the ability to selectively capture mRNA. Replacing test tubes with a closed microfluidic system will solve both of these problems. Currently isolation of mRNA is completed on the bench top through a series of steps, which include transferring the sample multiple times to new test tubes, containing different reagents. By packing a microtluidic channel with the polymer capture material. we will be able to change reagents by altering flows through the channel. This advance reduces time, and will virtually eliminate sample degradation. RNA in eukaryotic (mammal) cells is degraded by an enzyme called RNase. RNases are ubiquitous throughout the body and important for sample handling, since they are found on the surfaces of skin, hair and dust. Thus, isolated mRNA exposed to air for long period of time will be exposed to RNase, . and be degraded. Capturing mRNA's in a microfluidic channel will significantly reduce the exposure of the sample to RNases, and will yield improved purity and integrity. Microfluidic control is central to achieving the aforementioned goals. Recent work has been focused toward developing polymer architectures for effecting microfluidic control [2-4].

The goal of this project is to streamline this process to capture biotoxin genes, and make detection of a complex chimeric bioweapon in a period of minutes. To accomplish this goal, we have designed three experimental approaches. First, we will demonstrate that expressed genes from chimeric bioweapons can be captured, purified and concentrated on a microfluidic chip; second we will synthesize oligonucleotide copies (cDNA probes) of the captured genes using

recently developed polymer monolith microvalves. Third, the cDNA probes will be directed onto a microarray chip designed to detect biotoxin genes. This report will highlight the work on development of a microfluidic devices for microfluidic gene arrays for rapid genomic profiling, Preliminary results for preconcentration of mRNA in a porous polymer bed supplemented by a numerical analysis of a dimensionless anlaysis of the effect of heat energy convection on a microfluidic chip power consumption.

1. Alibek, K., *Biohazard,* ed. S. Handlernan. 1999, New York: Random House. 320.

2. Rehm, J.E., T.J. Shepodd, and E.F. Hasselbrink, in *Micro Total Analysis Systems.* 2001, Kluwar Academic. p. 227-229.

3. Kirby, BJ., TJ. Shepodd, and E.F. Hasselbrink, J chromatogr A, 2002. under review.

4. Hasselbrink, E.F., T.J. Shepodd, and J.E. Rehrn, Anal Chern, 2002. under review.

Chapter 1

1. Microfluidic gene array for rapid genomic profiling

Sensitive, accurate detection and portable identification of biological agents is critical to our ability to avert wide spread casualties from a bio-terrorist threat. The use of Gene or "micro" arrays has become a widespread bioanalytical technique for genomic profiling. Recent advances in microarray technology have led to diagnostic applications that are currently being developed for the detection of biological pathogens. Two major drawbacks to the current experimental format in which these assay are performed are the degradation sensitive long sample processing time required for probe generation and the extended time requirement for the aforementioned probes to be hybridized to the target DNA on the slide surface. The goal of these studies was to develop an array based microfluidic chip and platform that can be adapted to a hand portable device. We have also focused our efforts to develop a platform that is easily customized for rapid assay development in a wide application space. This will be accomplished by integrating two newly emerging and maturing technologies: microarrays and microfluidics. Microarray devised devices were first described in 1989 by Ekins et. al. [1] where antibodies were affixed to a solid substrate. Subsequent publications Fodor [2] and Schena [3] set the stage for the explosion of DNA, gene or Micro array technology. Since these landmark publications, various chip design platforms including oligonucleotide, EST, and cDNA have been developed along with an ever-expanding number of sources of content or synthesized genes for detection of a particular agent or expressed gene. These array technologies vary widely in their format as well as their application. High density oligonucleotide arrays, based on Fodor's publication [2] later became the base technology for Affymetrix (patent # 5,445,934). These oligonucleotide formats have extremely high density, with the capacity to perform measurements on over 350,000 DNA elements in a single experiment. While this technology has great measurement capacity, due the photolithographic manufacturing process using multiple masks, it is less well suited for integration with microfluidic systems.

A better suited microarray platform for microfluidic integration in a custom or robotically spotted cDNA microarray first described by Schena [3]. Sensitive, accurate detection and portable identification of biological agents is critical to our ability to avert wide spread casualties from a bio-terrorist threat. The use of Gene or "micro" arrays has become a widespread bioanalytical technique for genomic profiling. Recent advances in microarray technology have led to diagnostic applications that are currently being developed for the detection of biological pathogens. Two major drawbacks to the current experimental format in which these assay are performed are the degradation sensitive long sample processing time required for probe generation and the extended time requirement for the aforementioned probes to be hybridized to the target DNA on the slide surface. The goal of these studies was to develop an array based microfluidic chip and platform that can be adapted to a hand portable device. We have also focused our efforts to develop a platform that is easily customized for rapid assay development in a wide application space. This will be accomplished by integrating two newly emerging and maturing technologies: microarrays and microfluidics. Microarray devised devices were first described in 1989 by Ekins et. al. [1] where antibodies were affixed to a solid substrate. Subsequent publications Fodor [2] and Schena [3] set the stage for the explosion of DNA, gene or Micro array technology. Since these landmark publications, various chip design platforms including oligonucleotide, EST, and cDNA have been developed along with an ever expanding number sources of content or synthesized genes for detection of a particular agent or expressed

gene. These array technologies vary widely in their format as well as their application. High density oligonucleotide arrays, based on Fodor's publication [2] later became the base technology for Affymetrix (patent # 5,445,934). These oligonucleotide formats have extremely high density, with the capacity to perform measurements on over 350,000 DNA elements in a single experiment. While this technology has great measurement capacity, due the photolithographic manufacturing process using multiple masks, it is less well suited for integration with microfluidic systems. A better suited microarray platform for microfluidic integration in a custom or robotically spotted cDNA microarray first described by Schena [3]

This array format has demonstrated good correlation to standard quantitative genetic assays such as northern blot analysis [4]. Additionally, using a robotic spotter for array construction offers the capability to deposit high density DNA probes in complex architectures, such as microfluidic systems that also offer the flexibility to routinely alter content of the device to optimize assay conditions rapidly. Low density microarrays have also been recently been developed [5-7]. These devices make use of a plastic microfluidic channel to effect rapid target to probe hybridization times. The current studies were designed to extend this capability to develop on-chip sample purification and rapid sample labeling. We also aimed to produce a device that can perform these rapid hybridization reactions in a high density microarray format. In order to realistically manipulate this technology for practical purposes outside the laboratory, it is crucial to first determine the most efficient method of hybridizing a target sequence to a known probe sequence. In these experiments, different techniques of labeling mRNA and cDNA, including those with Cye dyes and Universal Labeling System (ULS) dyes, were performed. In order to further increase the speed in which these type of analyses can be performed, we have designed and fabricated a microfluidic microarray that will both decrease sample probe preparation time as well as target to probe hybridization time. The development of such devices will allow for the miniaturization of microarray instrumentation and make hand-portable microarray techonlogies a reality. We first focused on the fabrication of the high density microfluidic microarray that has the ability to detect thousands of individual elements in real-time. We have optimized the design of this chip to affect reduced hybridization times of target oligonucleotides, eliminate diffusion effects to optimize chip-based nucleic acid hybridizations, and to maximize signal to noise ratios in order to reduce the optical limit of detection. We have further developed porous polymer monolith material for the trapping of mRNA. Using this polymer we demonstrate the rapid trapping and release of purified mRNA. To reduce the time required for sample processing, we evaluated several commercial nucleic acid labeling protocols. These experiments demonstrate that it is possible to generate hybridization ready samples in less than twenty minutes. Finally we have developed the first generation fluidic and detection platform. This platform will allow for easy use of the fabricated microfluidic chips, and is capable of both performing fluidic control elements as well as optical detection. We are currently integrating thermocontrolling capability into the device. In this manuscript we demonstrate the individual processes, such as sample trapping and rapid hybridization, in order to translate these technologies to a completely integrated hand-portable device.

1.2 Materials and Methods

1.2.1 Reagents and supplies:

The following chemicals were used at various steps of experimentation and were used without further purification. 3-Glycidaloxypropyldimethoxymethylsilane, 99% pure Hexanes, Ethanolamine, sodium dodecylsulfate (SDS), NaCl, and Ethanol were purchased from Aldrich Chemical Co. DEPC water, 20X SSC, 20X SPPE buffers were purchased from Invitrogen. Rat oligo test set, spotting buffers, and epoxy coated microscope slides were purchased from MWG. PCR clean-up kits were purchased from Qiagen. The microfluidic manifold was constructed on site using Delrin and aluminum stock. O-rings were purchased from Apple Rubber. Microfluidic fittings made with PEEK were supplied by Sandia National Labs. Ulysis DNA labeling kits were purchased from Molecular Probes (Eugene, OR.). CyScribe kits for first strand synthesis and cytosine labeled Cye dyes were purchased from Amhersam biosciences. All other chemicals purchased were reagent grade or better.

1.2.2 Microfluidic chip fabrication:

Devices were optimized by experimental modeling and fabricated using standard photolithography techniques. Microfluidic chips were fabricated in fused silica which has the advantage of detecting fluorescent spots with a high signal/noise ratio [8]. Fabrication with fused silica allows for the use of multiple etch depths to normalize pressure gradients and reduce the diffusion distance for target analytes to the probe surface. This reduces hybridization time dramatically. Integration of microarrays with a microfluidic device was accomplished by designing and fabricating areas for performing sample clean-up, amplification and/or hybridization on a single chip. The construction of these arrays was accomplished using a multiple depth photolithographic etch process on two separate four inch quartz wafers which were subsequently bonded at 1100oC. Spotting of oligonucleotide probes to the microfluidic microarray chips is achieved by use of a robotic spotter that deposits the probes on to a custom addressable array. The robotic spotter has micron resolution to position the spotting pens within the microchannels. Further details of robotic spotting are provided in U.S. Patent Application Serial No. 10/701,097, "Microfluidic Integrated Microarrays for Biological Detection", filed November 4, 2003. After spotting, the microfluidic chip is cleaned and prepped following standard procedures (0.1% sarcosine, next 3x SSC, rinse in deionized water, and immersion in ice cold ethanol). Sample oligonucleotide probes, for example commercially available rat DNA probes from MWG Biotech (High Point, North Carolina) are attached to the microarray surface according to these processes.

1.2.3 Fluidic hardware and detection platform development:

Chip manifolds and supporting hardware were designed and fabricated to interface the microfluidic chips using an O-ring face seal. Detection of DNA microarray spots on the microfluidic chip was accomplished using a combination of light delivery using fiber waveguides with a CCD array detection platform for detection of fluorescent spots on the array surface. Using AA battery power to drive an 80mW LED, we are able to deliver light to the chip using a custom fabricated fiber bundle waveguide. This waveguide illuminates the channel where the DNA microarray is located. Oligonucleotide probes were then deposited in the open microchannels using a conventional arraying robot which was controlled by a custom script to

allow the deposition of probes in specific locations. These open channels containing the covalently linked oligonuceotides were then sealed with a polymer film in order to flow reagents to each spot on the device. The microarray is sealed using pressure or adhesion with an optically transparent chemically resistant plastic film. The microarray can be adhesively sealed by treating a chemically resistant clear plastic sheet (e.g., PDMS) with plasma oxidation to activate surface functional groups. Amineterminated silane was then applied to the plasma oxygen activated surface to enable an epoxy-amine sealing reaction between the amine coated surface of the plastic film and the epoxy coated fused silica chip. The plastic surface is then bound to the tops of the ridges between the channels and top side of the chip. Chips are housed in a manifold outfitted with o-ring seals designed to allow for one step chip alignment and to facilitate fluid connections to the outside environment. A Basler A102fc color CCD camera with a 2:1 pulled fiber optic was purchased from Videoscope International. Capillary tubing was purchased from Polymicro, inc.

1.2.4 Results for platform development

The microfluidic chips were designed to accommodate on-chip sample purification, labeling and array analysis. To accomplish this, specific regions for sample concentration and arraying are designed into a single device. Using this process we were able to create areas on a compact device (2.5x3.1cm), which can accommodate UV, patterned monoliths for sample processing (figure 1 B&C) and an array-spotting surface for post-fabrication DNA arraying. We designed (figure 1A) and fabricated (figure 1B) the first generation of microfludic microarrays. In this process, two wafers are etched then, bonded to produce the microfluidic chips. Fabricated Chips (Figure 1B) contained an open channel that is etched 15 um into the top surface of the microfluidic device (Figure 1B&C). This design allows spotting of DNA probes on the fabricated chip in an open channel. This architecture serves several purposes. Critical to the design of these chips is the ability to robotically spot DNA detection (probes) on the channel array surface after fabrication of the microfluidic chip. The shallow serpentine micro channel (15um height x 300 um wide) greatly reduces the hybridization time by minimizing the diffusion time of the sample and fluidically directing the target past each surface probe. The second set of channels are closed and are located between the two wafers. These channels house a monolith material that is used for sample purification.

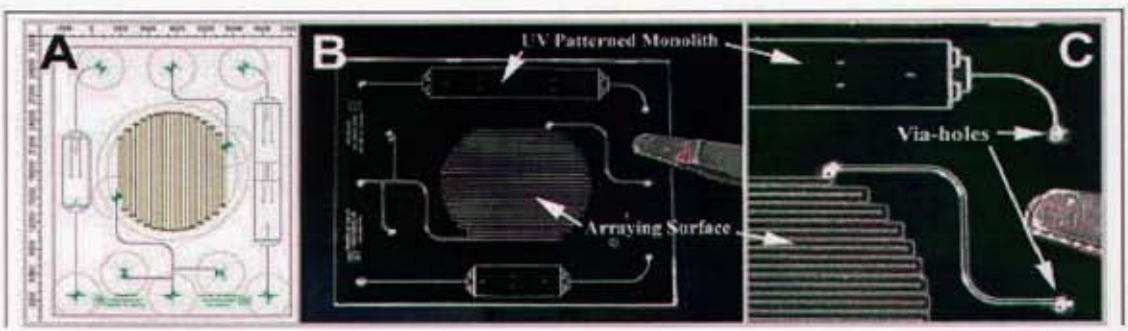

Figure 1.1. Fabricated microfluidic microarray. Masks (A) are designed to optimize hybridization conditions such the entire injected sample passes through the serpentine array surface (B&C) which is spotted post-fabrication. Using a double wafer etch process areas for containing monoliths are isolated from the array surface.

The Oligonucleotide trapping monolith is composed mainly of gyclidyl methcrylate (GMA) which has a porous structure with roughly 1-3mm pores (Figure 2A). The Gycidyl (epoxide) chemistry allows for post functionalizationof the polymerized monolith, as seen in Figure 2B&C. Polymerized monolith (Figure 2B), is notfluorescent at 488nm (blue light). In contrast when this monolith is post-functionalized (C) with any amine containing molecule (Oregon Green), the monolith appears as a fluorescent signal housed in the capillary (Figure 2B).

Figure 1.2. Porous polymer monolith are photoinitiated on-chip (A) and bind directly to channel walls. These formed monoliths have no native fluorescence (B), but can be post-functionalized to incorporate any primary amine containing molecule including fluorescent dyes (C), oligonucleotides, proteins, etc.

In order to trap oligonucleotitides the glycidyl methacryalte polymer was functionalized using an amine terminated oligodT which contained a C6-linker molecule. Once the monolith was polymerized in the channel the oligo dT solution was introduced in a Tris buffer pH 8.3. The monolith was then heated to 60°C for thirty minutes in a humid chamber. Unbound oligo dT was then flushed out using additional buffer solution. mRNA's (in a TE/SSC buffer) were then isolated by using a syringe and syringe pump to deliver flow through the porous polymer monolith. The hybridization in the monolith is rapid, occurring in less than two minutes (figure 3A). After a secondary flushing step using the binding buffer, which does not result in decreased fluorescence, the mRNA was eluted using the TE buffer as seen in Figure 3B.

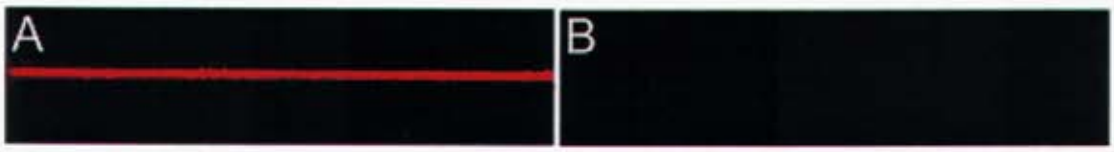

Figure 1.3. Oligonucleotide trapping monolith: These monoliths were functionalized with an Oligo dT. After functionalization the labeled mRNA was dissolved in the binding buffer was introduced to the column, which became bound (A). After a flushing step (not shown) the trapped olignucleotide was eluted from the column using a salt free TE buffer. This entire process was complete in two minutes.

To determine the optimal strategy for fluorescently labeling nucleic acids we tested three commercial protocols for use with our system. Depending on the labeling strategy the the intensity of the probe hybridization on the microarrays appears to have variability, while the patterns of gene expression appear identical between labeling platforms (data not shown). In general the direct incorporation of Cye labeled oligonucleotides produced more consistent labeled cDNA's. However, as previously reported their appeared to be significant bias in the incorporation of the Cy3 vs. Cy5 dye set into the synthesized cDNA's. We also found that this labeling technique is not well suited for the labeling of oligonucleotides for the rapid detection of genetic signatures. In contrast, we found that the direct labeling of mRNA was an efficient technique for obtaining labeled full length nucleic acids. We found that the expression patterns

between synthesized cDNAs (figure 4B) and direct labeled mRNA (Figure 4C) were identical. In addition, compared to the first strand (cDNA) method, the direct labeling of mRNA can be accomplished (including clean up) in approximately 10 minutes.

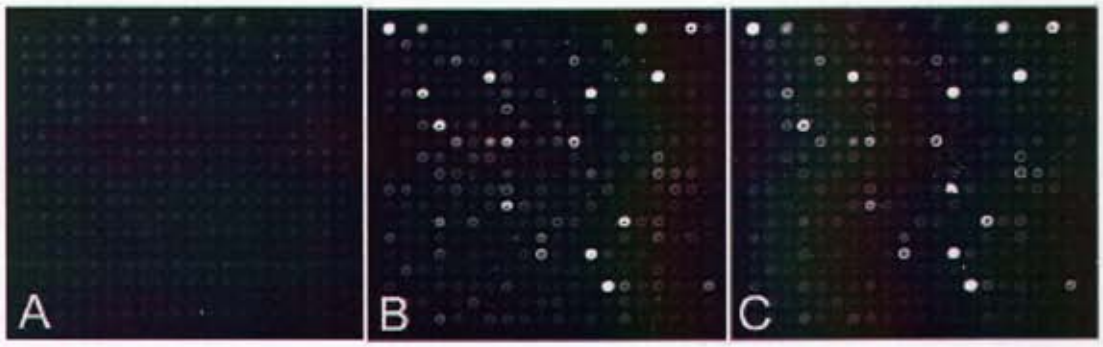

Figure 1.4. Comparison of first strand cDNA synthesis to direct mRNA labeling. Compared to control (A), 0.581ug alexa fluor 532 labeled synthesized cDNAs generated a robust and easily detectable signal (B). This was also the case for the direct labeled mRNAs as 0.372ug alexa fluor labeled mRNA generated a highly detectable gene expression profile (C).

In order to detect the hybridization of target to probe on the array surface a detection platform that utilizes a second collection fiber bundle array is place on the array surface (Figure 5A). When the microfluidic chip is illuminated the pattern of the array surface is easily observable through the fiber bundle (figure 5B). When this collection fiber bundle is mated to a color CCD camera the CCD camera is placed adjacent to the fiber bundle, which is placed on the plastic film for imaging the microarray (Figure 5A). Using this system it is possible to detect fluorescent spots on the array surface or presence of fluorescent dyes in solution in the channel. To observe the hybridization of mRNA in the microfluidic microarray chip, the chip was first spotted with oligonucleotides. The chip was then sealed using a compression plug which sealed a polymer film on the fused silica microarray chip. The labeled mRNA was pre-heated to 90°C, then introduced to the array which was held at a constant temperature of 42°C. The mRNA solution was then allowed to stand in the array channel for 5 min, after which addition aliquot of solution was passed into the array. After the second incubation period was complete the channel was imaged. We found the images collected prior to the hybridization (figure 5C) did not display any detectable mRNA target probe hybridizations. In contrast in when fluorescently labeled mRNA were passed through the channel were able to detect the hybridization of the target mRNA to the surface probe in less than twenty minutes (Figure 5D).

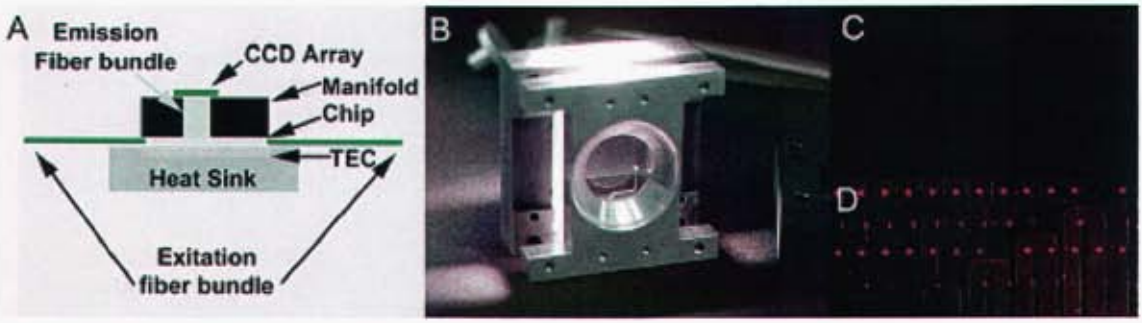

Figure 1.5. Design, fabrication and use of the microfluidic genearray platform. A manifold was designed to incorporate the use of excitation source, emission light collection using a CCD camera and thermocycling apparatus (A). The manifold and associated parts were fabricated to house the microfluidic chip and associated optics (B). Using this system to perform on-chip hybridizations we found mRNA labeled with Alexa fluor 647 annealed to the non-fluorescent spotted microfluidic genearray (C) in less 20 minutes (D).

Our results demonstrate the feasibility of fabricating high density microfluidic genearray chips with the capability of performing rapid genomic profiling. The chips are simple to use and easily customizable allowing for the rapid alteration of probe content. In addition the entire assembly is miniaturized and can be packaged in a hand-portable platform for field analysis of biological agents. Previous work by Lenigk, et.al demonstrated the feasibility of the production of microfluidic genearrays [7]. In these studies the number of probes was limited to capability of the device used to deposit oligonucleotide probes in the microfluidic channels. In the present study we have developed a custom spotting scripting software program that allows for the deposition of up to 4000 individual elements in selectable positions along the microfluidic channel. As demonstrated in the previous studies [6, 7], the use microfluidics dramatically decreases the time required for the hybridization to the oligonucleotide probes in the microchannel. In our studies we have seen hybridization times of less than twenty minutes. In addition we have developed an integrated platform for the easy use of the microfluidic chips. This system includes the development of sample trapping monolith polymers which have the ability to trap and elute fluorescently labeled mRNA in less than two minutes. These polymers were similar to those reported for use as a separation matrix for polycyclic aromatic hydrocarbons [9-11]. During the fabrication of the polymers in this study we took great care in preserving the glycidyl functionality of the polymerized monomer. After this initial polymerization a second reaction to functionalize the polymer was conducted. We found that we were able to extensively functional the surface of this polymer with any primary amine containing molecule. In this study this was limited to amine terminated fluorescent dyes, and Oligo dT. However, the flexibility of this polymer will make the functionalization of the surface chemistry capable of accepting any selective trapping molecule. Such chemistry will allow for the use of these columns as pre-selection concentrators for enrichment of a subset of genes of interest in following studies. We further evaluated the ability to rapidly label nucleic acids using a direct labeling method. We found that the direct labeling method held several advantages over the current standard first strand synthesis of cDNAs. First, because the reaction is simple, we found far fewer difficulties with contamination of labile mRNA samples. In addition, the method was far more rapid, only requiring a maximum of twenty minutes compared to the usual four-six hour procedure required for first strand cDNA synthesis. Finally, we found that the using this

enzyme free system was far easier to integrate with our assay platform as the labeling of trapped mRNA samples can be accomplished by only changing a single solution in the polymer monolith. Detection of the amplified microarray spots can be carried out using optical fiber bundle arrays, that both transmit and collect light. These fiber bundles deliver high intensity light from low power consuming and robust LED's. Detection is carried out with a high resolution CCD array capable of collecting signal from all elements on the microarray in parallel. The resolution of this CCD array makes it possible to detect all spots in parallel without the need for focusing optics. The CCD array/fiber bundle is be placed directly onto the arraying surface of the chip (figure 1B). This capability will make it possible to detect the presence or absence of thousands of threat genes in *real-time*. In summary, we demonstrate a first generation integrated microfluidic high density microarry platform (up to 4000 probes), that has the ability to house rapid sample preparation, hybridization, and detection of genomic samples. This allows sample preparation of less than 40 minutes including; direct labeling of mRNA's, trapping with porous monolith and elution for hybridization. Most importantly this device demonstrates the hybridization of mRNA's in a microfluidic channel in less than 20 minutes. In the future we plan to perform real time detection of probes using the fully integrated handheld portable device.

1.3 References

1. Ekins, R., F. Chu, and E. Biggart, Development of microspot multi-analyte ratiometric immunoassay using dual fluorescent-labeled antibodies. Anal. Chim. Acta., 1989. 227(1): p. 73-96.
2. Fodor, S.P., et al., Light-directed, spatially addressable parallel chemical synthesis. Science, 1991. 251(4995): p. 767-73.
3. Schena, M., et al., Quantitative monitoring of gene expression patterns with a complementary DNA microarray [see comments]. Science, 1995. 270(5235): p. 467-70.
4. Bartosiewicz, M., et al., Development of a toxicological gene array and quantitative assessment of this technology. Archives of Biochemistry and Biophysics, 2000. 376(1): p. 66-73.
5. Liu, Y. and C.B. Rauch, DNA probe attachment on plastic surfaces and microfluidic hybridization array channel devices with sample oscillation. Anal Biochem, 2003. 317(1): p. 76-84.
6. Liu, R.H., et al., Hybridization enhancement using cavitation microstreaming. Anal Chem, 2003. 75(8): p. 1911-7.
7. Lenigk, R., et al., Plastic biochannel hybridization devices: a new concept for microfluidic DNA arrays. Anal Biochem, 2002. 311(1): p. 40-9.
8. West, J.A.A. and B.J. Wiedenman, Development of a microfluidic integrated microarray for detection of chimeric biological weapons., in Sand# 8433. 2003, Sandia National Laboratories: USA.
9. Fintschenko, Y., et al., Chip electrochromatography of polycyclic aromatic hydrocarbons on an acrylate-based UV-initiated porous polymer monolith. Fresenius J Anal Chem, 2001. 371(2): p. 174-81.
10. Ngola, S.M., et al., Conduct-as-cast polymer monoliths as separation media for capillary electrochromatography. Anal Chem, 2001. 73(5): p. 849-56.
11. Yu, C., et al., Monolithic porous polymer for on-chip solid-phase extraction and preconcentration prepared by photoinitiated in situ polymerization within a microfluidic device. Anal Chem, 2001. 73(21): p. 5088-96.

This page intentionally left blank.

Chapter 2

2. Microfluidic polymeric constant flow-through mRNA sample preconcentrator

Sample preparation can be divided into four major steps, including 1) separation of sample from matrix through cell lysis and filtration 2) sample analyte preconcentration 3) derivatization and 4) biochemical pretreatment. The actual methodologies in implementing these steps vary according to the sample type and end analysis.[5] mRNA as a polynucleotide analyte has particular appeal. It represents the active portion of DNA, or the part of DNA that is actively engaged in phenotypic expression in an organism.[6] It is readily extracted from the eukaryotic cellular matrix due to the presence of a polyadenaline tail, thereby limiting competitive reactions in later analysis.

Most specific mRNA extraction is based on the interactions between a sequence of oligo dT's and the poly-A tail of mRNA. The use of functionalized cellulose to selectively extract polynucleotides was developed early in the 1960's.[7-10] The integration of polythymine deoxynucleotides specifically for the purpose of mRNA purification with cellulose columns, cellulose powders, polymeric beads, and magnetic beads were innovations that followed.[11, 12]

Methods for Polynucleotide extraction in microfluidic devices have been reported primarily using materials for nonspecific binding such as silica beads,[13] teflon membranes,[14] silica posts,[15] and gels with immobilized beads.[16, 17] These forms of extraction report somewhat higher efficiencies and faster extraction times, but none of them have been optimized for specific mRNA extraction. Further, the fabrication of the devices requires labor intensive packing of beads or other material and often results in irreproducible construction. A particular difficulty with the use of these materials is the absence of a stable covalent bond between the deposited material and the channel walls, which requires the use of frits to contain the deposited material. An additional difficulty for these methods is the need for multiple solvents or reagents, which complicate the use of the devices in robust field environments.

Examples of specific mRNA preconcentration and purification on chip are rare. Jiang and Harrison previously reported the ability to integrate mRNA isolation using magnetic beads on a microfluidic device.[18] In these studies the mRNA is first isolated using magnetic beads in a microfluidic chip, subsequently removed from the stream, and finally, the residue of nonhybridized material is washed away. This reported device requires 20 minutes of flow time and offers 26% efficiency.[18]

Recently, functionalized photo-polymerizable monoliths have been used as an alternative to traditional sample preparation methods for chemicals,[19] polypeptides[20, 21] and polynucleotides,[22] most of these being used in conjunction with capillary electrochromatography or HPLC. These materials have been reviewed extensively by Peterson and Svec.[23, 24] They provide high surface area for adsorption of the analyte of interest,[25] variable pore size and porosity based on concentration and type of porogenic solvent,[19] and are easily and cost-effectively created inside of microfluidic channels through UV-light exposure. They also exhibit good surface adhesion and make even contact with channel walls.[26]

Despite these advances, to date there are no sample preparation devices which truly take advantage of current technology and trends for preconcentration and purification of mRNA. In this paper, we report an mRNA sample preconcentration method which allows for facile incorporation into a microfluidic detection unit, and furthermore allows for constant flow through binding kinetics with the use of a single solvent. Using a photo-initiated monolith that is polymerized and functionalized in-situ, we were able to take advantage of the large surface area and controllable pore size inherent to monoliths. High efficiencies and fast hybridization times predict that the oligonucleotide functionalized porous polymer monoliths will be an ideal method for mRNA sample preparation in microfluidic devices.

2.1 Experimental

2.1.1 Reagents and supplies:

The following chemicals were used at various steps of experimentation and were used without further purification. 3-Glycidylpropyldimethoxymethylsilane(GMA), Ethyleneglycoldimethacrylate (EGDMA), 99% pure Hexanes, Ethanolamine, sodium dodecylsulfate (SDS), NaCl, and Ethanol were purchased from Aldrich Chemical Co. DEPC water, 20X SSC, 20X SPPE buffers were purchased from Invitrogen. PCR clean-up kits were purchased from Qiagen (Valencia, CA). The microfluidic components were constructed on site using Ultem (polyetherimide, GE Plastics, Southfield, MI). O-rings were purchased from Apple Rubber. Microfluidic fittings made with PEEK were supplied by Sandia National Labs or purchased from Upchurch Scientific (Oak Harbor, WA). Ulysis DNA labeling kits were purchased from Molecular Probes (Eugene, OR.) and used to label mRNA as suggested by the manufacturer. All other chemicals purchased were reagent grade or better.

2.1.2 Porous polymer monoliths:

Trapping of target oligonucleotides was accomplished using a UV cured porous polymer monolith which was fabricated in a fused silica capillary. Briefly, capillaries (Polymicro, Technology, Phoenix AZ) were pretreated with a mixture of 50% v/v distilled deionized water, 30% glacial acetic acid, and 20% Z-6030 (Dow Corning Midland MI). The solution was used to fill the capillaries which stood for an hour. Chips were then flushed with filtered buffer (10mM NaH_2PO_4 Buffer, pH 7.0) and dried. Monoliths are readily created by choosing a crosslinker, a functional monomer, a porogenic diluent, and a photo-initiator. For the se experiments the monomer mixture was optimized to contain (added in this order) 12.5 % v/v 10mM NaH_2PO_4, pH 7.0, 12.5 % ethyl acetate, 40% methanol, 10.5 % GMA, 24.5 % EDGMA, also containing either 2.5 mg Irgacure (Ciba Specialty Chemicals, McIntosh, AL) or 5.0 mg Azobisisobutyronitrile (AIBN). The solution was vortexed until the initiator was solublized and was passed through a hydrophobic filter. Prior to polymerization the capillary is flushed with the sodium phosphate buffer. The capillaries are then filled, and *photo* initiated at 365 nm using either a UV crosslinking oven (Spectronics Corporation, Westbury, NY) or using an Optilux™ 501 UV dental curing gun. Devices and capillaries were masked where appropriate.

2.1.3 Functionalizing PPM

After porous polymer monoliths are formed in the capillary, the material is flushed with phosphate buffer. These monoliths can be stored for several days or immediately functionalized

with a variety of chemistries. For our purposes we then either functionalized the PPM with primary amine terminated fluorescent dye molecules, typically performed in pure acetonitrile, or functionalized the polymers with the oligonucleotide complete with a 5'NH_3-C6 linked Oligo dT (30 or 40mer) having a 6-carboxyfluorescein covalently linked to the oligonucleotide near the 5' terminus. To perform functionalization, oligo dT (10-20ug/ul), was dissolved in a buffer containing 3X SSC and 0.05-0.1% SDS. The oligonucleotides were then denatured before introducing them to the native PPM. The covalent attachment of oligonucleotides was optimized at 120°C for 30 minutes. The attachment of the oligo dT was confirmed by imaging the fluorescently derivatized column.

2.1.4 Hybridization of mRNA to Oligo dT Monolith:

Stopped flow mRNA hybridizations were carried out by first performing a blocking step to prevent nonspecific binding using a blocking buffer containing 5-10mM Tris buffer pH 9.0, 0.05-0.1% SDS, 0.1mM BSA and ethanol amine. After blocking was complete 0.5-1.0 uL of mRNA containing sample was pumped through the capillary in order to remove the concentration gradient appearing from reagent mixing in the microfluidic T. During loading, the capillary was maintained at a temperature well above the theoretical melting temperature for polyadenaline and polythymine hybridization, to avoid mRNA annealing. Once the mRNA's were loaded, the capillary was removed from heat for a period of time ranging from 2-600seconds. After this incubation at room temperature, the monolith was flushed with 5uL of washing buffer and imaged using a fluorescent scanner. The average fluorescent intensity across the length of the capillary was noted for each hybridization time. A relative efficiency was calculated for each trial by using the expression $(W\text{-}C)/(L\text{-}C)$, where W is the fluorescent intensity of the washed and trapped mRNA, L is the loaded intensity and C is the control intensity with no mRNA. Bound mRNA's were then eluted with $\leq$5uL of buffer over 2 minutes at 90°C by placing the capillary directly on the heating block.

2.1.5 Continuous flow mRNA hybridizations:

These experiments were performed as described for the stopped flow hybridization, with a few minor alterations. We maintained the underside of the fitting at 95°C (using this temperature ensures compatibility with thermoelectric cooler limitations and is standard with PCR melting temperatures). The temperature on the top side of the fitting was measured with a k-type thermocouple and reported as 50 degrees C. Since steady-state thermal conduction over a homogenous material is linear in nature,[27] the middle portion of the microfluidic fitting could be calculated as approximately 72.5 degrees C. The flow rate was set at 0.5uL/min, providing an average fluid velocity of 3mm/s, or approximately 10 seconds of contact time for the mRNA to hybridize with the oligo dT's inside the column before passing through the other side. Following mRNA preconcentration, the capillary was immediately rinsed, with no incubation time between steps.

2.1.6 Imaging

Unless otherwise stated, all scanner images were prepared the same to not lose comparative qualities. The pictures within each figure were taken with the same scanner settings and were processed in Microsoft Office Picture Manager by manually adjusting the brightness and contrast and the color in order to convert from gray scale to color images. The brightness was increased

to 40, contrast to 80 and midtones to 100 for each picture. Then the color was altered by increasing the amount to 100 and the saturation to 100. The hue of the DNA wavelength pictures was changed to 100 to produce the green tint and the hue of the mRNA wavelength pictures was left at 0 to achieve the reddish tint.

2.2 Results & Discussion

We performed a variety of experiments to determine the optimal scheme for the deposition of glycidyl containing PPM in the capillary. These experiments terminated in the polymerization of a porous material that readily allows the attachment of various amine containing compounds (figure 2.1). The nodules in these polymer materials appear to be bound to the channel wall (figure 2.1A). We found that the polymerized monoliths were rigid and similar to previous studies,[20, 26] mitigating the use of frits to contain the polymer in the capillary channel and allowed the deposition of the polymer in a discrete location, such as in a microfluidic device. These polymers as seen in Figure 2.1A, have a very homogenous consistency, with a rough pore size of 2 to 3 um in diameter. The pore size can be optimized within the monolithic structure to allow uL/min flow at pressures ranging from 0 to over 2000 psi simply by varying the constituents in the porogenic solvent. For ease of use, we created monolithic structures which allowed flow at pressures under 200 psi by selecting methanol as the porogenic solvent. The monolith mix was photopolymerized inside a 75um inner-diameter (id) silica capillary. We estimate 65% porosity, in a polymer of 75um id and 3 cm in length. The total porous volume (porosity x capillary volume) is approximately 84 nL, assuming that full polymerization of all constituents occurred.

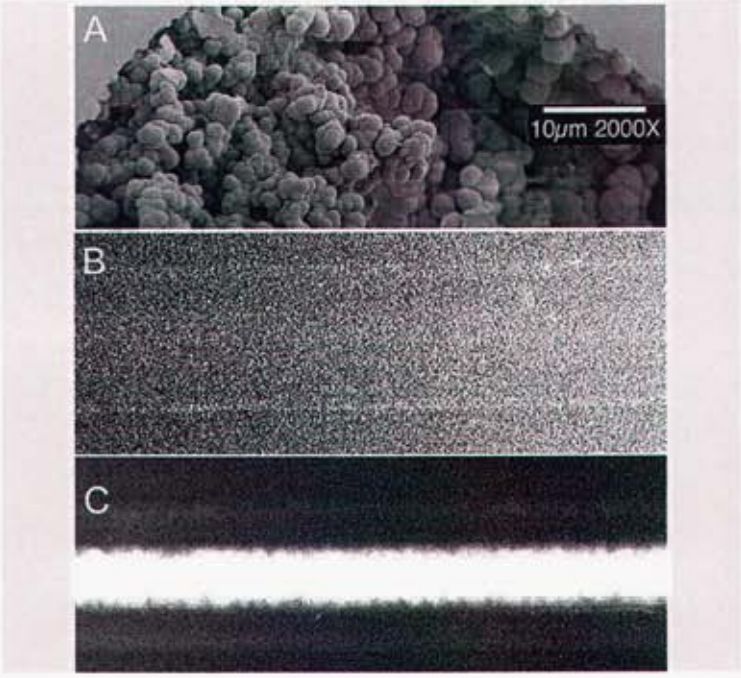

Figure 2.1. Photopolymerization and post-functionalization of the monolith material: Reveals SEM image of polymer consistency in the capillary (A) in addition to a high gain image with no fluorescent marker (B) in contrast to a low gain image with oregon green covalently bound to the PPM (C).

Following the optimization of the monolith material, we then optimized the ability to post-functionalize the materials. The GMA monomers selected for these studies allowed the attachment of a variety of biological entities provided the molecule could perform a nucleophilic attack on the intact epoxide ring on the synthesized PPM. Prior to functionalization there was no discernable fluorescent signal from the PPM. In figure 1B the gain was increased to allow the imaging of the capillary, which can be seen upon closer examination. To optimize the conditions for functionalization, we initially made use of a fluorescent dye. Oregon green caverdine was employed due to its high fluorescent intensity and the presence of a terminal amine on the dye molecule, enabling direct covalent reactivity with the GMA. In order to functionalize the PPM, the column was filled with Oregon Green caverdine and allowed to stand over night at room temperature. After this overnight incubation the columns were extensively flushed, typically three hours, to remove unbound species. Figure 2.1C is a fluorescent image of a capillary after functionalization with Oregon Green. In contrast to the control image (figure 2.1B), an intense fluorescent image is present. To ensure the chemistry was stable these columns were flushed (in the dark) in a variety of solvents and pH conditions for as long as one week. Continuous flushing for over a week, with a range of pH's from three to nine could not remove the Oregon Green once bound. This excessive flushing, however, was not necessary to remove the unbound species. It was determined that as little as 1-2uL of buffer would remove the excess or unbound fluorescence. Functionalized monoliths also maintained fluorescence for more than 6 months, demonstrating the robustness of the polymer once functionalized, which is ideal for the rugged requirements of field tests.

Having optimized the attachment of organic compounds to the polymer we then focused on the ability to functionalize the polymer monoliths with oligonucleotides (figure 2.2). As with the Oregon Green functionalization, the optimization of oligo dT binding was performed under stopped flow conditions in a 75μm i.d. capillary (figure 2.2A). The attachment of an oligonucleotide (30mer Oligo dT labeled with FAM) on the polymer was carried out in a range of temperatures and incubation times to optimize the functionalization. Initially, the oligo was introduced and allowed to incubate for 30 minutes at 60°C, and then flushed with 5uL of phosphate buffer. As noted previously, only 1-2uL of buffer were required to remove the unbound species. Each subsequent polymer was also flushed with the same amount of fluid following oligo dT incubation. As seen in figure 2.2B an extremely faint, if not undetectable, amount of Oligo dT is attached to the PPM. When the incubation time was extended to 60 minutes (figure 2C) there appeared to be an increase in the fluorescent intensity of the functionalized polymer. Beyond 60 minutes the incorporation of the oligo dT was not significantly improved with longer incubation times at this temperature ($p = 0.179$). In contrast, raising the temperature demonstrated the most dramatic effect on functionalization of the PPM. When the temperature of the functionalization reaction was increased to 90°C from 60°C the amount of fluorescent signal doubled with a p value of 0.838 for a t-test with the hypothesis that the increase in signal intensity at 90°C was equal to twice the increase in signal intensity at 60°C (figure 2.2D). As seen with the lower temperature, oligo binding increased over time. Between 30 and 60 minutes, the binding of the Oligo at 90°C was significantly increased by 1.23 units of fluorescent intensity per minute (significance of coefficient $p = 0.059$) more than doubling the fluorescent intensity seen at 30 minutes of incubation (figure 2.2E). This increase while much greater over time than the lower temperature also reached a maximum at 60 minutes with no further increase in oligo binding observed at time points greater than 60 minutes ($p = 0.911$). Maximal binding in the shortest time was found when the temperature of the functionalization was raised to 120 degrees. In contrast to previous trials at lower temperatures, increased

incubation did not result in increases (compare figures 2.2F & 2.2G) in fluorescent intensity ($p = 0.776$). The observed plateau in fluorescent intensity was not attributed to signal saturation since higher fluorescent intensities were observed on the capillaries prior to washing off the excess oligo dT.

Figure 2.2 contains magnified images of single capillaries, and as such is only representative data. Multiple capillaries were functionalized under each of the foregoing conditions. The intensity, representing oligo dT binding, spanning the length of the entire capillary image was averaged with each capillary for a given experimental condition. These averaged intensities taken with an 8-bit tricolor resolution were then plotted in a contour plot as shown in Figure 3. The fluorescent intensity at 90°C reaches a maximum around 60 minutes, which maximum is equivalent to the intensity of a capillary incubated at 120°C for either 30 minutes or 60 minutes. Thus, binding continues to increase with time after the first 30 minutes for temperatures below about 113°C and do not for temperatures over this. The average fluorescent intensity of capillaries bound at 120°C for 30 minutes were statistically identical to the average intensity of those incubated for 60 minutes, $p = 0.776$. For all further experimentation, the optimal binding conditions thus determined were used, that being 30 minutes at 120 degrees C. Higher temperatures and longer times apparently would not increase binding and would only increase the possibility of DNA or polymer degradation. Additional bindings under similar conditions did not result in significant increases in oligo functionalization.

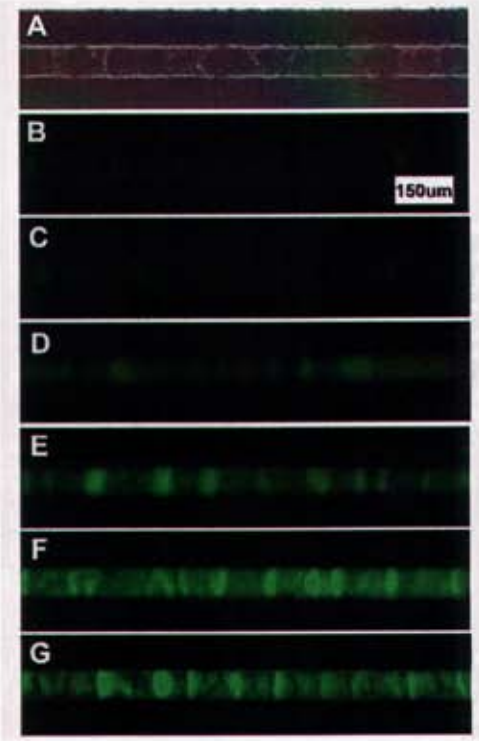

Figure 2.2. Oligo dT binding optimization: Shows a light microscope image of the capillary with monolith (A) together with oligo binding intensity for different times and temperatures. The images are for 60°C for 30 minutes (B) and 60 minutes (C), 90°C for 30 minutes (D) and 60 minutes (E), and 120°C for 30 minutes (F) and 60 minutes (G).

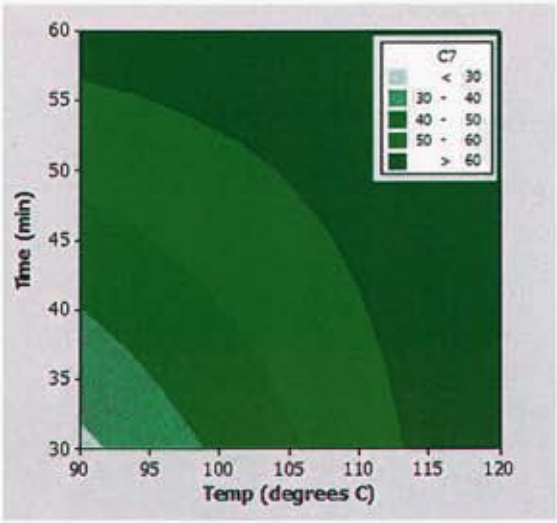

Figure 2.3. Contour plot of oligo binding: Reveals the conditions under which maximal binding of oligo dT's to the PPM occur. Incubation at either 90°C for 60 minutes or 120°C for 30 minutes results in maximal binding.

Like the dye functionalized PPM, we found that the Oligonucleotide functionalized monoliths were exceptionally stable both with various buffers and solvents. Over 200uL of pH 9.0 blocking buffer was flushed through the column at 120°C for more than an hour with minimally detectable oligo elution or degradation as indicated by fluorescent intensity and ability to trap and elute mRNA. In addition we found the functionalized columns were stable over time, as many of these devices have been stored dry over several weeks without significant decreases in fluorescent intensity. Taken together these results indicate that the functionalized polymers will be stable under the most severe of conditions, as required for field applications.

The primary objective of these studies was to demonstrate the ability to trap, concentrate, and select specific nucleotide sequences. With the monolith optimally functionalized, we then tested the ability of the Oligo dT columns to trap and release mRNA. An experimental configuration was set up using a microfluidic junction and syringe pumps to allow repeatable experimental conditions. The T-junction had two points of entry and one exit. In this arrangement one connection was used to load the mRNA samples, while the other, which contained the sample buffer only, was used to wash and elute the loaded sample. To the third connection a capillary functionalized with Oligo dT was inserted. This allowed precise control over the amount of time allowed between load, wash and elution steps. A heating block, verified at 90°C with a thermocouple, was placed within easy access to be able to apply and remove heat to the capillary virtually instantaneously. Figure 4 shows the results of injection of a single capillary volume of mRNA's and their subsequent trapping and release. Prior to loading the capillary with labeled Alexa fluor 647 mRNA, the capillary was imaged (Figure 2.4A). No flourescence was detectable in the absence of labeled mRNA. The gain and contrast were set so that the control would barely be visible and were kept constant for the remaining images. Immediately following the imaging of the unloaded capillary, approximately 1 ng (100nM) of labeled mRNA was then loaded into the capillary. The device was in contact with the heating block set at 90 degrees C to allow for denaturation of the nucleic acids (figure 2.4B). The capillary was then allowed to stand for 10 minutes after which the unbound material was flushed out of the column using 5μL of 1XSSC buffer (figure 2.4C). Compared to control, the bound mRNA (figure 2.4C) generated average 3

fold fluorescent signal increase (figure 2.4E). The fluorescence of the bound mRNA was considerably lower than imaged for the loading solution (~3 fold). In order to insure that the wash volume was sufficient, the column was flushed with more than 60 uL for the duration of an hour with no change in fluorescence. It has been ascertained that 1-2uL is sufficient to wash the mRNA in capillary. Following washing, the mRNA was eluted at 90°C with 2.5uL of 1xSSC in 5mM TE buffer over one minute (figure 2.4D). The fluorescent intensity of the eluted column appears to be the same as that of the control, indicating a nearly complete removal of bound mRNA from the capillary. However, as these images represent discrete segments of a capillary, the full range of intensities across the capillary were plotted against the capillary position in Figure 2.4E. As previously described the control had a low, almost undetectable, fluorescence intensity over the entire column. When the column was loaded we noted a high intensity fluorescence profile that appeared to decrease along the column length. This was a phenomena discovered to be due to a more dilute concentration caused from reagent mixing in the microfluidic junction. Further experiments allowed for a larger volume of mRNA's to be pumped through the microfluidic junction in order to eliminate this effect. The variation observed in concentration did not appear to effect mRNA hybridization. This high intensity signal decreased when the column was washed removing the unbound mRNA. We noted the intensity gradient observed with the loaded column was absent and the fluorescent intensity of the bound mRNA appeared to be consistent across the length of the monolith filled channel. Finally when the column was eluted the overwhelming majority of bound mRNA was denatured and removed. Figure 2.4E reveals that complete elution did not occur, but a majority of mRNA was successfully recovered.

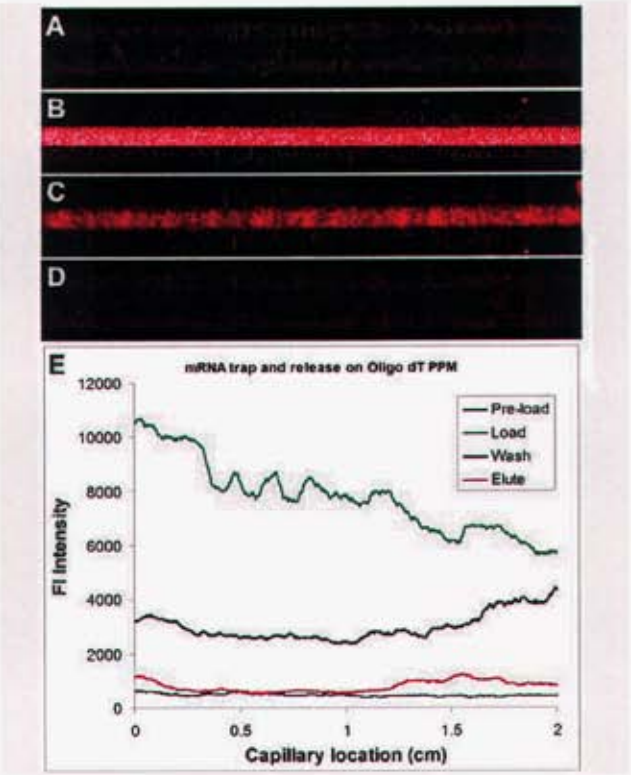

Figure 2.4. Batch trapping of mRNA: Demonstrates ability of PPM to trap mRNA from a single capillary volume. Shows the control with no mRNA (A) as compared with the loaded intensity

(B), the washed fluorescence (C), and the eluted intensity (D). While the images are only representative segments of the capillary, figure 2.4E is a graph of fluorescent intensity across the entire length of the capillary.

Although the trapping and elution of mRNA's from the monolith may seem self explanatory, we did note conditions which inhibit successful mRNA preconcentration and purification. For example, failure to denature the mRNA inhibits hybridization of the sample analytes to the Oligo dT functionalized porous polymer monolith (figure 2.5). In addition, failure to properly block the column has repeatedly resulted in irreversible binding to the polymer monolith (data not shown). Successful blocking of the column was best performed using a solution containing both ethanol amine and bovine serum albumin. Finally, selecting a proper salt concentration can facilitate finding a single binding/washing/elution buffer, thereby solving one of the many problems of automated microfluidic analysis in determining how and where to store the multitude of reagents.[5]

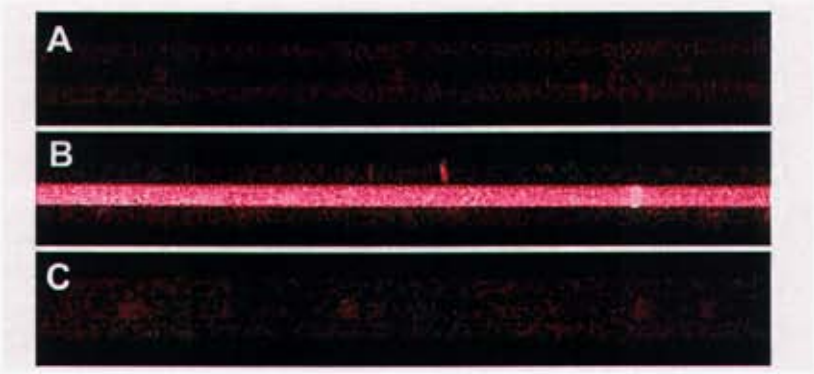

Figure 2.5. Failure to denature mRNA: Shows the control with no mRNA (A) as compared with the loaded intensity (B) and the washed fluorescence (C). There is no significant amount of trapping when mRNA is not properly denatured.

Having optimized the conditions for optimal binding, washing and elution of purified mRNA we then focused on determining and optimizing reaction kinetics (figure 2.6). We found in our experiments there was no visible trend in hybridization efficiency over time. In fact, the efficiency of trapping with a hybridization time of ten minutes appears to be the same, or perhaps a little less than the efficiency of trapping with an incubation time of two seconds. The other trials with hybridization times of 5, 10, 15, 30, 60, and 120 seconds together with the previously mentioned two trials appear to form a linear pattern with no slope (significance of coefficient from 2 to 120 seconds, $p = 0.962$) scattered about an average efficiency of 33%. The lack of any increase in efficiency with time demonstrates hybridization is complete at the initial time point measured, two seconds.

The exact same procedure was used to test and measure 40mer oligo efficiencies. The 40mers also demonstrated a linear pattern of efficiency with no apparent slope (significance of coefficient from 2 to 120 seconds, $p = 0.867$). In contrast to the 30mer Oligo dT, the scatter plot is centered about an efficiency of 50%. The ratio of the average efficiencies demonstrates that the 40mer functionalized monolith binds with 1.5 times the efficiency of the 30mer characterized monolith. We believe the results are due to a greater change in free energy than the 30mer in hybridization with its RNA counterpart.

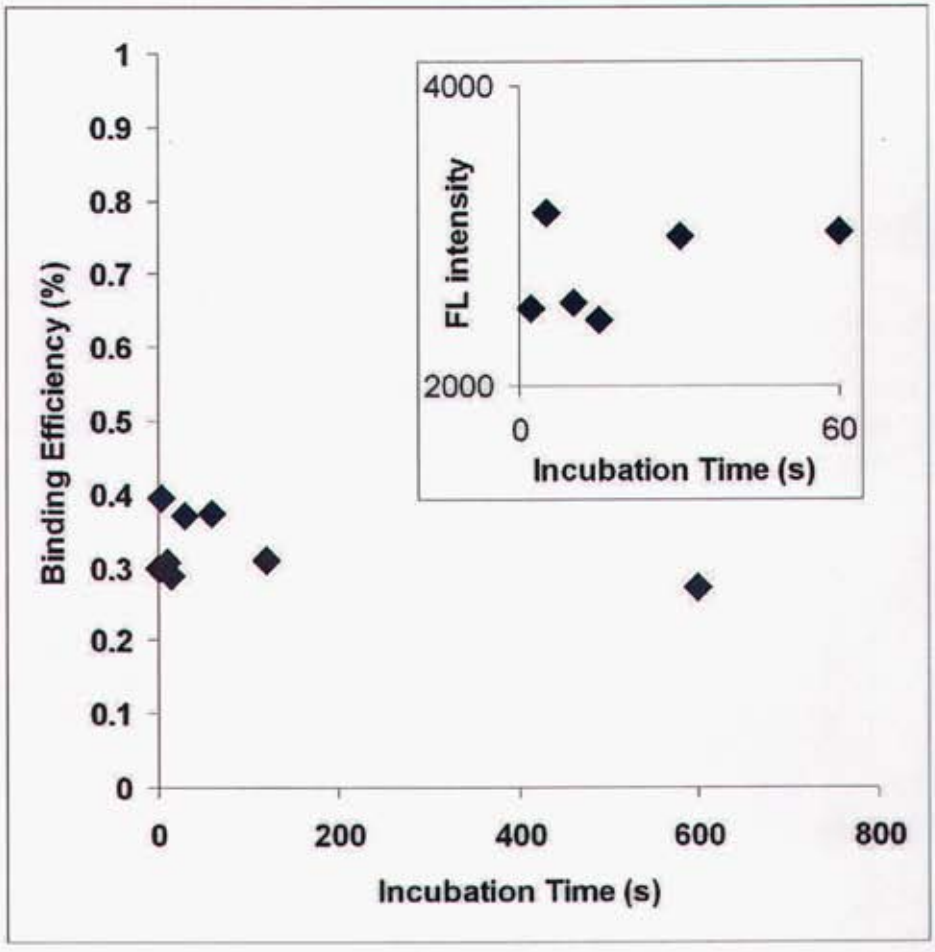

Figure 2.6. Kinetics of DNA hybridization: Experiment for a capillary containing 30mer and 40mer oligo dT's. The 30mer and 40mer oligo dT's are represented by the blue diamonds and range from 30-40% efficiency over 8 trials with no significant change for increased hybridization times.

Since both the 30mer and 40mer functionalized polymers demonstrated linear binding characteristics with a nonsignificant slope, it can be concluded that hybridization is already complete by the time the monoliths are washed, over a range of nucleotide probe lengths. Therefore, the kinetics of hybridization inside of the polymer are rapid, fast enough to allow constant flow through binding.

We then optimized the PPM for continuous-flow mRNA purification and sample preconcentration. For these experiments the intensity gain was set to the highest level without saturating the CCD array of the scanner during preconcentration (figure 2.7). In addition, contrasting the previous experiments where the capillary was heated, the microfluidic T was placed over a heater at 95°C (figure 2.7F). This allowed for RNA denaturation prior to introduction to the Oligo dT column. Similar to the previous experiments, the unloaded capillary appeared to contain no detectable fluorescence (figure 2.7A). To acquire the loaded image, the capillary was again heated to 95°C and loaded with the same solution used in figure 2.4B. In contrast, to figure 2.4B the fluorescence in figure 2.7B (the same solution) was nearly

undetectable, due to the decreased gain settings. The microfluidic fitting was then placed on the TEC and the mRNA solution was pumped through the heated fitting into the capillary. We observed in these experiments a small portion of capillary with no fluorescence, followed by an intense segment of brightness, which then gradually tapers down to a more faint level of fluorescence. The intensity of the fluorescence in the brightest region was approximately 30 fold higher than the initial loaded volume (figure 2.7B&E), demonstrating the preconcentration of mRNA during constant flow. The efficiency as the ratio of trapped mRNA over loaded mRNA was approximately 80%. Not only was preconcentration achieved, but the capture efficiency was greater for the constant-flow set up than it was for the batch reaction. This is significant as it will eventually lead to the ability to work with extremely dilute solutions and to concentrate them to a point that is detectable in real time. Furthermore these concentrated mRNA's could be eluted in a discrete volume of solution, only requiring 1.0 uL of buffer at 95°C, to release the entire sample (figure 2.7D), resulting in fluorescence measurements comparable to the control. As the images in figure 2.7 represent only discrete segments of the capillary, Figure 2.7E shows the intensity of the capillary along a larger portion of its length. A large fluctuation of mRNA binding is seen over the length of the capillary. Along the first few millimeters, the intensity is nearly identical to the control with no mRNA. The next few millimeters exhibit concentration around 30x of the initial concentration. Finally, the last 2.5cm (only half centimeter shown) fall to a constant intensity just over the initial concentration. These results were found to be identical over a number of Oligo dT functionalized capillaries (n>10). We believe the explanation for these three regions of differential binding in capillary lies with the temperature gradient within the microfluidic fitting and capillary. The first few millimeters of capillary are confined within the microfluidic junction or T that is heated to 72 degrees C at its center. This temperature is above the melting temperature, and consequently no mRNA's bind. The next section of the capillary is within the microfluidic fitting sticking out from the junction (see Figure 2.7F). This portion is not touching the TEC and is somewhat cooler than the microfluidic junction. Nevertheless, as it is in contact with the microfluidic junction, some heat is transferred along its length, resulting in a temperature that is still higher than the temperature of the surroundings, yet lower than the melting temperature. It is inside this fitting that the melting temperature is crossed, as exhibited by the sharp spike in fluorescent intensity in Figure 2.7E. As the temperature continues to fall across the length of the fitting, the efficiency of mRNA capturing begins to decrease. Finally, as the capillary emerges from the fitting, the temperature drops almost instantaneously to room temperature, reducing the mRNA hybridization efficiency for the remainder of the capillary. This explanation seems reasonable based on previous work on the thermodynamic nature of nucleic acid hybridizations.[7] Our results are similar to those found by Jiang and Harrison, *et al* who reported 26% efficiency in 20minutes of run time, not including denaturing and subsequent elution, along a 2.9cm channel that was 200um wide and 30um deep. They concluded from their results that a more efficient capture bed was needed to improve efficiency.[18] Here we report 80% capture efficiency for the flow through preconcentrator, with potential for near 100% with proper temperature optimization across the entire length of the capillary, in a photo-initiated monolithic bed, in less than 3 minutes which includes denaturing, loading, trapping, washing, and elution.

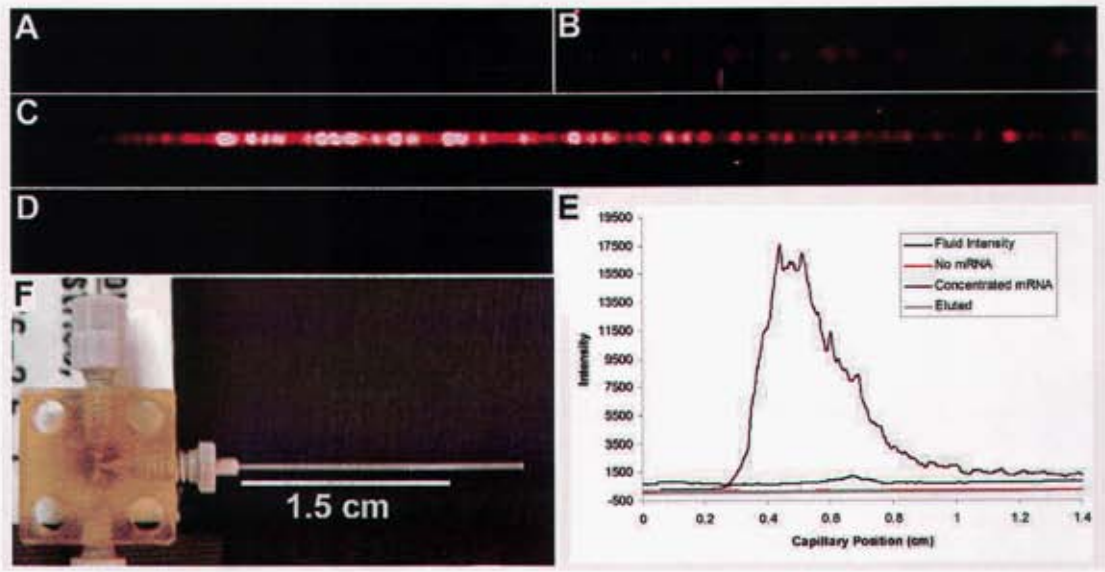

Figure 2.7. Constant-flow preconcentration: Depicts PPM ability to preconcentrate mRNA. The images are for the control with no mRNA (A), single volume of mRNA (B), washed following preconcentration (C), and eluted (D). While the images are only representative segments of the capillary, figure 2.7E is a graph of fluorescent intensity across the entire length of the capillary.2.7F shows the capillary set up with the microfluidic T resting on the TEC.

Having demonstrated the efficacy of preconcentration of purified mRNA we then tested the selectively of the hybridization reaction in a large background of DNA. Using a mixture of Alexa fluor 532 labeled DNA 4ug/ml and Alexa fluor 647 labeled mRNA 0.85ug/ml, we tested the ability of the Oligo dT functionalized column to selectively trap mRNA. As seen in figure 2.8A&B, the control images showed a low level of fluorescence in their respective channels. When one capillary volume of the sample solution was preloaded on to the capillary an intense fluorescent image was present in the green (DNA) channel, and significantly lower in the red (mRNA) channel (Figure 2.8C&D). Preconcentration of the mRNA was then performed (figure 2.8E&F). After passing 160 uL of the sample solution through the monolith trapping column the image collected from the green (DNA) channel (Figure 2.8E) appeared similar to the preloaded column, while the fluorescence in the red (mRNA) channel increased dramatically (Figure 2.8F). The loaded column was then washed with an additional volume of sample buffer which removed the unbound DNA (Figure 2.8G), while the bound mRNA (Figure 2.8H) remained hybridized on the functionalized column. Similar to previous experiments the bound mRNA was then eluted by increasing the temperature on the monolith column. This resulted in a dramatic decrease in the observable fluorescence on the column.

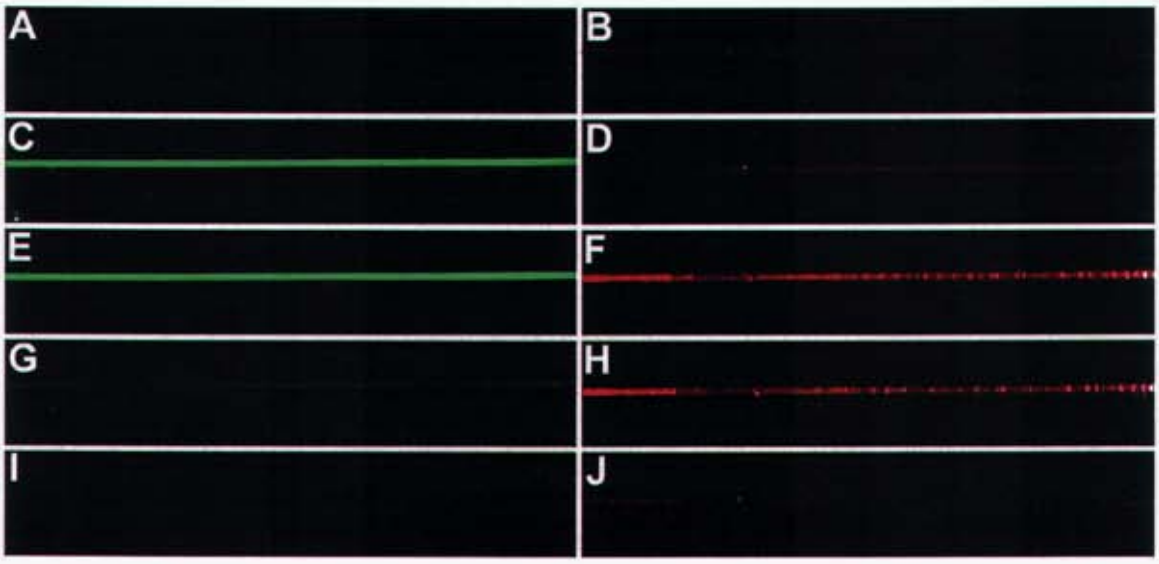

Figure 2.8. Selectivity of PPM preconcentration: Shows the selective adsorption of mRNA (red, right side of figure) over cDNA (green, left side of figure) for preload (A&B), a single volume (C&D), post preconcentration (E&F), washed (G&H) and eluted (I&J). Although DNA is significantly more concentrated than mRNA, only mRNA is left after wash step.

We believe that our results demonstrate the potential use of these materials as a sensitive single-step, real-time detection system. The 30mer kinetics experiments captured approximately 0.3ng of mRNA in 3cm of microfluidic capillary. This amount of mRNA contributed to a signal to noise ratio of nearly 50:1. Assuming linearity of fluorescent intensity increase between 0 and 1ng mRNA, we could theoretically detect as little as 18pg of mRNA in a 3cm capillary or 60fg (0.06amol or 36,000 molecules) mRNA in a 100micron segment of capillary with a 3:1 signal to noise ratio. We used a mRNA concentration of 100nM in our experiments, however, as noted previously we can preconcentrate mRNA at least 30x yielding a potential detection limit around 20fM. We also report the ability to reuse the sample preconcentrators. We have consistently used the columns for 10 or more trials, verifying completion of elution after each trial. During the course of the 10 trials, the efficiency did not decrease, and the baseline fluorescence after elution did not change noticeably (data not shown).

2.3 Conclusions

In conclusion, we have confirmed our original goal to create a photo-polymerized monolith in-situ, which allows constant flow sample preconcentration with temperature controlled binding and elution. We have discovered the means whereby trapping can be maximized and are taking the next steps by developing devices which will allow more precise temperature control inside different regions of the monolith. Other areas that still need to be explored include examining the efficiency of and overall binding capacity of the monoliths under competitive binding conditions. The rate of nonspecific binding needs to be identified as well. The potential of the monolith is great in that it allows low pressure installation of a binding substrate without need of a frit to contain it. It has the potential to bind as much mRNA with similar or higher efficiencies as standard batch processes s in minutes or even seconds and under constant flow conditions.

2.4 References

1. Selvaganapathy, P.R., E.T. Carlen, and C.H. Mastrangelo, *Recent progress in microfluidic devices for nucleic acid and antibody assays.* Proceedings of the IEEE, 2003. **91**(6): p. 954-75.
2. Saito, Y., et al., *Miniaturized sample preparation and separation methods for environmental and drug analyses.* Anal Sci, 2002. **18**(1): p. 7-17.
3. Verpoorte, E., *Microfluidic chips for clinical and forensic analysis.* Electrophoresis, 2002. **23**(5): p. 677-712.
4. de Mello, A.J. and N. Beard, *Dealing with real samples: sample pre-treatment in microfluidic systems.* Lab Chip, 2003. **3**(1): p. 11N-19N.
5. Lichtenberg, J., N.F. de Rooij, and E. Verpoorte, *Sample pretreatment on microfabricated devices.* Talanta, 2002. **56**(2): p. 233-266.
6. Campbell and Farrell, *Biochemistry.* 4 ed. 2003: Thomson Learning.
7. Bautz, E.K. and B.D. Hall, *The isolation of T4-specific RNA on a DNA-cellulose column.* Proc Natl Acad Sci U S A, 1962. **48**: p. 400-8.
8. Gilham, P.T., *Complex Formation in Oligonucleotides and Its Application to the Separation of Polynucleotides.* Journal of the American Chemical Society, 1962. **84**(7): p. 1311-1312.
9. Gilham, P.T., *The Synthesis of Polynucleotide-Celluloses and Their Use in the Fractionation of Polynucleotides.* Journal of the American Chemical Society, 1964. **86**(22): p. 4982-4985.
10. Gilham, P.T., *The synthesis of celluloses containing covalently bound nucleotides, polynucleotides, and nucleic acids.* Biochemistry, 1968. **7**(8): p. 2809-13.
11. Aviv, H. and P. Leder, *Purification of biologically active globin messenger RNA by chromatography on oligothymidylic acid-cellulose.* Proc Natl Acad Sci U S A, 1972. **69**(6): p. 1408-12.
12. Hornes, E. and L. Korsnes, *Magnetic DNA hybridization properties of oligonucleotide probes attached to superparamagnetic beads and their use in the isolation of poly(A) mRNA from eukaryotic cells.* Genet Anal Tech Appl, 1990. **7**(6): p. 145-50.
13. Chung, Y.C., et al., *Microfluidic chip for high efficiency DNA extraction.* Lab Chip, 2004. **4**(2): p. 141-7.
14. Dai, J., et al., *Electrokinetic trapping and concentration enrichment of DNA in a microfluidic channel.* J Am Chem Soc, 2003. **125**(43): p. 13026-7.
15. Christel, L.A., et al., *Rapid, automated nucleic acid probe assays using silicon microstructures for nucleic acid concentration.* J Biomech Eng, 1999. **121**(1): p. 22-7.
16. Breadmore, M.C., et al., *Microchip-based purification of DNA from biological samples.* Anal Chem, 2003. **75**(8): p. 1880-6.

17. Wolfe, K.A., et al., *Toward a microchip-based solid-phase extraction method for isolation of nucleic acids.* Electrophoresis, 2002. **23**(5): p. 727-33.

18. Jiang, G. and D.J. Harrison, *mRNA isolation in a microfluidic device for eventual integration of cDNA library construction.* Analyst, 2000. **125**(12): p. 2176-9.

19. Yu, C., et al., *Monolithic porous polymer for on-chip solid-phase extraction and preconcentration prepared by photoinitiated in situ polymerization within a microfluidic device.* Anal Chem, 2001. **73**(21): p. 5088-96.

20. Shediac, R., et al., *Reversed-phase electrochromatography of amino acids and peptides using porous polymer monoliths.* J Chromatogr A, 2001. **925**(1-2): p. 251-63.

21. Throckmorton, D.J., T.J. Shepodd, and A.K. Singh, *Electrochromatography in microchips: reversed-phase separation of peptides and amino acids using photopatterned rigid polymer monoliths.* Anal Chem, 2002. **74**(4): p. 784-9.

22. West, J.A.A., et al., *Microfluidic gene arrays for rapid genomic profiling.* Proceedings of the Society of Photo-Optical Instrumentation Engineers (spie) Conference on Lab-on-a-Chip: Platforms, Devices, and Applications; October 26-28, 2004; Philadelphia, PA, 2004. **5591**: p. 167-173.

23. Peterson, D.S., et al., *Dual-function microanalytical device by in situ photolithographic grafting of porous polymer monolith: integrating solid-phase extraction and enzymatic digestion for peptide mass mapping.* Anal Chem, 2003. **75**(20): p. 5328-35.

24. Yu, C., F. Svec, and J.M. Frechet, *Towards stationary phases for chromatography on a microchip: molded porous polymer monoliths prepared in capillaries by photoinitiated in situ polymerization as separation media for electrochromatography.* Electrophoresis, 2000. **21**(1): p. 120-7.

25. Yu, C., et al., *Preparation of monolithic polymers with controlled porous properties for microfluidic chip applications using photoinitiated free-radical polymerization.* Journal of Polymer Science, Part A: Polymer Chemistry, 2002. **40**(6): p. 755-769.

26. Ngola, S.M., et al., *Conduct-as-cast polymer monoliths as separation media for capillary electrochromatography.* Anal Chem, 2001. **73**(5): p. 849-56.

27. Welty, et al., *Fundamentals of Momentum, Heat, and Mass Transfer.* 4 ed. 2001, New York: John Wiley & Sons, Inc.

This page intentionally left blank.

Distribution List

1	MS 9292	Y. Fintschenko, 8324
1	MS 9403	T. Sheppod, 8778
3	MS 9292	K. Patel, 8324
2	MS 9018	Central Technical Files, 8944
2	MS 0899	Technical Library, 4536