A Microfluidic Approach to Enzyme Linked

Immunosorbent Assays

A Masters of Engineering Project

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by

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ABSTRACT

The Enzyme-Linked Immunosorbent Assay (ELISA) is a biochemical technique that is useful for diagnosing disease and infection by detecting antigens/antibodies in a sample. Studying assay design is important in designing an optimal and cost-effective assay which comprises short processing times, small reagent consumption, and portability. The primary objective of this study was to design and test a microfluidic chip to produce a rapid ELISA platform.

A microfluidic device integrating six straight channels and six nitrocellulose strips was used as a platform to produce a rapid and semi-qualitative dot ELISA. Typical dot ELISA processing times are on the order of hours, but has been reduced here to 35 minutes. The same six channel microfluidic device with polystyrene strips in place of nitrocellulose strips was explored for its ability to produce a quantitative sandwich ELISA. The device produced indistinguishable results due to high noise levels which are thought to be a result of non-specific binding.

BIOGRAPHICAL SKETCH

Travis Lee was born in Honolulu, Hawaii in 1983. He attended Cornell University in Ithaca, New York to obtain his Bachelors of Science degree in Biological and Environmental Engineering. Thereafter, he continued to study at Cornell University to earn his Masters of Engineering from the Department of Biological and Environmental Engineering.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

1. Introduction

The field of microfluidics has stimulated much interest in the development of miniaturizing laboratory equipment and processes. These processes offer lower reagent consumption, automation, portability, parallel processing, safer reactions, and cost effectiveness. There are a large number of advancements and patents available for microfluidic technology in DNA genotyping, capillary electrophoresis, chip design, separations, microchemical systems and microreactors. These advancements in micro-technology have provided numerous advantages over macroscale devices. For example, fabrication costs of these macroscale devices usually require more materials, bigger processing plants, and often require large permanent investments for fabrication. Additionally, microfluidic devices can have shorter processing times, real-time data collection/analysis, smaller footprint and fewer materials.

The immunoassay can detect proteins specifically and sensitively with high affinity through antibody-antigen interactions. The immunoassay has been around for some time and exists in two fundamental forms, homogeneous and heterogeneous (Bange, 2005). The homogeneous assay exists entirely in the solution phase and does not require separation of the antibody and bound target for detection. In a heterogeneous assay, the antibody and bound target need to be separated from non-linked/non-reactive material in order to be detected. Heterogeneous assays have a couple of advantages over homogeneous assays including better sensitivity and less cross-reactivity in the reaction due to separation of the antibody-antigen complex. In addition, heterogeneous assays are able to take advantage of large surface to volume ratios characteristics of micro-channel

1

geometries. The major disadvantage of a heterogeneous assay comes from non-specific binding of secondary or non-target molecules on reaction surface.

There are several types of heterogeneous assays; the assay of interest for this study and the most basic type is the sandwich ELISA. Typically, a monoclonal primary antibody is first immobilized to the micro-well and a blocking agent is added to saturate non-specific binding sites. A test sample containing the antigen of interest and other proteins is added. Due to the specific binding properties of antibodies, only the antigen and coating primary antibody bind. A secondary enzyme conjugate antibody bound to the antigen is then to form a sandwich structure followed by a substrate to react with the conjugated enzyme for colorimetric detection, see Figure 1.1.
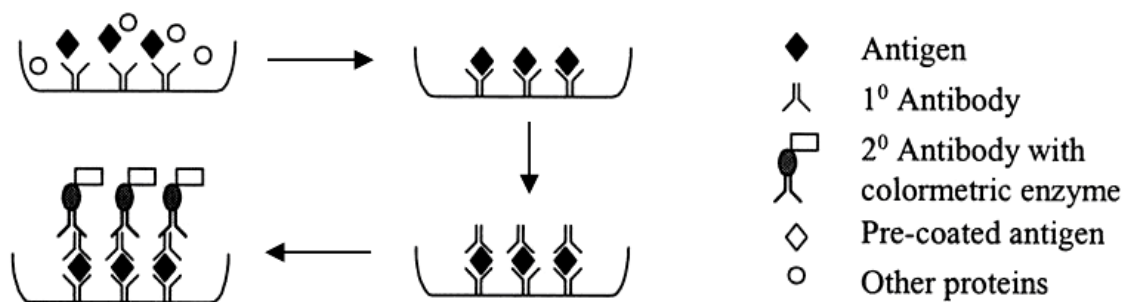
Figure 1.1. Sandwich ELISA schematic adapted from Lai et al., 2005.

Enzyme Immunoassays (EIA) are sensitive assays for detecting low concentrations of a target molecule. The targets of these assays can be antigens, antibodies, and small molecule chemicals. The applications of an immunoassay include viral infection/disease detection, measuring immune responses, and chemical detection.

The current methods of performing ELISA are based predominately on microtiter plate format and require relatively high volume reagent/sample consumption, heating, repetitive reagent dispensing, agitation and spectrophotometers for end point detection. It

also includes long incubation times and involves labor intensive operation. An interesting alternative to the standard ELISA is the dot ELISA. The dot ELISA can be done in a rapid fashion and detection can be done visually. The dot ELISA can be more sensitive than the standard sandwich ELISA (Zheng et al., 1990, Siavashi et al., 2004). Although, the dot ELISA is not quantitative like the standard ELISA, its ease of use and rapid detection make it an attractive approach.

An integrated microfluidic device can also be automated such that precision of the assay is independent of the lab technician's skill level. Automation allows for precise fluid control, real time data collection and analysis. Miniaturization and integration of a microlfuidic device provides the requirements necessary to produce a portable device for onsite testing or point-of-care diagnostics.

Other attempts at microfluidic sandwich ELISA have yet to obtain a short assay time from start to finish. To date there have been no microfluidic dot ELISA publications and thus the possibility has not been explored. The study herein investigates the application of a microfluidic dot ELISA and sandwich ELISA on a polystyrene chip. The set up used here has the ability to be controlled and automated via a personal computer, thus providing the potential for an integrated and portable device. The specific objectives of this study are:

1. To design and fabricate a chip that will exploit the inherent advantages of micro-channels in microfluidic devices.

2. Test the fabricated chips.

3. To optimize assay-processing conditions.

2. Literature Review

2.1. Background

All microfluidic EIA devices are challenged with the same fundamental properties of microchip fabrication and design. These properties are illustrated below.

1. *Material*. The options for device material are often limited by fabrication techniques. Materials play a critical role in antibody immobilization and also determine the range of detection methods that can be used.

2. *Surface Modification*. It may be required to provide the proper surface chemistry for specific antibody immobilization. The immobilization of a secondary molecule or analyte (non-specific binding) can significantly reduce immunoassay quality.

3. *Detection Method*. Detection of the antibody-antigen interaction effectively determines the qualitative and quantitative nature of an immunoassay.

4. *Design*. Channel design is critical for fluid transport and manipulation. The channel dimensions will also affect the surface/volume ratio which is critical for assay time by changing molecular diffusion distances.

These challenges have been addressed by many in the field of microfluidics, a review of some the techniques and developments are described here.

2.2. Materials

Many of the early materials of microfluidic devices were similar if not the same as those used in the microelectronic industry. The methods for producing micro-electro-mechanical-systems (MEMS) were initially modified to create microfluidic devices.

Thus, most devices were made of silicon and glass, but very few were constructed from polymers. Although a silicon based device can be fabricated with extremely good definition and a high aspect ratio it is expensive and not optically transparent (Bange, 2005). Consequently, it is not suitable for detection by immunoassays that rely on colorimetric, fluorescent, and other optical detection methods. Using polymeric materials such as polystyrene, polyethylene (PE), polyimide, polycarbonate, polydimethylsiloxane (PDMS), polyethylene glycol (PEG), polymethyl methacrylate (PMMA), polyethylene terephthalate (PET) and cellulose acetate (Rossier, 2002; Bange, 2005) offers practical alternatives for device materials. Many of the polymers are optically transparent and exhibit hydrophobic surfaces which are desirable for the immobilization of antibodies to channel surfaces. Once of the disadvantages of polymeric devices are its electrical properties which aren't preferred for electrophoretic or electrokinetic driven fluid transport.

2.3. Surface Modification

Several methods of surface modification have been applied for enhanced antibody immobilization or reduction of non-specific binding. Non-specific binding of molecules in a heterogenous assay reduces the quality by affecting signal/noise ratio. A three layer biopassivation technique utilizing biotinylated IgG, neutravidin, and biotinylated dextran were deposited onto channel surfaces and reduced non-specific binding (Linder et al., 2001). The highly hydrophobic surface of PDMS that interacts with apolar analytes was effectively suppressed. Linder et al. (2001) also suggested that the 3rd layer (biotinylated dextran) may be replaced with any biotinylated probe such as antibodies. A method

utilizing a supported and conjugated phospholipid bilayer is described by Yang et al. (2005). The conjugated lipid bilayer contained specific DNP (Dinitrophenyl)-conjugated lipids for anti-DNP antibody attachment. The lipid bilayers were shown to last up to several weeks and more importantly reduced non-specific adsorption by a factor of two (Yang, 2005). Surface modifications give rise to the possibility of covalent and non-covalent antibody–substrate interactions via functional surface groups. Silicon microchips with surface modifications such as glutaraldehyde (GA), branched polyethyleneimine (BPEI), linear polyethyleneimine (LPEI), and 3-aminopropyltrimethoxysilane (APTES) were presented (Yakovleva et al., 2002). These surface modifications are able to bind covalently and non-covalently to antibodies, but have not yet seen good precision. Chemical vapor deposition (CVD) has also been presented to develop functional surface groups on PDMS (Lahann et al., 2003). A layer of pentafluorophenol ester groups was produced within the channel for covalent binding of antibodies.

2.4. Detection

A glass charge coupled device (CCD) array has been presented for the detection of Cy-5 fluorescent labeled antibodies (Wadkins et al., 1998). The system uses a combination of evanescent illumination via a 635 nm laser and the CCD array to detect Cy-5 labeled antibodies. The Cy-5 antibodies were able to bind to and detect staphylococcal enterotoxin B (SEB) at 5ng/ml. Sato et al. (1999) described a highly specific method of detection for non-fluorescent substances in microfluidic channels. Thermal Lens Microscopy (TLM) utilizes two laser beams, an excitation beam and a

probe beam, to generate an optical signal that is detected with a photodiode. A disposable polymeric device with electrodes for ELISA assays has been created such that the presence of a redox enzyme-substrate reaction can be detected in the channel by the integrated electrodes (Rossier, 2001).

Choi et al. (2002) developed a magnetic bead approach coupled with electrodes for electrochemical detection and integrated microvalves for fluid transport on glass chip. Yakovleva et al. (2002) described a method for chemiluminescence in a silicon microchip for immunoassays. A luminescent reaction was performed in a channel using an enzyme-hapten tracer plus a luminol/PIP/H_2O_2 substrate mixture (Yakovleva, 2002). The chemiluminescence signal was detected with a photomultplier tube (PMT). Pre-coated polystyrene beads have also been applied to immunoassays on a glass substrate. Thermal lens microscopy is utilized for detection. The use of fluorescence polarization for detection of protein-protein binding interactions has been presented by Yadavalli and Pishko (2004). Tetramethylrhodamine isothiocyanate (TRITC) labeled antibody was used to detect polyaromatic hydrocarbon (PAH) in PDMS microchannels at the 10-40 nmole range (Yadavalli, 2004).

2.5. Channel Design

Lai et al. (2004) described a disk-like immunoassay which takes advantage of centrifugal forces and capillary surface tension to produce gated fluid movement at specific rotation speeds. The disk also takes advantage of an enzyme substrate reaction for colorometric detection. A chip design for analyzing multiple antibodies has been presented (Jiang et al., 2003). This chip utilizes channel geometries for mixing and

creating a serial dilution in successive micro-channels on PDMS substrate. A polycarbonate membrane is used to increase surface area for presentation of antigen and interaction with antibodies. Detection of HIV-1 membrane proteins gp41 and gp120 was confirmed using a secondary fluorescent antibody for UV detection (Jiang et al., 2003). A significant time reduction was not apparent in this set up.

3. Materials and Methods

3.1. Chip Design - Dot ELISA

The microfluidic chips are designed and fabricated at Kionix from polystyrene, the most widely used material for biological assays. The chip comprises two layers of polystyrene sheets and a layer of thin film. Polystyrene films were obtained from Plastic Suppliers, Inc. A CNC mill is used to fabricate channels and cut the plastic pieces; a proprietary solvent lamination technique is employed to assemble these polymeric devices.

The thin polystyrene film is used to fabricate circular diaphragm membrane valves. A cross-sectional view of a single valve is shown in Figure 3.1; in both the closed (A) and open (B) positions. A pressure difference created via a pneumatic pump is delivered to the chip diaphragm chamber, this allows the membrane to open and close the microfluidic channel.
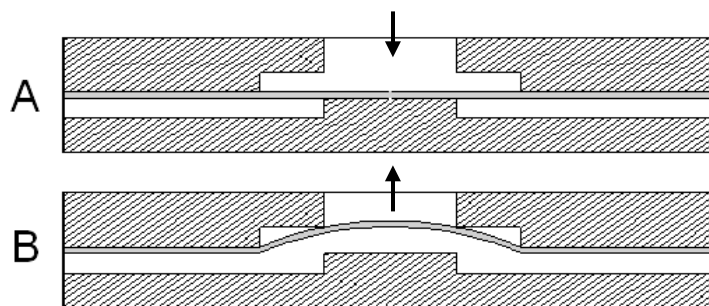
Figure 3.1. A membrane diaphragm valve in closed (A) and open (B) positions.

If one uses a series of three valves (inlet, driver, and outlet) and the operation of these valves are properly sequenced, a pumping system can be created as shown in Figure 3.2. Typically, these systems contain inlet and outlet valves that are smaller than the

9

driving valve. Altering the actuation sequence, fluid flow can be directed in both directions.

The advantages of this system come with the ability to control the pneumatic pumps with a personal computer. A computer program can be used to control the delay time, the time a valve stays open, and thus control the rate fluid is moved.

Figure 3.2. Six actuations required to produce flow through three valves marked A, B, and C.

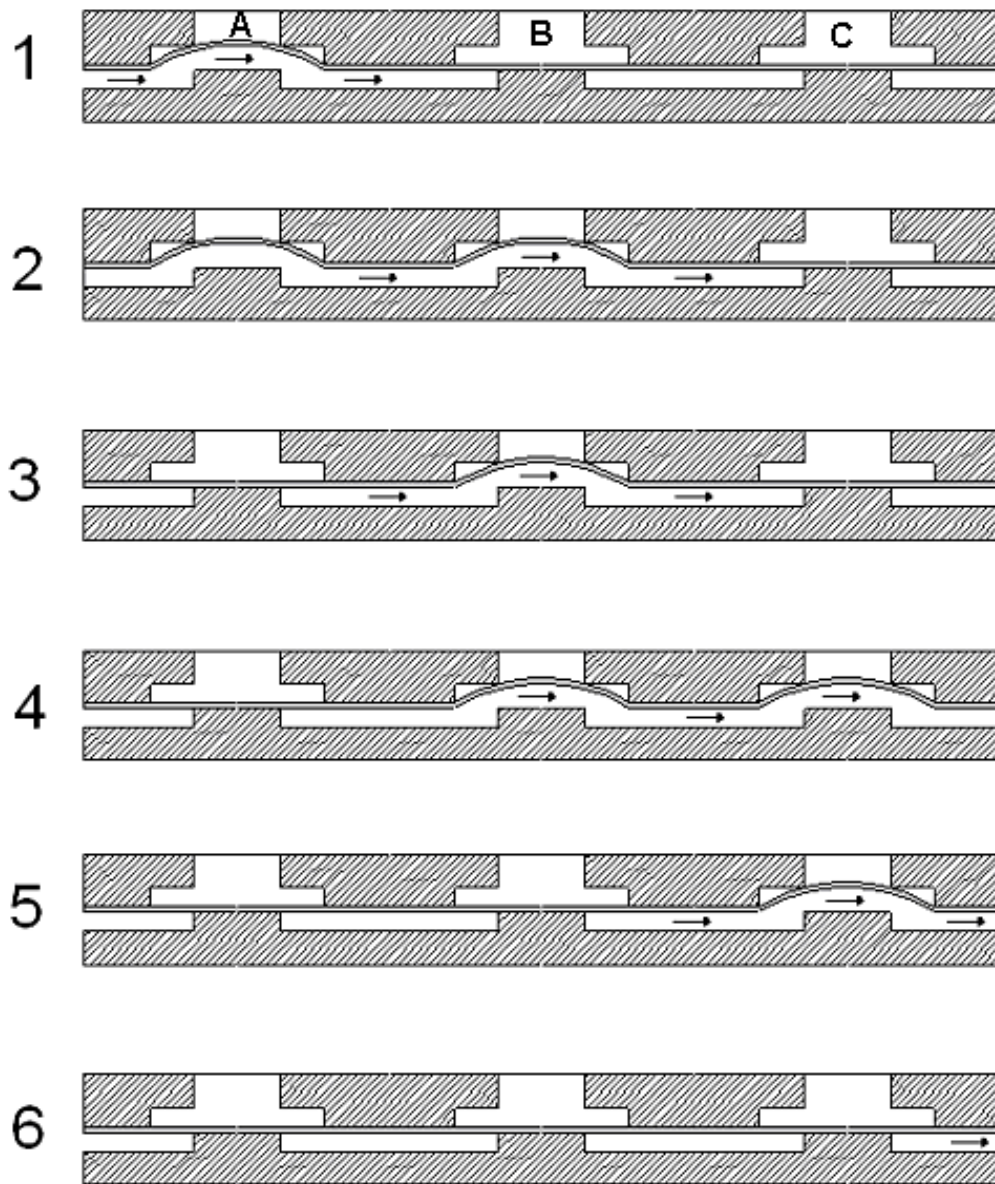Nitrocellulose has been used in many dot ELISA set ups because its large binding capacity derived from its porous three dimensional structure and charged backbone, but it has never been tested on a microfluidic platform. Nitrocellulose paper was cut to a diameter of 2mm using a laser cutter. The dots were assembled onto an adhesive strip with holes atop the nitrocellulose dots as illustrated in Figure 3.3. The holes allow for fluid delivery to the top and bottom of the nitrocellulose dots. The holes under the nitrocellulose dots are especially important for diffusion of antibody via the top and bottom of a dot.
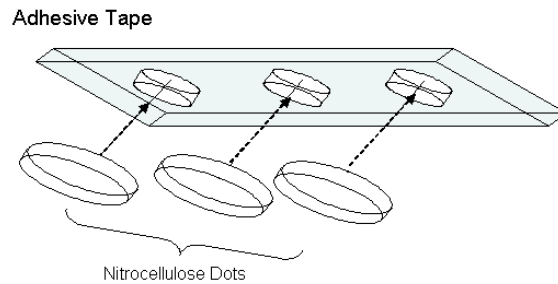
Figure 3.3. Nitrocellulose dot assembly to adhesive tape.

A straight channel design was applied for its ability to slide the strips in and out of the chip easily. The channel depth was 500μm. A six-channel chip with the planar membrane pumping system is depicted in Figure 3.5.

3.2. Chip Design - ELISA

Polystyrene Strips

The chip design for the ELISA utilized the straight channel design and pumping mechanism just described above, it was also used to test the ability of a standard

sandwich ELISA on chip. The design for the standard ELISA, replaced nitrocellulose strips with 50μm thick polystyrene strips of 2mm wide and 40mm long.

Polystyrene Beads

To further test the ability of a sandwich ELISA, a chip utilizing polystyrene beads was designed to significantly increase the surface area within a channel. Polystyrene beads, 90μm in diameter, were purchased from Polysciences, Inc. A rectangular microfluidic channel with a weir structure was designed such that entry of polystyrene beads was possible while the movement of beads was restricted to a solid barrier. These channels did not employ the use of the valve pumping system due to time restriction. The flow was generated by manually aspirating the fluid in the channel. A cross-sectional view of this chip is shown in Figure 3.4 and a typical channel fabricated with polystyrene beads is shown in Figure 3.6.
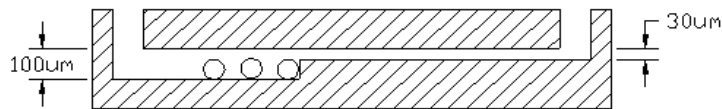
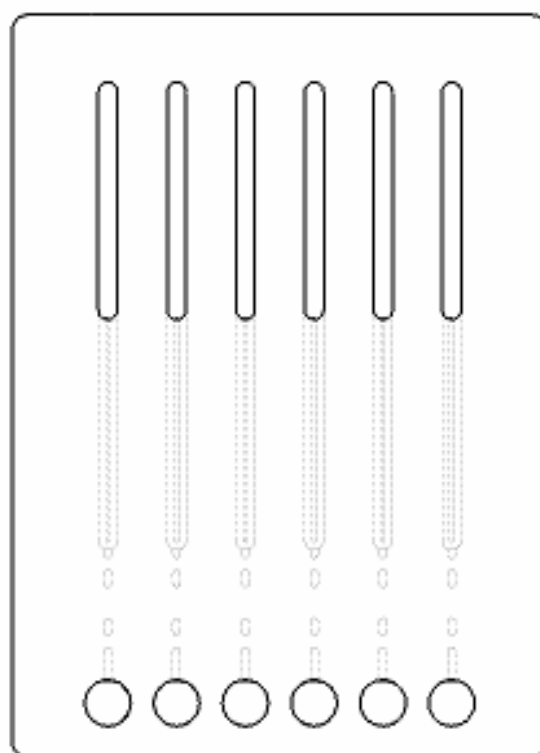**Figure 3.4. Cross-section of polystyrene bead channel design.**

Figure 3.5. Straight channel chip design for use of nitrocellulose and polystyrene strips.
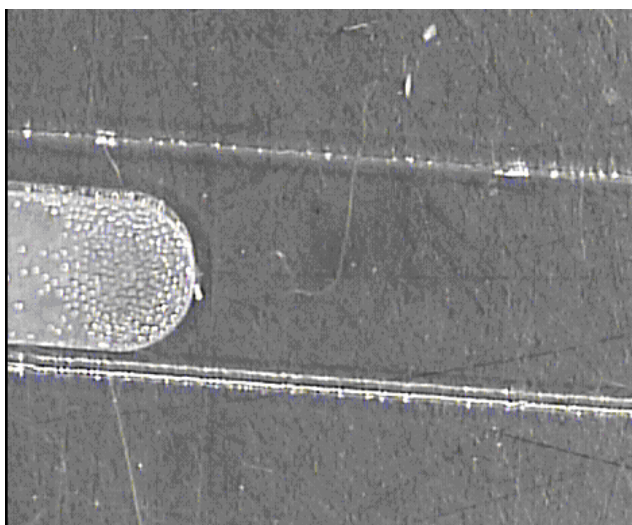
Figure 3.6. A straight channel (16mm x 3mm x 35μm) with polystyrene beads.

3.2 Reagents Preparation

The phosphate buffers (PB) used in these experiments was made according to the proportions described in Table 3.1 and Table 3.2. The solutions used in this experiment as suggested by the ELISA kits were .05% Tween 20 and phosphate buffer solution at 10mM and pH 7.4. Phosphate buffer preparation for ELISA tests were determined by the following methods (Segel, 1968):

To make a 10mM PB buffer solution at pH = 7.4 I applied the Henderson Hasselbalch equation which is written as

$$pH = pKa + \log\frac{[A^-]}{[HA]}$$

where [A⁻] is the deprotonated form and [HA] is the protonated form. Rearranging we have

$$\log\frac{[A^-]}{[HA]} = pH - pKa$$

Rearrange for [A⁻]/[HA]

$$\frac{[A^-]}{[HA]} = 10^{pH-pKa}$$

Now substituting the desired pH and the known pKa we have,

$$\frac{[HPO_4^-]}{[H_2PO_4^{2-}]} = 10^{7.4-7.21} = 1.5488 = \frac{1.5488}{1} ratio$$

$$[H_2PO_4^-] = 1/2.5488 = 0.392 \Rightarrow 39.2\%$$

$$[HPO_4^{2-}] = 1.5488/2.5488 = 0.608 \Rightarrow 60.8\%$$

Therefore, for a 1 Liter solution at 10mM we have,

$$10\,mM \times \frac{1M}{1000\,mM} \times MW \times Fraction$$

$$[KH_2PO_4^{2-}] = 10\,mM \times \frac{1M}{1000\,mM} \times 136.1\frac{g}{M} \times 0.392 = 0.534\,g$$

$$[K_2HPO_4^{-}] = 10\,mM \times \frac{1M}{1000\,mM} \times 174.2\frac{g}{M} \times 0.608 = 1.06\,g$$

Table 3.1. A list of all the chemicals used to create the buffer solutions used in this experiment. It also lists the pKa values that are useful in making the buffer solution.

Chemical Name	Formula	Molecular Weight	Manufacturer	pKa at 20°C 1	2	3
Potassium Phosphate Monobasic (Crystals)	KH_2PO_4	136.09	Mallinckrodt	2.12	7.21	12.67
Sodium Phosphate Monobasic	$NaH_2PO_4 \bullet H_2O$	137.99	Fisher Scientific Company	2.12	7.21	12.67
Sodium Phosphate Dibasic Anhydrous (Granular)	Na_2HPO_4	141.96	Mallinckrodt	2.12	7.21	12.67
Potassium Phosphate Dibasic (Crystals)	$K_2HPO_4 \bullet 3H_2O$	228.2	Sigma Chemical Company	2.12	7.21	12.67
Potassium Phosphate Dibasic Anhydrous	K_2HPO_4	174.18	Fisher Scientific Company	2.12	7.21	12.67
Polyoxyethylene Sorbitan Monolaurate (Tween 20)	$C_{58}H_{114}O_{26}$	1225	Sigma Chemical Company			

Table 3.2. Amounts of chemicals for different buffers

0.01M PB buffer, pH 7.4	g solute/L solvent (1L| 500 ml)	
Potassium Phosphate Monobasic (KH_2PO_4)	0.533	0.276
Potassium Phosphate Dibasic ($K_2HPO_4 \bullet 3H_2O$)	1.387	0.694
Potassium Phosphate Dibasic Anhydrous (K_2HPO_4)	1.059	0.503
Sodium Phosphate Monobasic ($NaH_2PO_4 \bullet H_2O$)	0.541	0.27
Sodium Phosphate Dibasic Anhydrous (Granular) Na_2HPO_4	0.863	0.432
100 ml 0.05% Tween 20	**ml Tween 20 | ml distilled water**	
Polyoxyethylene Sorbitan Monolaurate (Tween 20)	0.05	99.95

3.3 Dot ELISA Protocol

The following protocol was followed to perform the dot ELISA assay. A pump delay between each actuation used during all steps was 150ms unless otherwise specified.

1. Spot nitrocellulose strip with ~1-2μl of 0.005mg/ml of monoclonal antibody (mAb). (Off chip)

2. Incubate for 10 minutes or until dry.

3. Insert strips into the microfluidic channels and wash with 60μl PB pH 6.5.

4. Add 0.05% BSA (Bovine Serum Albumin) blocking buffer.

5. Incubate for 10 minutes.

6. Wash 3 times with 60μl PB pH 6.5.

7. Add 30μl secondary antibody (1:400 dilution of 0.7mg/ml)

8. Add 25μl TMB (Tetramethyl benzidine) substrate.

9. Incubate.

    a. For 5, 10, 20, and 35 minutes.

    b. Cycle at 5, 10, and 15 minutes. The cyclic procedure is a repeated 4 pumps forward and 4 pumps backward. A pump delay of 1000 ms is used for the cyclic incubation. For the 6 actuations we have a delay of 1000ms which is 6 seconds for one pump, therefore one cycle is 48 sec.

10. Wash with 200μl of distilled water.

3.5 Sandwich ELISA Protocol

Thyroid stimulating hormone (TSH) ELISA kit containing standard antibodies, enzyme conjugate, enzyme substrate (TMB) and stop solution were a gift from ChemUX BioScience (South San Francisco, CA). Mouse monoclonal anti-TSH antibody (Cat. No. ab1989-1) was purchased from Abcam (Cambridge, MA).

The initial ELISA experimental conditions including the incubation time of each step were referred to ChemUX standard protocol for its kit with modifications for a flow system. The test variables are located in Table 3.3 and Table 3.4. The assay procedure was as follows:

1. Dilute mouse monoclonal antibody (mAb) to a final concentration of 0.005mg/ml (5μg/ml).

2. A microcentrifuge tube (0.5 ml) was filled with diluted mAb and polystyrene strips added to the tube. Strips were incubated overnight.

3. Prepare the standard solutions with samples provided in the Test Kit from ChemUX.

4. Prime the chip prepared with 10mM, pH7.4 PB buffer at room temperature for a few minutes, try to remove any tripped air bubbles inside of the channel.

5. Dispense 40 μl of the standard solutions prepared in Step 1 to the sample inlet hole.

6. Turn pump on and run until the channel is fully primed by the standard sample solution.

7. Incubate the assembly at room temperature for 30 minutes.

8. Rinse the assembly with distilled water.

9. Dispense TMB, a Chromogenic substrate that produces colored substance under enzymatic conditions, and incubate at room temperature for 10 minutes.

10. Add Stop solution to the sample inlet hole and incubate for 5-15 minutes.

11. Take image of color change.

Table 3.3. Surface Coating Experiment Variables

Exp. No.	BSA Blocking			Washing	
	$V_{blocking}$ (μl)	$V_{washing}$ (μl)	P_{delay} (ms)	Time (min)	P_{delay} (ms)
1	35	150	400	4	150
2	35	150	400	4	150
3	35	150	400	4	150

Table 3.4. TSH Assay Protocol Variables.

Exp. No	Standard/Conjugate			Washing		TMB/Stop Solution		
	V_{std} (μl)	T_{flow} (min)	P_{delay} (ms)	$V_{washing}$ (μl)	P_{delay} (ms)	V_{TMB} (μl)	$T_{flow/inc}$	V_{Stop}(μl)
1	30	12	1000	150	400	20	5 min	15
2	35	12	1000	150	400	20	5 min	15
3	40	12	1000	150	400	20	5 min	15

4. Results and Discussion

The results for both the dot ELISA and sandwich ELISA on a microfluidic platform are discussed here in and the figures are presented at the end of this section.

4.1. Dot ELISA

Preliminary tests of the dot ELISA on standard nitrocellulose strips and on the prepared dot nitrocellulose strips show the feasibility of using these strips on a microfluidic platform see Figure 4.1. The strips show alternating positive and negative controls. The positives are indicated by a dark purple color, which is retained within the nitrocellulose disks. There is some background noise visible, typical of dot ELISA assays, but the results are clearly distinguishable. These strips were then tested on the microfluidic chip and were found to work well using the standard assay procedures. The assay was repeated with varying incubation times of the secondary antibody see Figure 4.2. From the results, we see that an incubation time of 10 minutes is optimal when compared to the background noise level.

A decrease in noise was desired and thus the washing step was changed. A washing step using a pump delay of 90ms was employed and a cyclic incubation procedure was also added. Once the channel was filled with secondary antibody the fluid was shuttled back and forth with a pump delay of 150ms for a specified amount of time. The results show that an incubation time of 10 minutes is ideal here as well, but a rapid washing step has reduced the background noise levels see Figure 4.3. A summary of the experiments and variables tested are presented in Table 4.1. Thus, the total processing time was ~35 minutes.

The appearances of faint lines in the positive results are believed to be the result of bubble formation in the channel. The formation of bubbles in these channels is an important factor, as it can reduce local volumes of fluid near the nitrocellulose strip. During fluid flow, these nitrocellulose strips have a reduced amount of reagent supplied to it, thus resulting in a local decrease of available antibody. This may cause a reduction in the colorimetric signal ascertained by the faint lines visible in the results. A typical bubble can be seen in Figure 4.4. The formation of bubbles was noticed to come primarily after the addition of a blocking buffer. This may be due to the difference in fluid properties (i.e., viscosity) of the blocking buffer and washing buffer. There also appears to be residual blocking buffer in the channel after the incubation step and the interface between the two fluids combined with the hydrophobic nature of polystyrene may be the cause of bubble formation, but has not been proven.

Table 4.1. Dot ELISA experimental variables.

Exp No.	Washing (pump delay ms)	Incubation (min)
1	150	5
2	150	10
3	150	20
4	150	35
5	90	5
6	90	10
7	90	15

4.2. ELISA

The straight channel design was successful in producing a rapid dot ELISA, but the results can only be qualitative in this assay. The color formed in nitrocellulose strips is intense but doesn't diffuse out of the strip and color intensity is not quantifiable. The ELISA, however, can be quantitative because color formation is developed in solution

and can be quantified with precision and accuracy via a spectrophotometer. The straight channel design was kept, but a polystyrene strip was utilized in replacement of the nitrocellulose strip to produce color in solution. Although, the strip has a smaller surface area, previous work (Lee, 2004) has shown good colorimetric detection using similar surface areas. Additionally, the enzyme-substrate reaction occurs at the surface of the polystyrene strip, thus all of the color formation remains in solution and is transported to the end of the chip for quantitative analysis. The test was repeated three times and the same results were obtained each time. Figure 4.5 presents a typical result which shows a high level of background color such that the positives are indistinguishable from the negatives. The fluid flow generated in the straight channel was greater than that observed with the nitrocellulose strips and thus a rapid washing step was not necessary. The level of background noise is typically much higher in microfluidic devices than in microwells because of their sensitivity, thus reducing background noise levels remains to be a problem (Bange, 2005).

To further investigate the level of noise seen within the straight channel a parallel experiment was conducted on the standard 96 microwell plate and for polystyrene strips (see Appendix for methods). The results show that the polystyrene strips actually exhibit a slightly stronger colorimetric detection but also show a greater level of noise (Figures 4.6 and 4.7). Since noise levels are greater on the microscale it is then no surprise that we could not distinguish between the positive and negative controls. The absorbance values for the standard plate and polystyrene strips are low because they were read on a microfluidic chip with a 100μm depth, which is considerably less than the 5mm deep microwells. In Figure 4.6 it is clear that the polystyrene strips produce an intense color

change represented by higher absorbance values, but are less distinguishable at the low concentrations. Thus, the potential for an ELISA on chip is shown, but a better understanding of the surface/antibody interaction is required to reduce non-specific binding and thus noise levels.

Polystyrene bead test results show that relatively good color levels were obtained in the channel, but again non-specific binding of the antibodies to the channel appears to be a problem. The positive and negative controls remain indistinguishable, see Figure 4.8. The channels from left to right are (-), (-), (+), and (+). In Figure 4.8, the negative controls at left appear to have a weaker color, but much of that color is seen further up in the channel near the polystyrene beads. A major problem observed while testing the polystyrene beads, is the formation of bubbles that get trapped in and around the beads. This was a recurring problem in these chips. The surface tensions of these bubbles are strong on the micro-scale, and thus cannot be broken down by an increase in flow. Some of the color variation is also attributed to the inability to control the specific number of beads in each channel.

A couple other polymer materials were also explored as potential materials from which strips could be made. These include polystyrene mesh and a polycarbonate mesh. An ELISA test was done manually on two plastic meshes to determine their feasibility and is shown in Figure 4.9. The results show that although a small color change is seen, a dramatic color change does not occur. This was shocking at first because I expected that a large surface area would enhance the color. This attenuation of color change is believed to be the result of the materials properties rather than the reagents because the same reagents from previous experiments were used. The polystyrene used in the mesh

is not the same as the polystyrene used in the plastic sheets. However, information concerning the polymeric structure is often held confidential by the manufacturer, as is the case here.

Other problems noticeable in previous studies of ELISA on chip include process times that have not been significantly reduced. It should be noted that these studies have used PDMS (polydimethylsiloxane) and PMMA (polymethyl methacrylate) primarily. Many previous studies have also used idealized antibody-antigen reaction systems (Lin, 2004). It has been noticed that as faster processing times are achieved it comes at a cost which is seen in expensive detection equipment.

For future studies and analysis a more quantitative method of describing the results should be taken. It has been shown that you may be able to analyze the pixel strength in a digital picture to qualitatively analyze the results (Lin, 2004). This type of detection and analysis is ideal because it's easy to use, portable and has the ability for point of care diagnostics. The pixel strength can be calculated and compared to a standard ELISA test on a microchip by simply taking pictures of the assay. The standard assay would serve as a way to measure the sensitivity of the results collected. However, it is important that the lighting remain exactly the same for all pictures taken or reference pictures would need to be captured at every experiment for calibration purposes. These pictures would then need to be analyzed with some simple computer software.
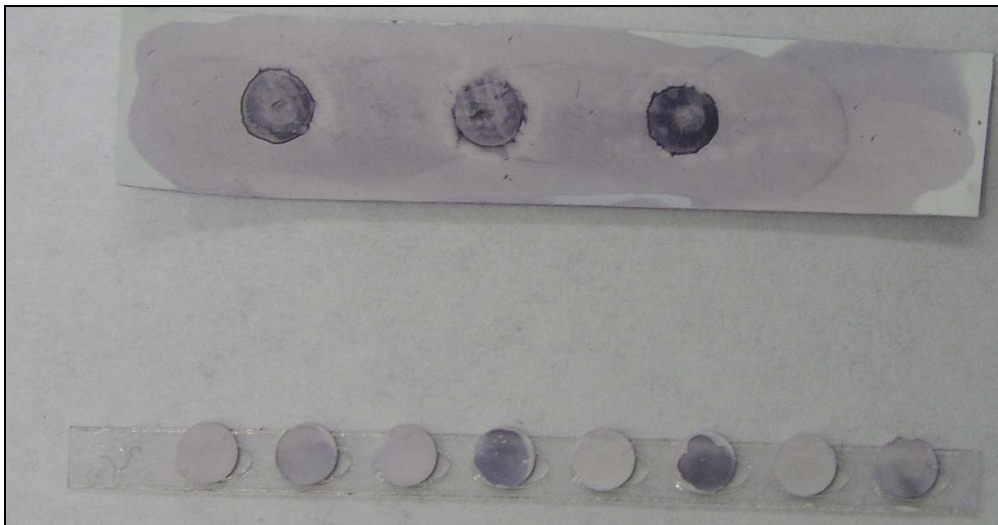
Figure 4.1. Elisa-spot reference strips with spotted nitrocellulose dots and strip.
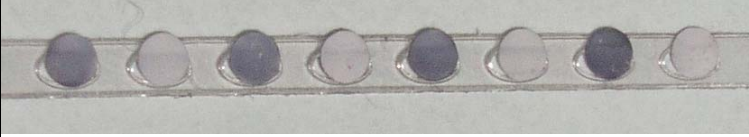
Incubation Time	Results
35 minutes	
20 minutes	
10 minutes	
5 minutes	

Figure 4.2. Dot ELISA results for different incubation times.
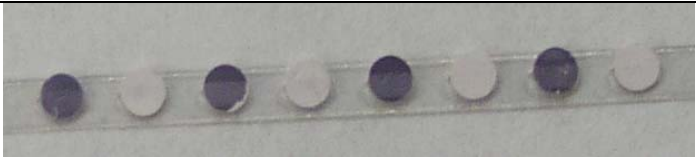
15 minutes	
10 minutes	
5 minutes	

Figure 4.3. Dot ELISA using with a cycled incubation and rapid washing.

Figure 4.4. A polystyrene channel with bubble formation in the channel.
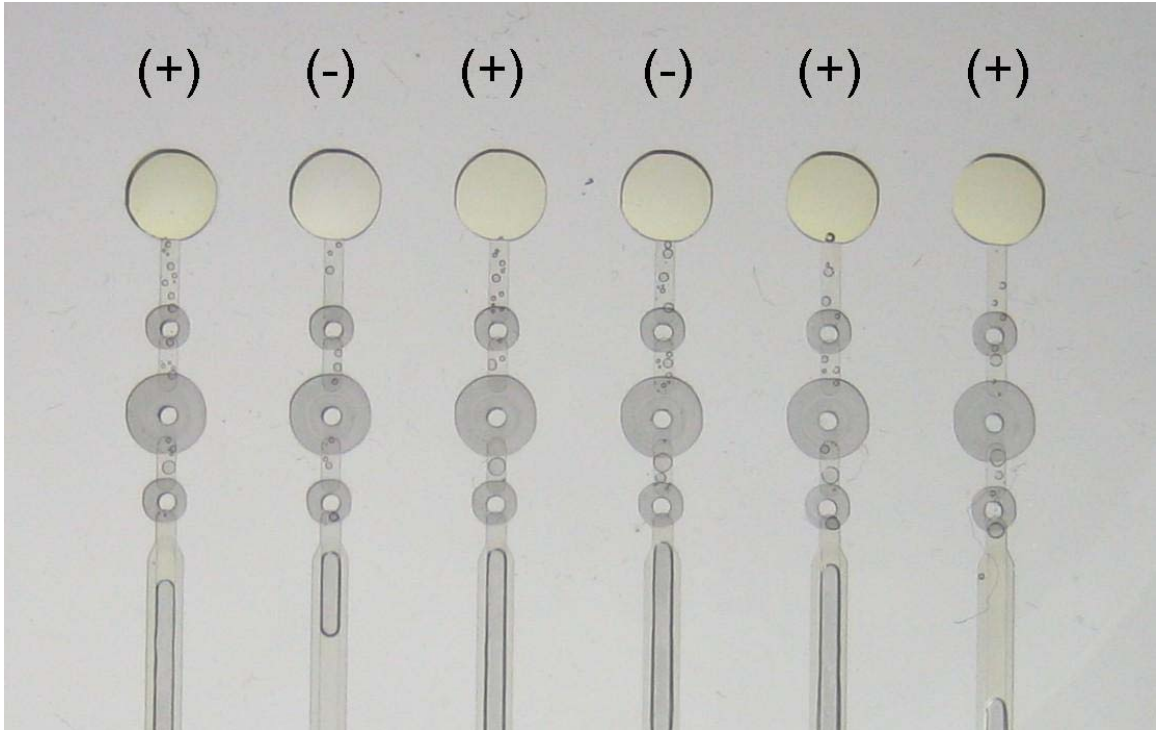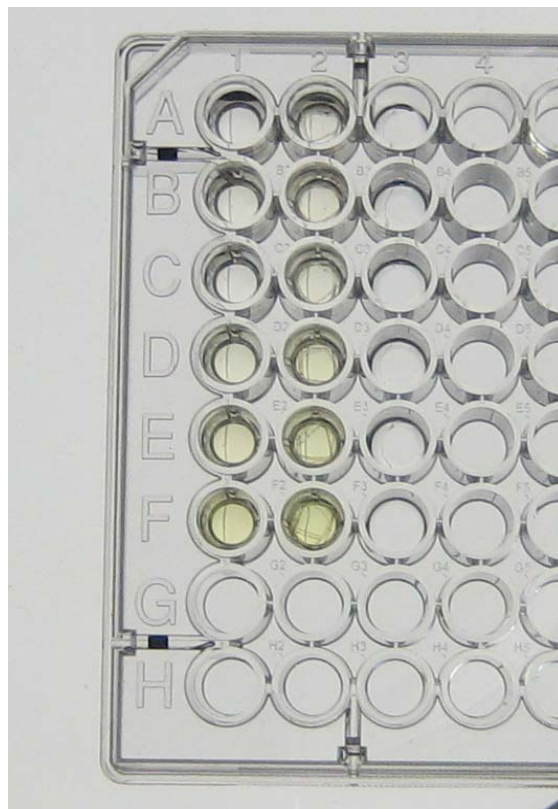
Figure 4.5 Sandwich ELISA results.

Figure 4.6. TSH ELISA results for 96 microwell plate and polystyrene strip comparison.
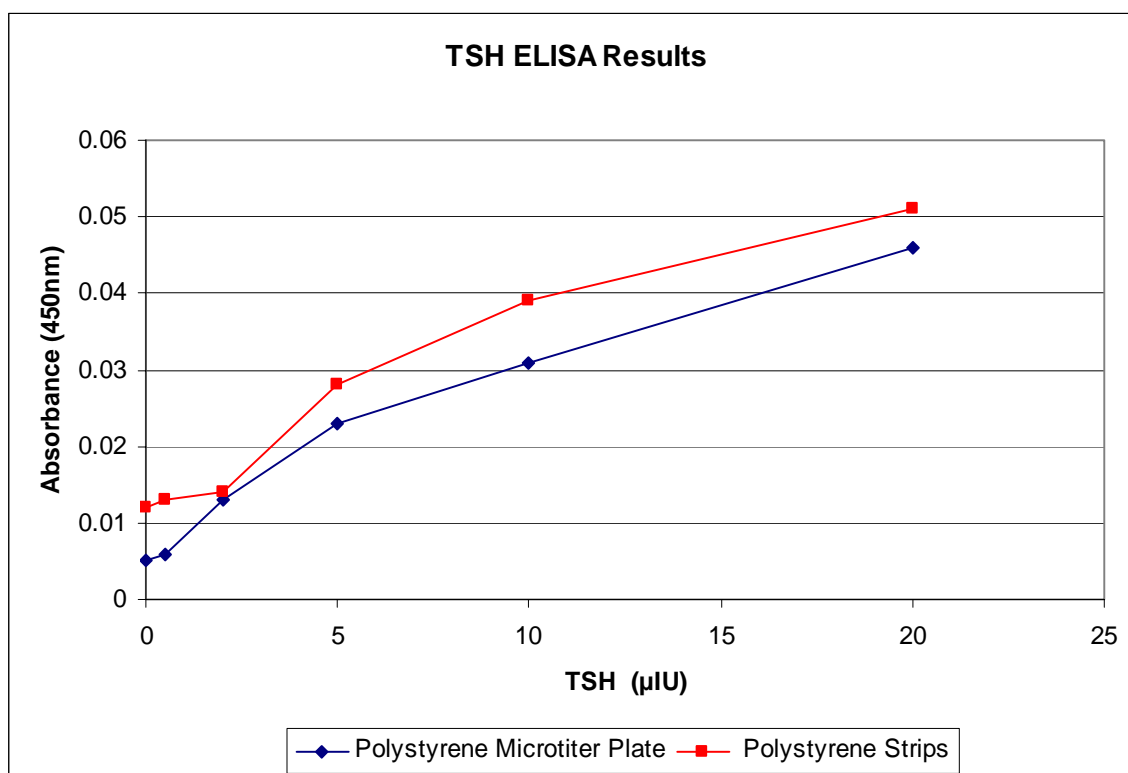
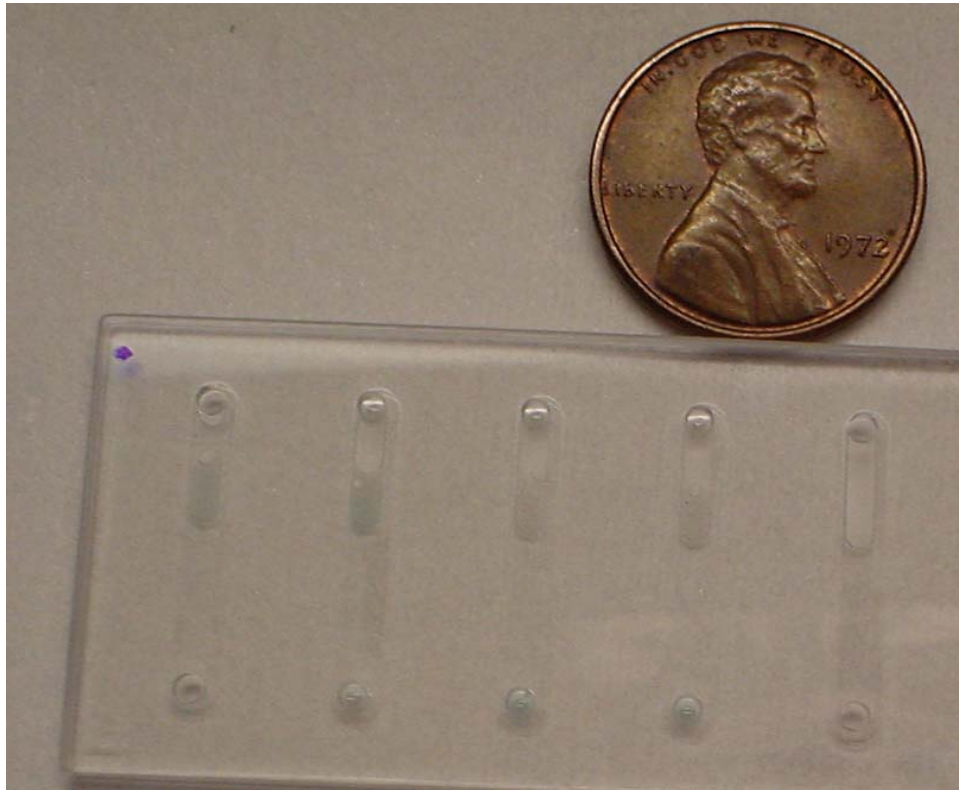**Figure 4.7. Absorbance measurements for the polystyrene strip and 96 microwell plate comparison.**

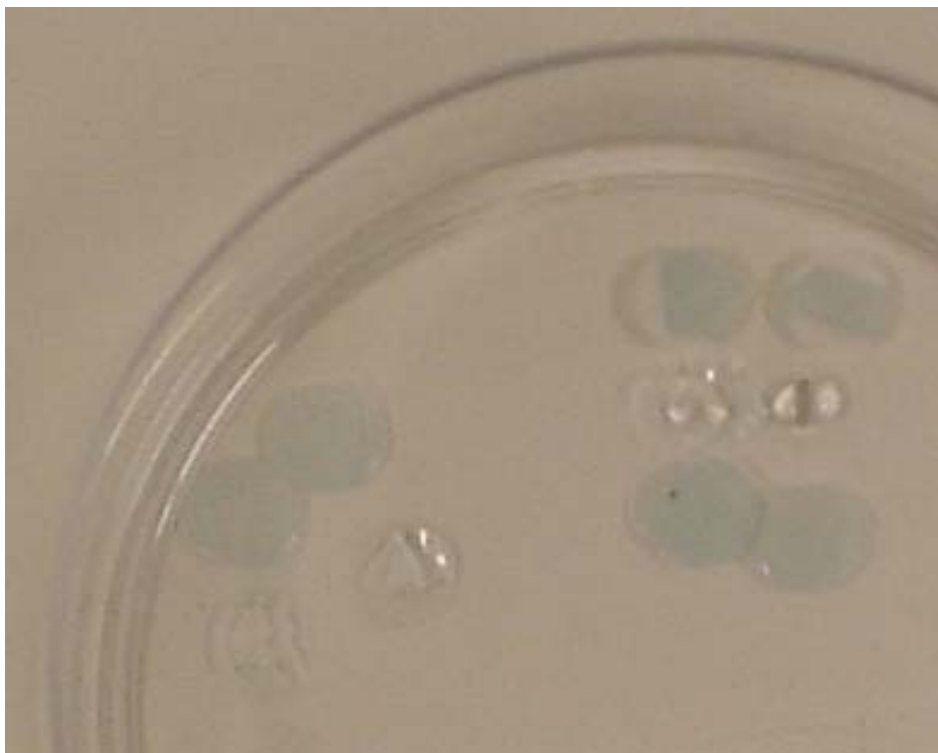**Figure 4.8. ELISA assay on a straight chip with polystyrene microbeads.**

Figure 4.9. Preliminary testing of polymer meshes.

5. Conclusion

The following conclusions have been drawn from this study:

1. A fast dot ELISA method that can semi-qualitatively analyze samples while using low reagent consumption is described. The total processing time was reduced from an order of hours to 35 minutes. A practical visual method that does not require special electric components on chip or optical devices for end-point reading has been used. These chips have the potential to be prototyped and produced in an economical fashion. The materials used to produce these chips are cheap and thus a disposable device is possible. Furthermore, small chip dimensions and controller footprint allow system portability and rapid point-of-care use in the field conditions.

2. A sandwich ELISA with the ability to produce quantifiable color changes has been demonstrated on chip as previous studies have shown (Rossier, 2001, Yakovleva et al, 2002, Jiang, 2003, & Lin, 2004). The difference in this study is that the device was made completely from polystyrene, but exhibited similar problems (background noise, contrast) with non-specific binding as observed in other microfluidic ELISA studies. A better understanding of the immobilization process on different types of polymer materials should aid in the improvement of these devices.

6. References

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7. Appendix

7.1. Polystyrene Strips and 96 well plate comparison Procedure

Four different concentrations of standards (0.5, 2, 5, 10, 20 μIU) plus one negative control were used in this experiment.   The polystyrene strips were put into one set of wells to run the experiment before the addition of TMB solution. The strips were then transferred into a new set of wells to ensure that color formation is from the polystyrene strips only.

The following steps were followed in the experiment:

1. Add 100μl of 5 μg/ml primary TSH antibody into each well,

2. Incubate for 80 min,

3. Wash 4 times with 200μl of PBS pH 7.4,

4. Add 115μl of blocking buffer to each well,

5. Incubate for 45 minutes,

6. Wash 4 times with 200μl of PBS pH 7.4

The steps according to TSH ELISA kit are as follows:

1. Dispense 50μl of standards, specimens, and controls into appropriate wells,

2. Dispense 100μl of Enzyme Conjugate Reagent into each well,

3. Thoroughly mix for 30 seconds. It is very important to have complete mixing in this step,

4. Incubate at room temperature (18-22°C) for about 60 minutes,

5. Remove the incubation mixture by flicking plate contents into a waste container,

6. Rinse and flick the microtiter wells 5 times with running tap or distilled water,

7. Strike the Wells sharply onto absorbent paper or paper towels to remove all residual water droplets,

8. Dispense 100μl of TMB solution into each well. Gently mix for 5 seconds,

9. Stop the reaction by adding 100μl of stop solution to each reaction well,

10. Gently mix for 30 seconds. It is important to make sure that all the blue color changes to yellow color completely,

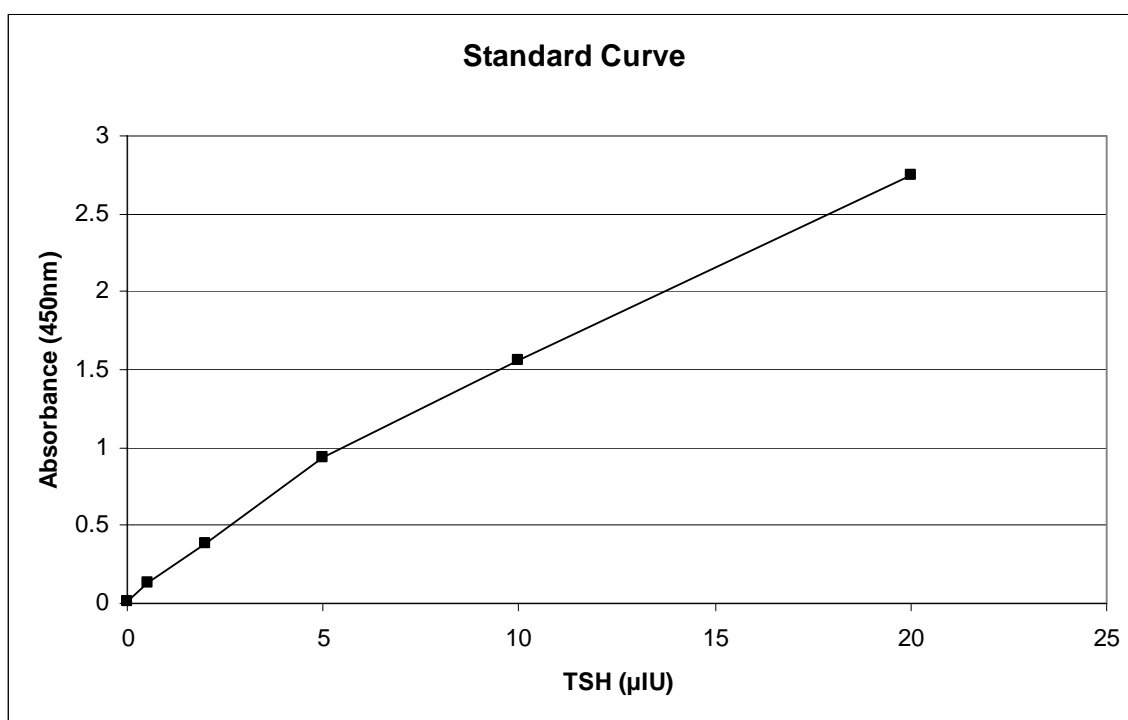11. Read with spectrophotometer at 450 nm

Figure 7.1. Standard absorbance curve adapted from ChemUX BioScience protocol.

7.2. Adsorption Kinetics

To achieve a shorter assay time, it is logical to reduce incubation times. An introduction to the kinetics behind the incubation step in the assay procedure is presented herein.

Adsorption of antibodies and immunoglobulins, such as IgG and IgA, at solid-liquid interfaces is dependent on several factors which include hydrophobic and electrostatic interactions, pH of solvent, 3-dimensional structure of protein, and the surface properties to which it's being adsorbed. The adsorption of proteins to solid surfaces can lead to alterations in the 3-dimensional structures of antibodies. The rate of conformational changes and severity of these changes vary greatly with time. Adsorption on hydrophobic surfaces is favored by interactions that counteract the electrostatic repulsions. For the purposes of this study, a short immobilization step is utilized (on the order of minutes), therefore long-term conformational changes will be ignored.

Adsorption of antibodies can be described in several ways depending on the conditions of the environment. The adsorption isotherms can be modeled as a Langmuir Isotherm given by

$$CS = \frac{Kc}{1 + Kc}$$

where CS = equilibrium solute concentration in the solid phase, K = partition coefficient and c = the concentration of solute in solvent.

Adsorption isotherms are characterized by a steep initial slope and an immediate plateau (saturation). The amount of desorption is higher for hydrophilic surfaces and pH levels that stray away from the isoelectric point of the solvent (Giacomelli, 2002). A change in pH can alter the conformation of the antibody or

immunoglobulin and thus affect the adsorption. In a previous study polystyrene exhibited the thickest layers of adsorbed immunoglobulin when compared to silica, methylated silica, gold, silanized mica, and gold coated mica (Giacomelli, 2002).

Antibody adsorption to polystyrene is dependent on the following (Crowther, 1995):

- Ratio of the surface area being coated to the volume of the coating solution.
- Diffusion coefficient of the attaching molecule
- Concentration of the substance being adsorbed.
- Temperature
- Time of adsorption.

The surface area to volume ratio and the diffusion coefficient of a protein are the two key factors affecting adsorption of proteins to solid surfaces in a microfluidic channel. Antibodies added to a micro-well in a microtiter plate, will diffuse from regions of high concentrations to regions of low concentrations. Adsorption of antibodies to the micro-well surface will decrease the antibody concentration locally. Therefore the immobilization step is a diffusion based process. The diffusion coefficient of the antibody may be estimated by the Stokes-Einstein relationship:

$$D = \frac{kT}{6\pi\mu r_h}$$

where r_h = hydrodynamic radius of particle, k = Boltzmans constant, μ = solvent viscosity, T = temperature (K), D = diffusion coefficient, where the $6\pi\mu r_h$ term is the drag (friction).

The time its takes for an antibody to diffuse to the solid wall of a well, is proportional to the distance that it has to travel. Therefore, the rate at which the antibody reaches a solid surface in a microfluidic channel is much higher than that observed in a microtiter plate well. For a rectangular channel with dimensions 40μm x 100μm x 20mm the time it takes an antibody to reach the side wall is on the order of a few seconds, see Figure 6.2.
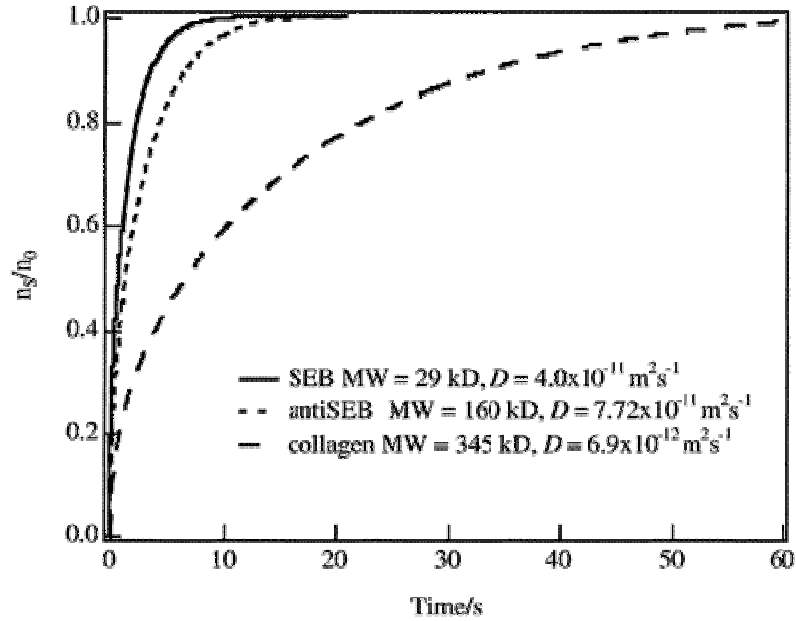

Figure 7.2. The ratio of total molecules that contact a mcirochannel surface as function of time based on Fick's second law of diffusion (Rossier, 2000).

In addition, the microfluidic channels have a characteristic flow, which incorporates convective diffusion into the immobilization process. The movement of proteins to the wall surface in laminar flow can be described by stagnation point flow (Buijis, 1996). It has already been shown by simple calculations that enzyme amplification in channels can be upwards of 70 times more efficient than microtiter plates (Rossier, 2001).

41

7.3. Equipment

The following list of equipment was necessary to conduct the experiments previously described.

1. Mitutoyo Absolute: CDI Chicago, Chicago Dial Indicator Co., Des, Plaines IL, 8"x12" Granite Base, P/N CD1812-1 Accuracy: .0001, Serial Number: 2355.

2. pH meter. Fisher Scientific. Accument. Basic AB15

3. Scale. Mettler Toledo. AB104-S. Max 110g e = 1mg. Min 10mg d = 0.1 mg.

4. TSH Enzyme Immunoassay Test Kit. (Cat No. CL10303, Lot No. 112702 – 02.) Chemux bioscience, Inc. South San Francisco, CA 94080, USA.

5. Abcam. Mouse Monoclonal to Human TSH 1.3 mg/ml. (ab1989-1. lot:806032P)

6. Human Chorionic Gonadotropin (HCG). Enzyme Immunoassay Test Kit. (Catalog Number: 10002)

7. Model HRL Dry-Film Roll Laminators. Think and Tinker Ltd.

8. Hot Plate Stirrer. Labonco.

9. Nano SU-8 Photoresist. Negative Tone Photoresist. Formulations 50-100. MICROCHEM. Newton, MA.

10. Microscope Camera. Warner Electric. Chicago, IL USA. (Model: 100B)

11. CNC Sherlin Mill Model 2000. Sherline Products Inc., 3235 Executive Ridge, Vista, CA.