Development of an Integrated Capillary valve-based Preconcentrator and a Surface-based Immunoassay

by

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Abstract

A new generation of integrated preconcentrator and immunoassay was developed. A novel, self-aligned method for patterning Nafion resin was developed and applied to create a preconcentrator. In a parallel effort, a surface-based immunoassay was developed and optimized for use with the preconcentrator.

Experiments were carried out with R-phycoerythrin (R-PE) to characterize the performance of the integrated preconcentrator and immunoassay. The immunoassay originally has a detection limit of 1ng/mL of R-PE in 1xPBS. When coupled to the preconcentrator, this limit has been improved to 0.01ng/mL, a roughly 100x improvement. Discussions of the differences between this and previous generations of concentrators are also presented in this thesis.

Thesis supervisor: Jongyoon Han
Title: Associate Professor of Electrical Engineering and Computer Science

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Contents

Chapter 1

Introduction

Plasma proteomics and their implications in clinical diagnostics

Plasma, or the liquid component of blood with the blood cells removed, remain one of the most important and clinically obtainable specimens. It also represents the largest and deepest human proteome that's present. Specifically, plasma contains abundant proteins such as albumin, tissue leakage proteins such as tissue damage markers, and local signaling proteins such as cytokines [1]. Given the diversity of proteins present in the serum and plasma, it's not surprising that many clinical tests rely on the accurate detection and quantification of serum/plasma proteins. Tissue leakage proteins are important because a small amount of proteins leaked into the plasma from an organ can represent a significant pathological state in the local organ. For example, cardiac myoglobin is present in a range of 1-85ng/mL in healthy individuals, but the concentration rises to 200-1100ng/mL in a person with active cardiac infarction. [1] An important class of local signaling proteins is called cytokines, which includes indictors of inflammation or infection. These proteins act as local signaling agents between cells, but are detectable in plasma at much lower concentrations after it has been diluted into a systemic level [1]. While genomic techniques, such as DNA or mRNA microarrays, have made significant inroads into both biological research and clinical diagnostics, genomic expression does not always correspond to the protein content found in serum or plasma. The reasons for this include post-translational modifications and protein-protein interactions. This made plasma protein levels the ideal candidate for diagnosing/detecting protein-based biomarkers. [2]

The large dynamic range of proteins raise an interesting challenge – the ability to design a technology that can detect high abundance proteins as wells as low abundance ones would certainly be useful in a clinical setting. The ideal system would be able to measure protein levels down to sub pg/mL concentration, while at the same time maintain the ability to detect higher abundance molecules. Also, recent research has shown that complex, systemic diseases such as cancer affect multiple protein products simultaneously, and measuring a single biomarker has not been as effective an indication of disease progress as previously thought. [3] As most sensors have a limited dynamic range of around 4 to 5 orders of magnitude, they don't have the capability to cover the entire proteome (about 10 orders of magnitude), and a better method to improve sensitivity and extend the dynamic range is needed. We are currently attempting to direct efforts in the lab to address this problem by locally increasing the concentration of low abundance proteins and thus resulting in a larger dynamic range available for detection and to improve the detection of clinically relevant markers.

Current clinical diagnostics techniques and equipment

Current methods of quantifying concentrations of proteins in biological samples involve various immunological methods as well as technologies that are derived from analytical chemistry, such as mass spectrometry.

The immunoassay remains one of the most popular methods for detecting pathogens in clinical samples and in research labs. Immunoassays rely on antibodies that are immobilized to a solid phase/surface to capture antigens that are suspended in solution/the mobile phase. Antigens that react with immobilized antibodies would be captured and immobilized together with the antibodies on the surface of the chip. A wash step follows to remove unbound antigens and the bound antigens can be quantified using fluorometric, electrochemical, or radioactive methods. [4]

Enzyme-linked Immunosorbent Assay, or ELISA, takes the concept of the immunoassay one step further by adding an amplification step after the antigen binding step. Instead of a secondary antibody with a simple fluorescent/radioactive tag, the secondary antibody is tagged with an enzyme, such as Horseradish peroxidase or alkaline phosphatase. After the secondary antibody is attached, substrates for the enzyme is flowed mixed with the bound complex. The enzyme attached to the antibody would react with the substrate and generate a signal in the form of a fluorophore; since the enzyme would continually generate additional fluorescence over time, the original signal is amplified over time and allow for more sensitivity detection of analytes in solution. [4]

A second technology that's currently used for detecting pathogens is mass spectrometry. Proteins carry specific mass signatures because of the unique combinations of components contained in their structure, and mass spectrometry achieves detection of specific proteins by comparing the signature to an established database of different proteins. The advantage of mass spectrometry is that it can analyze a number of different proteins at the same time, i.e. profile the protein contents in human serum. A number of complex, systemic diseases are usually best characterized by a panel of different proteins. Diseases such as cancer alter the concentration and conformation of a number of proteins, and the expression profile of proteins often contain useful information about disease progress and details of the disease itself.

These techniques are now quite well established in the diagnostics industry and have become the standard for clinical diagnostics tools. However, most of these techniques require labor-intensive work, are prone to human error, and are time consuming. The development of microfluidics assays, or "lab-on-a-chip" devices holds the promise of alleviating many of these problems, and some of these development would be detailed in the next section.

Microfluidics immunoassay, SPR, electrochemical methods, and more

There has been tremendous progress made on the design, fabrication, and usage of microfluidics devices over the past decade. Microfluidic devices offer the advantage of miniaturization, which reduces reagent consumption, and automation by integrating multiple processes. These devices reduce the frequency of human errors and ensure better repeatability as well as better sensitivity. Microfluidics-based assays also have the advantages of decreased assay time and thus opening the door to the use of these assays in a point-of-care setting.

A variety of materials are used to fabricate microfluidics devices, including silicon, pyrex, and polymers such as PDMS and PMMA. Regardless of the material used, antibodies need to be attached to the microchannel to capture analytes of interest. There are a number of ways to immobilize antibodies in a microfluidics channel – by attaching them to the microchannel surface or by immobilizing antibody-loaded beads in the microchannel. A number of different protocols, including physiosorption, chemisorption, and covalent linkages, have been investigated to immobilize antibodies onto the channel surface [5]. Similarly, a number of different bead trap designs have been proposed in the literature, including weir shaped structures that physically trap beads and chips that integrate magnets to traps and release beads. [6]

Other assay technologies that have been developed on microfluidics platforms include Surface Plasmon Resonance (SPR) and a number of electrochemical techniques. These technologies boost advantages such as label-free detection, real time kinetics monitoring, and excellent sensitivity. SPR works by measuring changes in a material's refractive index to light. Molecular binding events, such as antigen binding by antibodies that are bound on the surface, changes the surface's refractive index and thus reports a signal to the sensor. [7] Electrochemical methods include potentiometric (voltage sensing), amperometric (current sensing) and others. Typically, electrodes or microchannels are functionalized with antibodies, and binding events would cause a change in the resistivity of the buffer and thus transduce a signal to the sensor. [8] These sensors have the advantage of being inherently more integrable with microelectronics than sensors that rely on fluorescence readouts. However, the lack of good and established amplification methods limited the popularity of electrochemical sensors.

Miniturized systems often integrate sample preparation processes as well as the assays. In the next section, signal amplification and preconcentration processes that can be integrated with the assays are described and reviewed.

Microfluidic Signal Enhancement– pre and post binding

Due to the biological significance of low abundance proteins, a host of amplification methods have been developed both in microfluidics. Signals from molecular recognition events can be amplified in two ways: before binding and after binding. Most of the pre-binding signal enhancement focuses on increasing the number of molecules that would bind to capture antibodies, and post-binding signal enhancement increases signal output from molecules that are already immobilized. In this section, techniques amendable to use in microfluidics chips are discussed along with the technologies developed in our lab

There are a number of ways to preconcentrate sample molecules before they react with the capture antibodies. Field amplified stacking works by having a low conductivity buffer sandwiched between two buffers with higher conductivity. When a voltage is applied, the high electric fields in the low conductivity buffer stack the molecules against the boundary of the zone. [9] This is similar to isotachophoresis, where a gradient of molecules with different electrophoretic mobilities are stacked with respective to a gradient generated in steady state. These techniques establish a concentrating zone using properties of the buffer, which requires tedious buffer exchange and limitations of buffer compatibility. One of the solutions is to use electrokinetic manipulations, such as that developed in our lab, to perform preconcentration. This technique also doesn't require the use of prefabricated membranes or special structures in the microchannels.

Post binding signal enhancement increases signal generated by captured molecules by increasing their fluorescence intensity or other readout signals. The classical post-binding amplification method is ELISA, which relies on enzymatic activities to progressively increase the signal strength after binding of the secondary antibody. Recently, Schweitzer et. al. have reported the use of rolling circle amplification (RCA) as a sensitive and specific way to amplify signals from bound molecules [10]. The advantage of pre-binding amplification is that both sensor sensitivity and binding kinetics would be improved, leading to both faster and more sensitive assay.

Though not discussed here, new sensor designs and detection mechanisms also help lower detection limits and dynamic range of diagnostic technologies. Ultimately, pre-binding and post-binding signal enhancement technologies can be combined so that the detection limit can be pushed to even lower than either one can achieve by itself.

Outline of thesis

In this thesis, I will describe the development of a surface-based immunoassay for detecting both fluorescent and non-fluorescent molecules present physiological ionic strength. Subsequent chapters would detail biomolecules immobilization, device fabrication, and device characterization. More specifically, Chapter 2 will be devoted to various surface chemistries and functionalization schemes. Device fabrication and Nafion membrane formation would be discussed in Chapter 3, and I will describe device characterization and immunoassay enhancement in Chapter 4. Finally, future outlook and research directions are proposed.

Chapter 2

Biomolecule Immobilization

Introduction

Separation technologies are at the heart of immunoassays. In heterogeneous immunoassays, unbound analytes that are in solution need to be separated from analytes that are bound to capture agents, such that only bound analytes remain at the signal generation and detection stage [13]. If there's a failure to remove all of the unbound analytes, the quality of assay would suffer as these unbound analytes would interfere with subsequent introduction of secondary antibodies or detection.

There are multiple methods to perform separation of bound/unbound analytes. Liquid phase separation involves the use of electrophoresis, size exclusion chromatography, or precipitation to separate antibody-bound analyte from unbound analytes [13]. Most of these methods are nonspecific and can be affected by the concentration of protein in the sample, resulting in poor predictability and performance. [13] Another method for separation is for capture agents to be immobilized on a solid/insoluble support structure. Since captured molecules would no longer be in free solution, it becomes possible to specifically separate the analyte that are bound to the solid phase from those that remain in solution.

A number of methods have been derived for the immobilization of biomolecules on surfaces, and remain an active area of research today. A number of classical antibody immobilization techniques have been developed for use in immunoassays in both traditional and microfluidic formats. Some of these methods draw inspirations from the technology used in DNA microarrays [18] as the development of protein/antibody microarrays have been a big driver in the development of these surface chemistries. These techniques have been consequently adopted for use in some microfluidic immunoassays. However, immobilization of proteins proved to be more difficult than DNA because of fundamental biochemical differences between the two. First of all, proteins differ from each other in terms of structure, affinity for analytes, and interaction with the solid phase. A number of proteins also partially or completely denature when coupled to a solid phase, further compounding this problem of protein attachment. Non-specific binding of analytes to either capture agents or the solid phase support adds noise to measurements and requires special blocking steps or chemistries to minimize. Fortunately, a number of more sophisticated methods have been developed to bind proteins to solid phases. These methods aim to provide better detection sensitivity by providing oriented antibody attachment,

Silicon, glass, and polymers are the most popular materials for the construction of microfluidic devices. Of these materials, polymer materials are gaining more widespread use because of its low-cost and ease of fabrication [13]. As such, a major part of this section would be devoted to modification of our PDMS/glass microfluidic devices. We found that the best

performances have not been achieved with complicated immobilization schemes, but rather with simpler ones on our system.

Covalent attachment methods

Covalent attachment of proteins to the solid support involves the reaction of one or more amino acid residues on the protein side chains with suitable functional groups on the modified surface [5]. The advantage for covalent attachment is that the binding of protein to surface would be strong and irreversible at a variety of conditions. Covalent attachment also allows antibodies to be attached in a specific orientation by modifying the structure of antibodies so that that would be attached at the hinge region [12]. Self-assembled monolayers (SAMs) have emerged as a strong candidate for use in functionalization of biosensor surfaces. SAMs have the advantage of being uniformly arranged on a surface for given reaction parameters and thus given well characterized support structure for protein attachment. Unfortunately, most reactions in creating self-assembled layers have stringent requirements on the cleanliness of surface and reagents, making it more difficult to adapt the system to our use [12]. Covalent attachment of these molecules can also change the surface properties and introduce unwanted side effects. For example, silanization using amine-terminated silane SAMs creates reaction sites for protein attachment, but also makes the channel more hydrophobic and difficult to fill.

Silanization

As glass slides are used as part of our microfluidics system, silanization is chosen over use of gold-based thiol SAMs to ease fabrication and characterization. Silane molecules can be deposited on clean glass slides with active silanol groups [11]. For our experiments, a protocol from reference [11] is followed. An amino-terminated silane, Aminopropyltriethoxysilane (APTES, Sigma-Aldrich, MO) is used without further purification. Glass slides (VWR international, PA) are first immersed in Nanostrip (a mixture of sulfuric acid and hydrogen peroxide) for 30 minutes to remove organic residues and to activate the silanol groups on the surface. The glass slides are then immersed in 5% APTES in acetone for 30minutes. The slides are taken out afterwards, rinsed with DI water and ethanol, and cured in a vaccum oven overnight at 80 degrees Celsius. Following silanization, slides are reacted with glutaraldehyde for 2 hours and then incubated with the antibody solution for 8 hours. Slides are stored in PBS prior to use.

Two sets of experiments are carried out with silanized glass slides: bead immobilization and protein attachment. Shown below are some of the results done with the previously mentioned method. Sivagnanam and coworkers have previously shown that small magnetic beads can self assembly on glass slides silanized with APTES [15], and this method can potentially be used to immobilize functionalized beads in our system. Ultimately, we decided against using a covalent attachment method because of some of the drawbacks listed below. Glass slides immobilized with fluorescently labeled IgG (Invitrogen, CA) have areas of non-uniformity that probably resulted from non-uniform surface roughness and cleanliness of our glass slides. Silanized glass slides also cannot be easily bonded to PDMS pieces (Indeed, silane-adsorption is used to prevent PDMS from sticking to glass/silicon oxide on masters); this fact, combined with the multiple processing steps required for silanization, makes the devices more difficult to use and fabricate. Silanization also makes the surface hydrophobic (water contact

angle changed from 35 degrees to >70 degrees [12]). This has two effects on our devices: bubbles tend to form in hydrophobic areas of the device, making it more difficult to fill the microchannels; the Nafionmembranes made on these devices also tend to be unstable as the glass surface becomes hydrophobic after silanization. Finally, it is difficult to locally pattern silane patterns without the use of photolithography, which complicates the process. For these reasons, a non-covalent method of protein attachment has been chosen for use in our system.

Physical absorption

A number of other interactions, other than covalent reactions, exist for attaching biomolecules to biosensor surfaces. Physically absorbing proteins and antibodies on solid support is the simplest method to immobilize proteins and has been commonly used in immunoassays today. These methods rely on non-covalent forces, such as hydrophobic interaction between proteins and the solid phase, and electrostatic forces to achieve protein immobilization. Under this category, two different methods have been explored: (1) direct absorption and (2) bioaffinity based system.

Direct absorption

Direct absorption of antibodies has become well characterized with its use in Enzyme Linked Immunosorbent Assays (ELISA). In ELISA applications, the polystyrene on microtiter plates provides a hydrophobic surface for protein molecules to attach to. This surface is mirrored by the PDMS and glass channel walls in our devices. Once protein molecules absorb to the channel walls, they change their conformation to expose hydrophobic regions for bonding to the solid surface [16]. Typically, these proteins act as a protector layer as well to prevent non-specific absorption of subsequent proteins to the sidewalls. The ease of direct absorption comes at a cost: most proteins attached this way are randomly oriented and may not be functional. Indeed, some reports have pointed out that up to 90% of antibodies directly absorbed on surfaces lose their activities [16]. Proteins also have a chance to desorb from the surface once they are immobilized, resulting in less accurate assay results and decreasing repeatability. Fortunately, recent reports have pointed out that > 85% of the proteins would stay immobilized for at least 16 hours once absorbed, even in the presence of detergents [16]. The stability of proteins absorbed depends on the concentration of protein solution used for absorption and the condition the surface is placed in. It is believed that protein absorption to the surface happens in multiple phrases [16]. Proteins first form a relatively uniform monolayer by incubation the solution with the solid phase. As the incubation time increases, additional layers of proteins can be deposited can be formed on top of other layers. These layers are unstable and their formation should be avoided. Another factor that affects the quality of surface generated is the concentration of protein solution used for the incubation. Generally, an optimal concentration can be found for the specific material system with experimentation settings as shown in the subsequent sections.

Bioaffinity systems

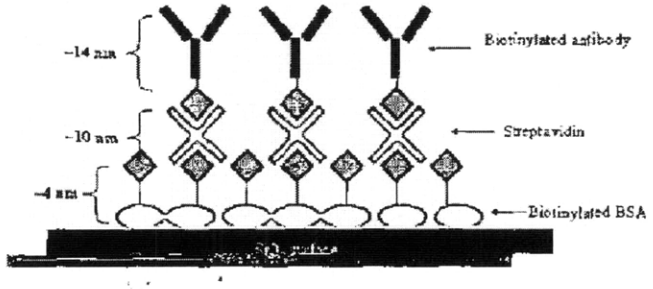
Many antibodies used in assays are biotinylated for coupling to avidin. Bioaffinity systems, such as the biotin-avidin system, provide a few advantages over random physical absorption of antibodies. Interaction between various components in the system is specific, so

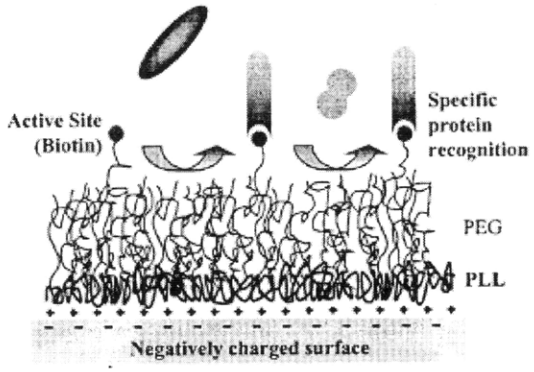
that only antibodies that are biotinylated would be immobilized to the surface; the biotin –avidin also allows for flexible movement of antibodies, thus allowing for some degree of orientation. The biotin-avidin is also carried out in mild conditions without use of harsh solvents [5]. An important advantage for the avidin-biotin interaction is that it is one of the strongest non-covalent interactions known [5], and thus reaction kinetics is quick.

There are multiple ways to utilize the biotin-avidin system. The first one, shown in Figure 1a, first uses physical absorption to immobilize a biotinylated molecule, such as biotin-BSA, on the solid phase surface; avidin can then be attached specifically to the immobilized molecules and a biotinylated antibody can be attached to the avidin afterwards [5]. Physical absorption of avidin to the PDMS surface can also be done and can potentially be easier to use as it skips a step. In our experiments, neutravidin is used in place of avidin for a few reasons. Neutravidin molecules tend to resist non-specific absorption better than avidin molecules, a property that is important for minimizing fouling; we also found that neutravidin produces a more uniform binding surface than avidin when coupled to the concentrator (data not shown). We have experimentally verified the sensitivity and specificity of both the biotin-neutravidin-biotin sandwich and the neutravidin-biotin pair, and have found them to be similar (data not shown).

A relatively new technology takes inspiration from the development of polyelectrolyte multilayers (PEMs). It involves a self-assembled layer of poly-l-lysine molecules on metal oxide surfaces. Poly-l-lysine (PLL) molecules are polycationic molecules that carry a positive charge at neutral pH. Most oxides, including silicon oxides and glass slides, carry a negative charge at neutral pH. Because of this, PLL-based molecules would self-assembly onto the surface of oxides from free solution via electrostatic interaction. Recently, researchers at Zyomyx have successfully grafted poly ethylene glycol (PEG) molecules onto PLL molecules and used it as a protein repelling layer [17]. The same group has further developed the technology to include biotin molecules on the PLL-g-PEG monolayer for further functionalization. These PLL-G-PEG/biotin monolayers offer the ability to uniformly pattern the surface with biotin molecules at a specific density, while the PEG molecules on the polymer offers strong non-specific absorption repulsion [17]. In this scheme, avidin or one of its derivatives (streptavidin or neutravidin) can be immobilized onto the biotin molecules, and a biotinylated antibody can in turn be attached to that (Figure 1b). While this scheme of protein immobilization provides a number of benefits, it is unsuitable for use in our system as will be explained in the following section.

a)





b)

Figure 1 Various ways to use the biotin-avidin system to attach biomolecules

a) Biotinylated molecule-avidin-biotinylated capture agent sandwich [19]
b) PLL-G-PEG/Biotin system showing a monolayer of poly-l-lysine conjugated to neutravidin and biotinylated capture agents [17]

Combining the two

Physically absorbed antibodies have the advantage of being easy to fabricate and integrate into our system, but may not offer the best results for analyte capture. Since the antibodies are positioned closely to the solid walls, steric hindrance can affect the ability of these antibodies to bind biomolecules and decrease the sensitivity of the sensor. [5] The antibodies are also randomly oriented, resulting in some antibodies losing their activities. One method to overcome this is to attach a spacer molecule to the antibody and to use that spacer molecule to attach to the surface [5]. Biotinylated antibodies with a long spacer molecule would be a suitable candidate for attaching to immobilized neutravidin molecules and are readily available for a number of different molecules of interest. Neutravidin molecules are multi-valent (each neutravidin molecule can bind up to 4 biotinylated antibodies) as well, making it less likely to lose all its capture function after physically absorbing to the pdms surface. To illustrate the difference between physically absorbed antibodies versus that absorbed using a biotin-streptavidin linkage system, a comparison of two pictures, both incubated for the same amount of time, exposure setting, and concentration of RPE, can be seen in Figure 2.

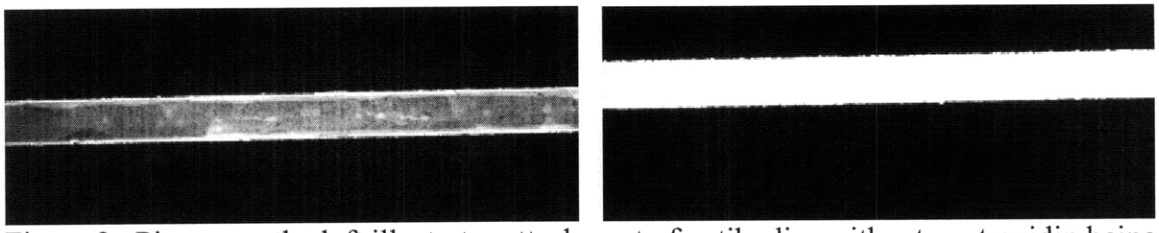


Figure 2. Picture on the left illustrates attachment of antibodies without neutravidin being coated on the microchannels first, whereas the picture on the right illustrates a neutravidin-biotinylated sandwich. The channels are both incubated with a 10ug/mL R-PE solution and quantified using a 250ms exposure time. Average intensities: left: 982; Right: 4095 (saturation of sensor)

Use in concentrator system

It is essential that the surface chemistry chosen works well when integrated with the concentrator. Since the concentrator relies on electrokinetic flow and electric current conduction, the velocity and flow profile of electrosmotic flow would be important. The velocity of electrokinetic flow depends on surface charge and surface roughness. In the PLL-G-PEG system, the surface charge of glass has been turned positive due to the presence of PLL, which is highly positively charged. These positive charges shield the negative charges present on the glass surface, reversing the flow of molecules and causing instabilities at the membrane junction. The brush-like nature of PLL-G-PEG layer also reduces the electrosmotic flow velocity by increasing surface roughness. While physically absorbed proteins would decrease electrosmotic flow velocity as well, they do not cause the same changes of the same magnitude. Given similar assay performance and more stable concentrator operation, the method of physically absorbing neutravidin followed by biotinylated antibody is chosen and would be used throughout this thesis.

Optimizing immobilization conditions

We performed a set of experiments to optimize reaction conditions with the physically absorbed neutravidin-biotin sandwich. The system is comprised of a layer of physically absorbed neutravidin followed by biotinylated anti-RPE antibodies. Since the interaction between these molecules and channel walls is based on hydrophobic forces, most of these molecules are immobilized onto the PDMS side walls (data not shown). PDMS plates with multiple wells (made by bonding two pieces of PDMS pieces) are used for testing various reaction parameters. The surface of each well is incubated with varying concentrations of neutravidin for 1 hour, followed by incubation of biotinylated anti-RPE for 30minutes. The second functionalization step is shortened to 30 minutes because of the fast biotin-neutravidin reactions. RPE molecules are then incubated for 1 hour in the wells; the wells are then rinsed with PBS-T buffer and their fluorescence intensities measured.

The parameters varied include initial neutravidin concentration, concentration of anti-RPE antibodies, and varying concentration of RPE analytes. We found that neutravidin concentration needs to be above a certain threshold level to produce a uniform surface and intensity. Though a higher concentration of neutravidin results in shorter incubation time, multilayers of proteins can form and this produces a surface unsuitable for use in our system. The observed results are consistent with previously published results [16]. Concentrations of biotinylated anti-RPE above 10ug/mL didn't produce noticeable differences in reaction time or fluorescence intensity of the final results. This is likely due to the high affinity constant of biotin for neutravidin; this leads to fast binding kinetics and equilibrium even with a lower anti-RPE concentration. The results of these tests are listed in table 1.

Neutravidin concentration	Anti-RPE at 0.1mg/mL		Ani-RPE at 0.01mg/mL	
	R-PE 1μg/mL	R-PE 100ng /mL	R-PE 1μg/mL	R-PE 100ng /mL
1mg/mL	2116	391	2391	405
0.1mg/mL	**2736**	**413**	**3153**	**430**
0.01mg/mL	1778	275	1824	323

Table 1. Various experimental conditions and their results are shown in this table. All conditions are exposed at 500ms with the LED system. The numbers in bold denotes the sample that displayed the highest intensities. Intensities are taken by selecting an area of uniform illumination, devoid of defects, and taking the average intensities across the area.

The intensities of captured R-PE molecules increase with increasing concentration of neutravidin molecules used. The fluorescence intensity is highest when a ~0.1mg/mL neutravidin is used for functionalization; RPE intensity decreases when neutravidin concentration is further increased at the incubation step. The differences in measured fluorescence intensities also seem to be more pronounced at high RPE concentration then low ones. Several possible reasons exist for these differences. For low neutravidin concentrations, equilibrium would take a long time to be established. This leads to incomplete functionalization at the time reactions were stopped and results in lower densities of molecules being attached to the surface. At high concentrations, formations of protein multilayers and steric hindrance can start affecting the subsequent binding of biotinylated anti-RPE and RPE molecules. The varying concentration of anti-RPE antibodies did not affect the resulting fluorescence intensities as much as the concentration of neutravidin molecules. This is likely due to the specific and fast interactions between biotin and neutravidin molecules, making a change in anti-RPE molecules minor. Reactions at different concentrations of RPE are limited by different factors. At high concentrations, reaction is limited by both the concentration of analyte and the density of capture antibodies, thus the binding capacity of the surface would significantly affect the results. At lower concentrations, reaction is limited mainly by the availability of analytes in solution, and thus the surface bound capture antibodies would play less of a role.

These coatings are also tested for their ability to resist non-specific absorption. A similar system [19], comprising of a biotin-streptavidin-biotin sandwich layer, can prevent non-specific absorption because of the steric hindrance it presents to molecules. Similar tests have been performed in our system using microfluidics channels because the high surface area to volume ratio of these systems; this property should lead to higher non-specific absorption in them and thus presents the worst case scenario. It also more closely resembles the systems used in our final experiments. In our experiments, no visible residual (compared to background) has been produced by a sample of 10ug/mL of R-PE in 1x PBS when the surface has been functionalized. On the other hand, patterns of absorbed R-PE can be seen on bare PDMS (Fig. 3). This showed that our system can effectively resist the attack of high abundance proteins that are not relevant to our experiments. This is particularly important when non-fluorescent proteins are used.

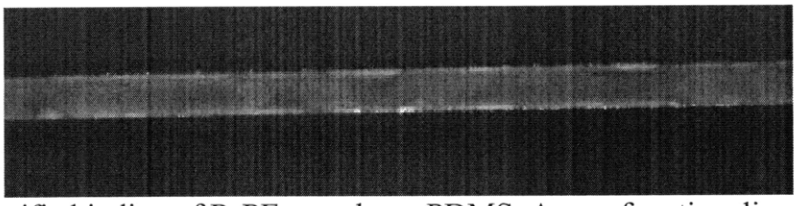


Figure 3. Non-specific binding of R-PE sample on PDMS. A non-functionalized, hydrophobic PDMS surface is highly susceptible to non-specific binding, as can be seen in this picture (average intensity: 475, 250ms exposure)

Selective protein patterning

Each of the above functionalization methods, by itself, would coat the entire microchannel with capture molecules. Depending on the width and depth of the microchannel (i.e. volume of the channel and ratio of volume to surface area), analyte molecules present in the sample solution can be entirely taken up before they arrive at the preconcentration zone. Sample depletion can be seen in Figure 4c. The channel shown in this microfluidic device has been coated with neutravidin and anti-RPE antibodies. In this experiment, sample is injected from the top right corner, turns around, and exits at the bottom right corner. A gradual decrease in fluorescence intensity can be seen as the flow progresses from inlet to outlet.

Sample depletion is immensely detrimental to the performance of the preconcentrator; the preconcentrator relies on packing sample molecules into a small zone for detection; therefore, the larger the capture area is, the larger amount of molecules would be captured outside of the detection region and the less efficient the system becomes. The difference between a selectively coated device and a device with a channel entirely coated with capture molecules is shown in Figure 4b and 4c. In this experiment, a 100ng/mL solution of R-PE in 1xPBS is used as the analyte, and the solution is concentrated using devices of the same design for 30 minutes. After 30minutes, the devices are flushed using PBS-T and examined with the microscope. Ideally, we would want the system to have only the area around the Nafion junction to be functionalized with capture molecules.

A number of methods exist for selectively patterning proteins at specific locations. Photolithography can be used to pattern [14] proteins at specific locations, but this involves the use of harsh chemicals and would add complexity and cost for our system. Two simpler methods would be discussed in the following sections: microcontact stamping and hydrophobic valve-based patterning.

The pictures shown below are of devices after concentrating a 100ng/mL R-PE solution in 1xPBS. In Figure 4a, Capture antibodies have been selectively coated near the Nafion junction, bound by the hydrophobic microvalves. As sample flows in from the top channel, a localized binding can clearly be seen near the Nafion junctions. Note that binding is limited to areas bound by hydrophobic microvalves. In Figure 4b, the entire channel is coated with capture antibodies. As most of the molecules are depleted before they reach the Nafion junction, a very small amount of R-PE molecules is actually concentrated. As a result, very small concentrated, localized binding can be seen.

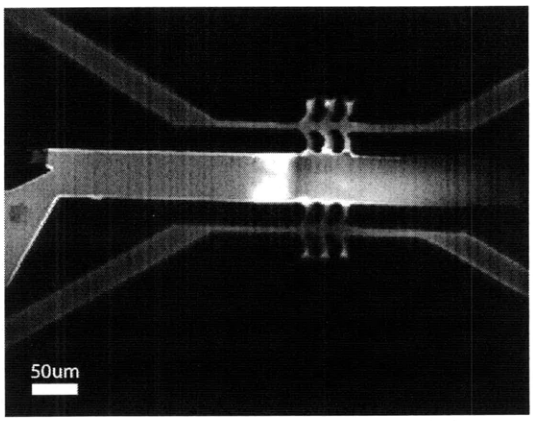


Figure 4a. RPE solution flows in from the top left corner into both bottom left and to the right hand side. Notice that the top left area beyond the capillary valves appears much darker compared to the detector area and the functionalization channels. Average intensity in concentration zone (100ms exposure): 967

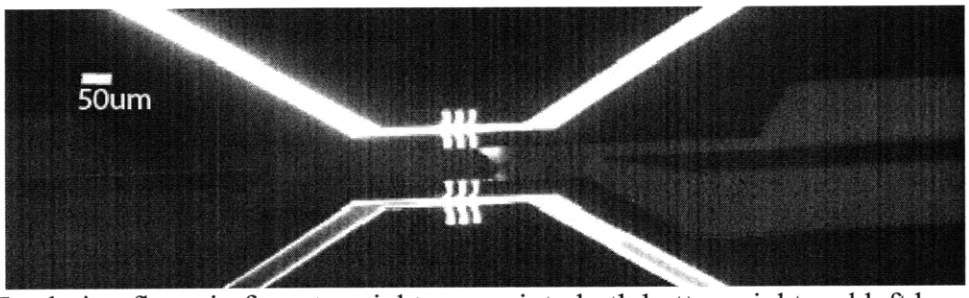


Figure 4b. RPE solution flows in from top right corner into both bottom right and left hand side. We can see that binding was happening in the top channel (where sample came in) and sample depletion has reduced the intensity of the concentration zone. Average intensity in concentration zone (100ms exposure): 154

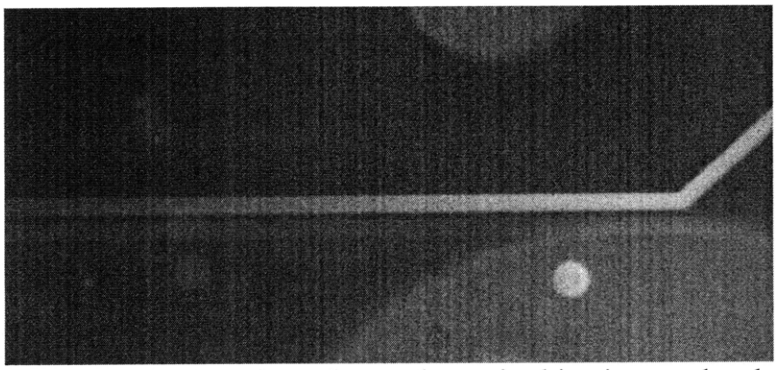


Figure 4c: Two interconnected microchannels are shown in this picture; the channels are coated with anti-RPE antibodies. A 100ng/mL RPE solution was injected from the top right corner, and exits in the lower right corner. This picture is taken after the channels has been flushed with PBS 1x, and shows a decrease in intensity along the sample flow path. This reflects the depletion of RPE molecules in the sample solution along the channel.

Microcontact stamping

Microcontact stamping is a specific example from a set of techniques known as "soft lithography" [34]. The stamps used typically have raised features on specific parts (the parts that would make contact with the surface) which can be used to pattern proteins or other molecules. A stamp can be generated via standard PDMS fabrication techniques, namely: first, a master in the shape of the desired mold is created in Su-8. Su-8 is used here to allow for the high aspect ratios required for stamp fabrication. PDMS is then cast on the master, resulting in am inverse replica of the master. This stamp can then be used to transfer ink to the substrate.

In our experiments, PDMS stamps are first oxidized in an air plasma machine for 15 seconds to make it hydrophilic. The stamp is then incubated in the desired ink (we tried PLL-G-PEG/biotin, protein A, and antibodies) for 20 minutes to allow for diffusion of ink into the stamp. The stamp is then blown dry with a nitrogen gun and the stamp is immediately dropped onto the cleaned glass slide. Conformal contact is achieved with the stamp's own weight and is maintained for around 2 minutes. The stamp is subsequently removed and the substrate rinsed with DI water to wash off any loosely bound molecules and then dried under a stream of nitrogen.

We were able to successfully print PLL-G-PEG/biotin [18] using a protocol from ETH, protein A, and antibodies. Figure 5 illustrates results obtained from one of our stamping experiments. After stamping, rinsing, and drying, the glass slides are ready for bonding with a PDMS substrate. Since exposure to plasma would result in destruction of the patterned molecules, the patterned glass slides need to be protected during plasma treatment. This can be done by placing a PDMS piece on top of patterned areas. Alternatively, we have verified experimentally that plasma treatment of glass slides is not required for them to bond irreversibly with plasma-treated pdms pieces.

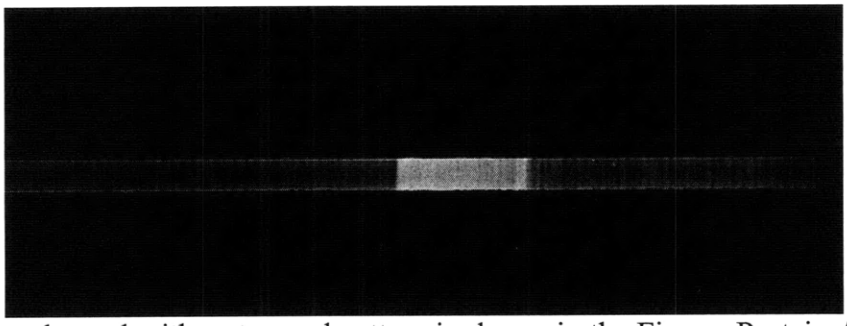


Figure 5. A microchannel with a stamped pattern is shown in the Figure. Protein A was stamped onto a glass slide and the glass slide is bonded with a pdms microchannel. FITC labeled IgG molecules is shown to selectively bind to the stamped area.

Further surface modifications are then performed within the microchannels after bonding. For stamped PLL-G-PEG/biotin, this involves the attachment of neutravidin and biotinylated anti-RPE. For stamped protein A, an IgG molecule would be attached at this point. Experiments are then performed on the system for reliability and sensitivity.

Since the patterned molecules present an area of weakness in bonding, there's a reliability concern with stamped and bonded devices. Liquid in microchannels can leak along these areas and move into other channels when pressure or electric fields are applied (Figure 6a). While this does not happen 100% of the time, it occurred at a high enough frequency to interfere with the

reliability of the assay. Changes to the geometries of the stamp, such as having a number of disconnected circles, should help alleviate this problem.

A bigger problem with this method is discovered when it is used with the preconcentrator. A device stamped with protein A is used in the experiment with a sample of fluorescently labeled IgG molecules. In this case, the preconcentrator is used to enhance binding of IgG molecules to the stamped protein A molecules. However, instead of being enhanced, a comparison between the area where molecules concentrated and the stamped area outside of the concentration zone reveals that these areas, where binding is supposedly enhanced, actually has a weaker fluorescence intensity (see Figure 6b). Several repeated experiments have yielded similar results.

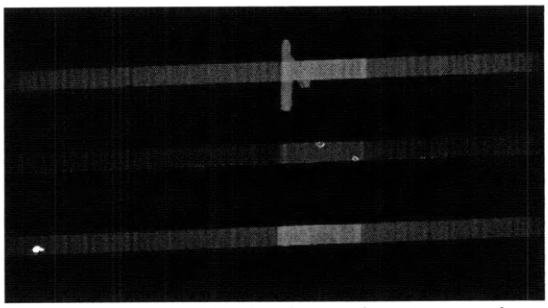


Figure 6a. Leakage of fluorescently labeled IgG along the stamped area can be seen in this figure.

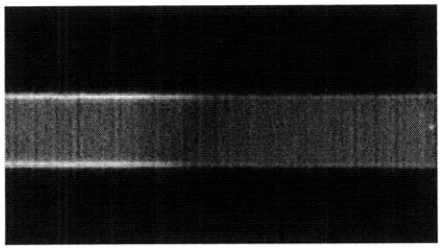


Figure 6b. Picture showing decreased intensity at preconcentration zone compared to fluorescence intensity elsewhere.

There are a few possible explanations for this phenomenon. Our main hypothesis has to do with effects of voltage on the immobilized biomolecules. Because of glass's hydrophilic nature, biomolecules adhering to it do so mainly do so via electrostatic forces and Van der Waals interactions, with hydrophobic forces playing secondary roles. At neutral pH, glass surface carriers negative a negative charge. When a voltage is applied across the microchannel to generate a depletion and concentration polarization, a high voltage drop is created across the depletion zone [36].

Several things can happen in this condition:

1. This high voltage drop can possibly disrupt the interaction between the absorbed biomolecules and the surface, causing detachment of immobilized molecules. Since the electric field at the depletion region is a lot higher than the electric field in the rest of the microchannel, most detachment of biomolecules would happen around the Nafion junction/depletion region. Thus, the areas that are supposedly enhanced by preconcentration would actually process a smaller binding capacity after preconcentration. As a result, these areas would generate a smaller amount of fluorescence after the channel is flushed with buffer.

2. With our concentrator system, all charged species are collected near the junction. Since we are using phosphate buffered saline in our model system, a large amount of salt ions would accumulate in the concentrator region as well. High concentrations of salt can cause shield charges between surfaces. The higher the concentration of salt is, the more effective the charge shielding becomes. The lost of charge interactions can cause damage to protein bonds, protein structure, and interfere with binding of biomolecules. A high concentration of salt effectively decreases interaction between charged species including the ones that are bound to the solid phase. Perhaps this is causing smaller than usual amount of biomolecules to remain bound to the surface.
3. A third possible explanation is that the vortexes created near the depletion zone have the potential to disrupt the stability of absorbed molecules. As these vortexes represent locally fast electrokinetically driven flow, they have greater power to move charged molecules and thus uproot molecules that are adhering to the glass slide.

Due to these reasons an alternative method of surface patterning to localize protein immobilization needs to be employed in our system.

Hydrophobic microvalves

Two requirements would need to be satisfied for the attachment methods to work well with our system. It needs to be able to withstand the effects of electric fields, and preferably simple enough for it to be adapted to changes in device geometries and formats. In our experiments, we found hydrophobic interactions to be a viable option. When a protein solution is incubated in a pdms/glass microfluidic system, most of the proteins stick to the PDMS side walls instead of the glass slide. This is expected because of the hydrophobic nature of PDMS. Our experiments have also shown that biomolecules adhering to PDMS are quite stable, and do not get flushed away even under high electric fields or high salt conditions such as those present in the preconcentration experiments. This can probably be explained by the fact that hydrophobic interactions are less reliant on the electrical properties of the proteins and the immobilized proteins are less likely to be dislodged by electrical effects.

A number of methods to selectively immobilize proteins on PDMS have been reviewed elsewhere [14]. However, most of these methods involved complicated chemical procedures and we are not sure how compatible with our Nafion infiltration procedures. We chose to use a simple physical absorption method for PDMS, which is straightforward, and provides stable attachment of biomolecules. The protocol used is listed as below:

Step 1: Fill channel with PBS buffer
Step 2: Replace PBS buffer with a 100μg/mL Neutravidin solution in PBS; incubate for 5minutes under constant flow
Step 3: PBS buffer flush
Step 4: Replace PBS buffer with a 10μg/mL Biotinylated antibody solution in PBS. Incubate for 5minutes under constant flow
Step 5: PBS flush

This coating method provides detection sensitivity down to 1ng/mL for R-phycoerythrin molecules, using a LED lamp (Cairn Research OptoLED) and various exposure settings under a 20x magnification.

To selectively coat the antibodies on PDMS, a hydrophobic surface, we needed a set of valves. A number of microvalves are considered, but these add significant complexity to the

design. Instead, we exploited the hydrophobic nature of PDMS. A hydrophobic surface would normally resist aqueous solution unless a certain pressure or surface tension overcomes this resistance. By combing the hydrophobic forces with geometrical changes in the microchannel network, we can fine tune the amount of pressure required for liquid to enter specific channels and essentially create a system of valves. Known as hydrophobic microvalves [20], these valves are formed by placing narrow constrictions (areas with high hydraulic resistance) in channels followed by an expansion (areas with low hydraulic resistance) into a wider channel. This design establishes a pressure barrier at the narrow region; when a pressure lower that that required for overcoming the narrow region is applied, flow would be stopped at the junctions and redirected into areas that require lower pressure to sustain the flow. Figure 7 from reference 20 shows the response of a hydrophobic microvalve used for metering flow.

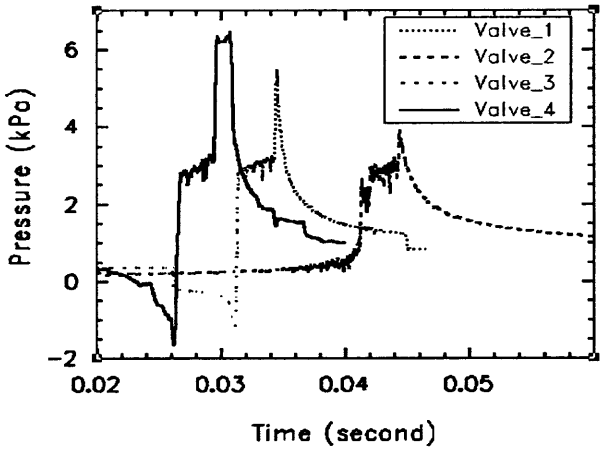


Figure 7. Pressure response of various hydrophobic microvalves. A large pressure drop is required before the valve allows fluid to pass through. Reprinted from Ref 20.

Hydrophobicity of the material system used is critical for the valves to function. This is because in a hydrophilic system liquid flow would be directed by both applied pressure and capillary pressure, and capillary pressure has a chance of overcoming the pressure barrier imposed by the geometrical changes. Additional capillary valves can be included to stop this from happening if the design is to be used with a hydrophilic material system. In place of geometrical changes, hydrophobic coatings can be used to limit liquid flow; however, this adds another layer of complexity to our fabrication procedures and is thus not used.

We would like to only selectively coat the areas around the concentrator with antibodies. To do this, a separate channel for functionalization would need to be connected to the sample channel, and two microvalves can be used to prevent fluid from leaking into the sample channels. In our design, the top channel is used for sample delivery, and the bottom channel is used for functionalization as seen in Figure 8a. The two channels overlap at the concentrator region where samples would enter the zone with coated areas. The operation of the device would be shown in more details in Figure 8a-c. Good pressure control is required for controlling liquid flow in the channels, as once liquid enters the constriction area, it becomes really difficult to reverse flow of the liquid (dewetting is difficult). Since negative pressure would not drive liquid into unwanted areas, it is used to fill the functionalizing channel, and at the end positive pressure is used to direct liquid into the other channels.

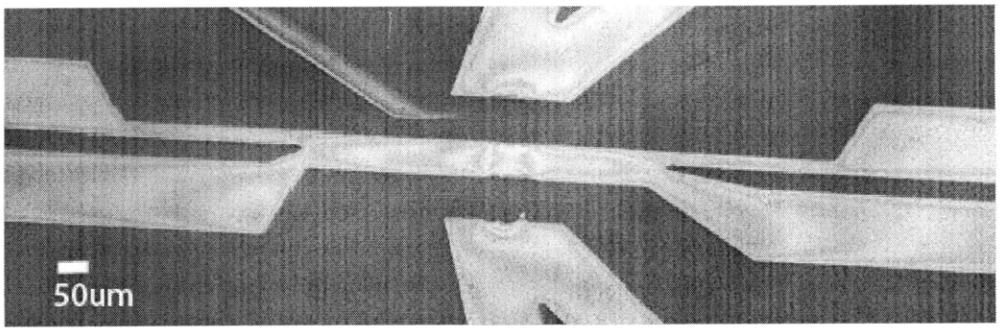


Figure 8a. Initial device – the channel in the middle is the sample channel; it is connected to the functionalization channel and a set of hydrophobic microvalves

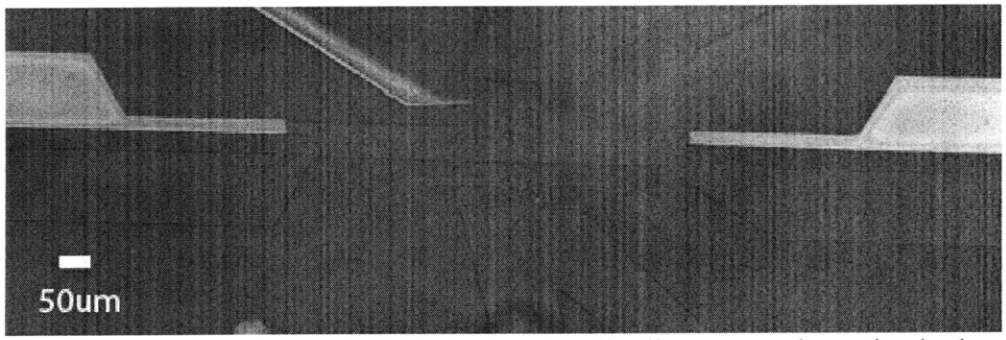


Figure 8b. Solutions of Neutravidin and biotinylated antibodies are used to selectively coat the area around the Nafion junction using the functionalization channels. The liquid, as seen in the figure, is stopped by the hydrophobic microvalves.

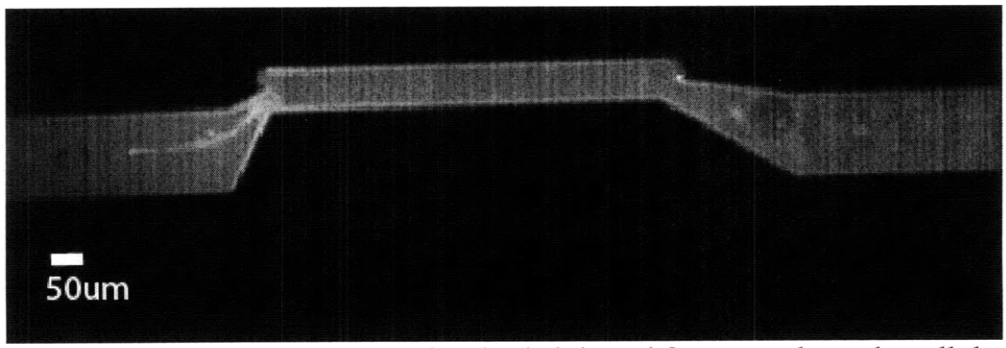


Figure 8c. A 10ng/mL solution of RPE molecules is injected from top channel to all the channels. This figure was taken after a PBS flush removed all the unbound molecules. Even though RPE solution has been incubated everywhere, it can be seen that only the coated sections of the channel has captured RPE molecules.

Chapter 3

Device Fabrication

All devices are fabricated at the Microsystems Technology Laboratories (MTL) with the help of its staff members.

PDMS Device fabrication

The fabrication details of these devices are detailed elsewhere [20]. Briefly, silicon masters are used to form molds for later pdms molding. Here, the masters are made using a dry etching process instead of a SU-8 process. Dry-etched wafers tend to maintain better long-term stability, have well-defined features, and are easier to fabricate. The table below this section offers a process flow and more detailed explanations on the fabrication of the microfluidic devices.

	Process step	Remarks
1	Piranha clean	
2	Photoresist deposition	Can use either spinner in TRL or coater6 in ICL
3	Photoresist exposure	
4	Develop photoresist	See above
5	DRIE etch	Etch depth characterized by Detak tool
6	Strip photoresist	Asher in ICL provides the best results

Table 2. Process flow for fabrication of Silicon masters for PDMS molding

After PDMS chips are made from inversed replica molding, an ion-exchange membrane needs to be integrated into the device. Ion-exchange membranes belong to a class of material used to separate ions by preferentially transport. A positively charged ion-exchange membrane only transports negatively charged ions, and vice versa. These membranes are used in various applications such as electrodialysis, separation, sensors, and fuel cells. Typically, these membranes are made of polymer networks having side groups that are highly charged. Due to the presence of these charged side groups, these cross linked polymeric molecules selectively allows ions to pass through [25]. Nafion is a prominent example of an ion exchange membrane. Nafion is an ionomer having a number of sulfonic acid side groups on a Teflon backbone, leading to an acidic material that is negatively charged under most operating conditions [25]. Because of this negative charge, Nafion is a cationic ion-exchange membrane. The exact structure of Nafion is still under active investigation, but is believed to comprise of big pores connected by and smaller ones. Nafion comes in membrane form as well as in a resin solution. In our experiments, Nafion

resin (Sigma-Aldrich, MO) of 5 wt % and 20 wt% and a number of different blends were created by mixing the two to arrive at the desired viscosity for fabrication.

The integrated Nafion membranes are used to produce ion depletion and concentration polarization phenomenon in our devices. Since the pores of Nafion are negatively charged, they would accept only cations; electrons and negatively charged ions are repelled by the membrane. This effect is very similar to that seen in nanochannel systems previously developed in our lab [26]. Ion depletion zones and concentration polarization can be created by applying a voltage across Nafion membranes that bridge two microchannels. Due to Debye layer overlap, the negative space charge would lead to preferential transport of counter ions (in this case positively charged ions). When enough counter ions have been separated from co-ions (negatively charged ions), it becomes energetically unfavorable to further separate the two; instead, both counter ions and co-ions are pushed away from the membrane. This results in a zone near the membrane that is devoid of any ions. This zone is known as the ion depletion zone, and charged species attempting to enter this zone would be repelled by the built-in electric fields in the zone. When a flow is used to bring in additional analytes, these molecules would be trapped at the boundary of the depletion zone. This results in the increase in local concentration of analytes near the depletion zone.

Remark: Ionic strength and ion depletion

Previous experiments of ion-depletion and concentration have been performed in low ionic strength phosphate buffer [26] with a 40nm thick nanochannel system. The relationship between ionic strength and Debye layer obeys an exponential relationship; as ionic strength increases, the "thickness" of the Debye layer decreases exponentially, resulting in significantly less overlap between Debye layers. This corresponds to a decrease in ion selectivity, requiring a slower flow rate and resulting in a slower concentration effect. When the ionic strength becomes high enough such that Debye layer overlap becomes negligible, the ion depletion effects would not start at all. Therefore, a smaller characteristic dimension would be required to induce ion depletion when the ionic strength increases. However, it is difficult to fabricate nanochannels with a high throughput [27 – need to change this in ref section]. Certain polymeric materials, such as ion-exchange membranes, have pores on the order of nanometers and make for good candidates for use in place of nanochannels made with traditional fabrication techniques. These materials can often have lower costs and have higher throughput as well. The pore size and charge of the pores can be tuned by using different material systems, degrees of crosslinking, and other parameters [25].

Ion-exchange membrane – Nafion in microfluidic devices

Our lab has developed a number of preconcentrator devices using a PDMS/glass material system combined with Nafion as the ion-exchange membrane. In this section we will discuss some of these and their characteristics.

In the simplest form, ion-exchange characteristics can be achieved with a junction breakdown mechanism in the PDMS/glass bonding interface [22]. By applying a high voltage between two disjoint microchannels, nanoscopic cracks along the interface can be formed that connect the two. These cracks emulate the function of nanochannels in the glass/silicon device;

similarly to the nanochannels, these only work at low ionic strength buffers due to the large characteristic dimension [22, 36].

An improved method of fabricating PDMS/glass preconcentrators involves the use of surface-patterned Nafion on glass slides. 5% Nafion resin solution is injected into a microchannel that is reversibly bonded to a glass slide, and is then flushed out with the use of negative pressure [23]. This method produces a Nafion membrane that is about 200nm thick and is quite uniform over the length of the junction. PDMS pieces can then be bonded to the patterned glass slide via plasma-activated bonding. This method provides for a good preconcentration performance, with a ~1000 fold improvement within a 1 hr period. This method also works with high ionic-strength buffers such as phosphate buffered saline (PBS), as the pore sizes of Nafion are much smaller than those made with the above-mentioned methods and are much more highly charged due to the sulfonic acid side groups. However, due to the planar nature of the fabrication method, devices made with this Nafion junction do not perform well with pressure driven flow, probably because of the limited current throughput allowed with the membrane.

Finally, Nafion junctions can be fabricated in pdms pieces instead of glass slides. Sung Jae Kim and coworkers have reported a method to produce a self-sealing vertical Nafion junction in pdms devices [24]. In this method, a thin cut is made into the PDMS piece so that the cut spans the initially unconnected sample and grounding channels. Nafion resin solution is then injected into the cut location and allowed to dry. The modified PDMS piece can then be bonded to a glass piece by plasma activated bonding. The advantage of this method is that the height of the Nafion membrane can be as large as, or greater than the channel height used for the sample channel, eliminating the bottleneck in current throughput. As the ionic current through the Nafion membrane is directly proportional to the cross-sectional area, this method can potentially provide >1000 times greater ionic conductivity than the planar Nafion junctions. Experimental results also show that it would be possible to use pressure-driven flow in these devices [24]. Using pressure driven flow is preferable because of the higher flow rate compared to electrokinetic driven flow, and also because pressure driven flow is easier to control in microfluidic devices. This type of device can potentially have higher throughput as well, as it remains operational for channel widths as wide as 1000 microns (compared to 100 microns of the other previously mentioned devices).

We can draw some conclusions from these previous generations of devices. Firstly, Nafion is a much better material choice than plain glass or PDMS- surfaces for the purpose of building preconcentrators. This is probably due to the higher ion selectivity, higher density of pores, and also the potential for building vertical and high-aspect ratio ion-exchange membranes. Secondly, we can see that the cross sectional area of the Nafion junction has a direct impact on the performance of the preconcentrator. While both of these fabrication methods have been in use in our lab, reliability of the resulting devices is often dependent on the skill of the user/operator. For example, in the surface patterned Nafion method, different amounts of negative pressure applied at the outlet would cause the membrane to have varying thickness and uniformity. Another example would be the cutting step of the vertical sealing method. Precise alignment of the blade to the desired location for the membrane needs to be done in order to ensure correct operation. This can be particularly troublesome when one wants to increase the density of concentrators. Since both surface patterning and self sealing methods require manual alignment for placement of the Nafion membrane, they are often composed of long lines to facilitate alignment in that direction. This limits the ability to have dense concentrator networks

in close proximity and limits the potential to multiplex concentrators to detect different analytes. These shortcomings can be remedied by an approach that's self-aligned, less dependent on operator skill, and can be integrated into other material systems for mass production (using techniques such as hot embossing/injection molding, etc.).

Capillary valves

In order to minimize dependency on the operator and eliminate the need to align patterned Nafion membranes to other features, some other technique to localize the Nafion resin would be required. The new method introduced in this thesis utilizes a technology known as a capillary valve.

Capillary valves belong to a class of microvalves known as passive valves [28]. A number of microvalves have been designed for pdms/glass microfluidic devices. Based on the operating principle, these valves can be divided into passive and active valves. Active valves are those that depend on user input to modulate the status of the valve, where passive valves are those that operate according to certain preset conditions that are built into the design of the valving system. Out of the passive valves, we have mechanical and non-mechanical valves [28]. Since we want to design the system to be broad, compatible with a range of material systems, and requires minimal user input, we chose the capillary valve for use in our system.

Capillary valves come in a variety of shapes and forms, including the capillary burst valve, check valve, and delay valve. As we want to retain liquid in selected areas only, we are most interested in the capillary burst valve. In a capillary burst valve, a sudden geometrical expansion of the microchannel traps the meniscus at the beginning of the expansion [29]. In our systems, this expansion is used to confine Nafion to an interconnecting channel between two sample channels (see Figure 9), and the design would need to provide for that function.

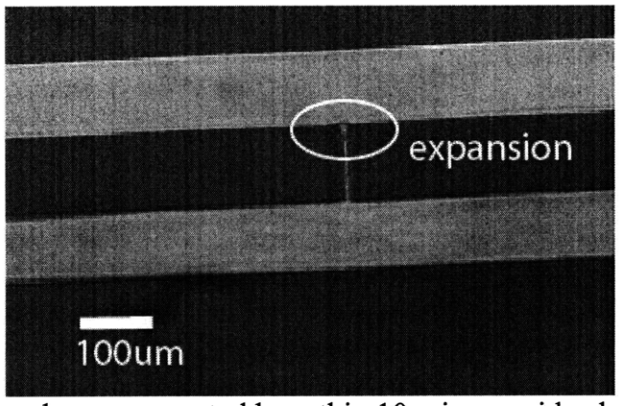


Figure 9. Two microchannels are connected by a thin 10 micron wide channel. The sudden expansion from the thin channel to the wide microchannel constitutes a capillary valve for Nafion membrane formation.

The calculations shown here assume no surface treatment on our channel surfaces, which is true for all of our devices. For the liquid to be pinned at the interface, the curvature of the liquid meniscus in a system undergoing capillary flow needs to be altered so that the filling front is stopped [30]. The capillary pressure in a rectangular channel can be estimated by calculating the interfacial energy of the liquid-gas-solid phase system [30]. Since the aspect ratio in our

system is high (~10) we can approximate the meniscus as a 2D dimensional entity. The capillary pressure in the system can be obtained by calculating the change in total interfacial energy [30]:

$$P = -\frac{\partial E_T}{\partial V} = \sigma(\cos\vartheta_c \frac{\partial A_{sl}}{\partial V} - \frac{\partial A_{la}}{\partial V})$$

Where E_T is the total interfacial energy, V is volume of liquid, σ is the surface tension, ϑ_c is the contact angle between the advancing liquid and the walls of the capillary system, and A_{si} and A_{la} are the areas of the solid-liquid and liquid-air interfaces respectively. A filling front advancing in a straight channel would have an angle $\alpha = 90 - \vartheta_c$ between the meniscus and the channel side wall. At a junction with abrupt opening having an angle β, the filling front would stop advancing if $\beta \geq \alpha$ as the change in angle is too large for the meniscus to overcome [30]. The final form of the pressure barrier is given in reference 30 and provided here [30]:

$$\Delta P = \frac{2\sigma}{w}\left(\frac{\cos\vartheta_c - \frac{\alpha}{\sin\alpha}\sin\beta}{\cos\beta + \frac{\sin\beta}{\sin\alpha}\left(\frac{\alpha}{\sin\alpha} - cons\alpha\right)}\right)$$

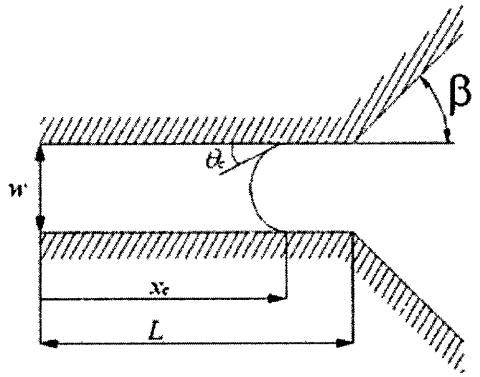


Figure 10. This picture provides some of the illustrations referenced in the previous paragraph. Using this equation, and using the contact angle for oxidized PDMS as the sidewalls of our system, we calculated the maximum allowable pressure for holding Nafion in our devices to be about 10kPa; this means Nafion can be injected by hand without the use of specialized instruments. Reprinted from reference 30.

High aspect ratio membranes with capillary valves

High aspect ratio membranes can be beneficial for reasons mentioned in the previous paragraphs. Here, we seek to use the principles behind capillary valves to create membranes of the same height as the microchannels. Figure 11 shows some early conceptions of such a device. Here, a single gate device comprising of two microchannels, one with a 50 or 100 microns width (the sample channel) and the other with a 100 microns width (the ground channel), is presented. The two channels are connected to each other by a 10 micron wide, 50 micron long channel. This connecting channel can be made in two shapes: a notch shape and a rectangular shape (see Figure 11). The notch shape makes it easier to fill the channel with Nafion due to the smaller angle that the meniscus needs to change the curvature by.

It turns out that 5% Nafion resin solution does not form good membranes. As Figure 4 shows, Nafion transforms from a dark, opaque looking solid (Figure 12a) to a hollow-looking shell after the annealing step (Figure 12b). The reason for this happening can be explained with a comparison to bead packing. Nafion resin can be compared to beads being suspended in a solution, and while in this form they are free to move around. When the solution is being heated and dried, an evaporation driven self-assembly process [31] begins to order the beads into a tightly packed formation. In Nafion resin, we speculate that the final spacing of Nafion molecules have to do with the initial concentration of Nafion in solution. If the initial concentration of Nafion resin is low, there aren't enough Nafion molecules in the solution to fill the entire channel after drying. this results in the hollow-looking picture in figure 4b. As this is an evaporative driven process, heating the devices might improve the final structure. We tested the effect of annealing temperature on the devices, ranging from overnight drying at room temperature to up to 95 deg. C on the hotplate for 1 hour. We found that the annealing temperature does not have a significant effect on the quality of the membrane and the preconcentration performance. This might be possible due to the inherent fast evaporation of Nafion (since the alcohols in the solvent used evaporate quite quickly), leading to similar structures whether the devices are heated or not.

When this hollow looking membrane is used for preconcentration in low ionic strength buffer, the results prove to be satisfactory: ion depletion and concentration polarization effects typically start at around 50V-100V, and electrokinetically driven flow can be used to drive preconcentration. However, when high ionic strength buffer is used, the hollow membrane starts failing and fluorescent molecules can be seen passing through the junction to the ground channel (Figure 13).

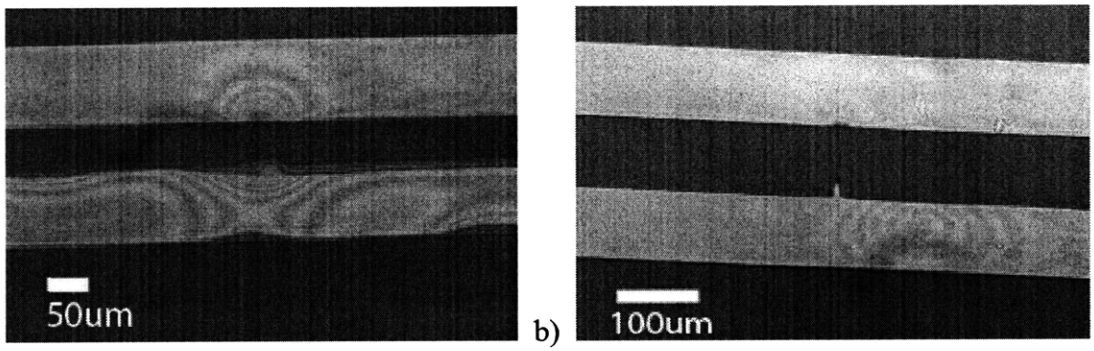


Figure 11. The left figure shows a notch shaped capillary valve filled with Nafion resin and the right figure shows a rectangular shaped capillary valve with Nafion resin.

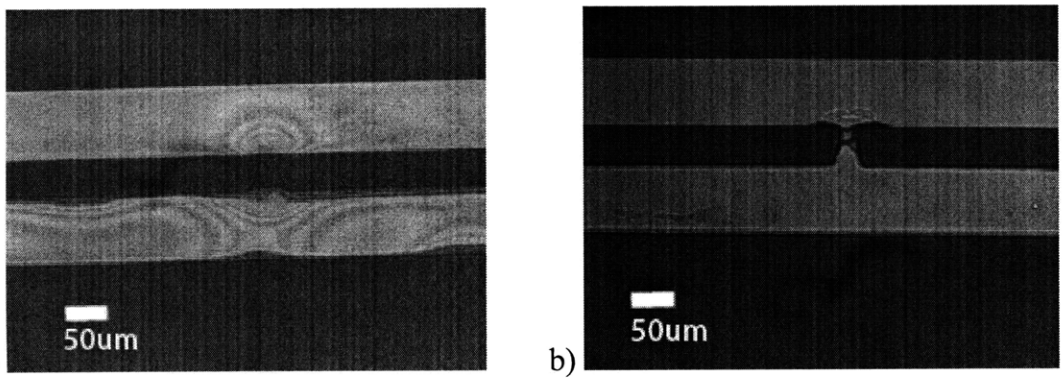


Figure 12. The wet and dry states of 5% Nafion are shown in these pictures. Picture 4a was taken immediately after Nafion was removed from the ground channel, whereas Picture 4b was taken after the device was left on the hotplate overnight. The dried Nafion residue in the notch appears porous and is structurally unstable.

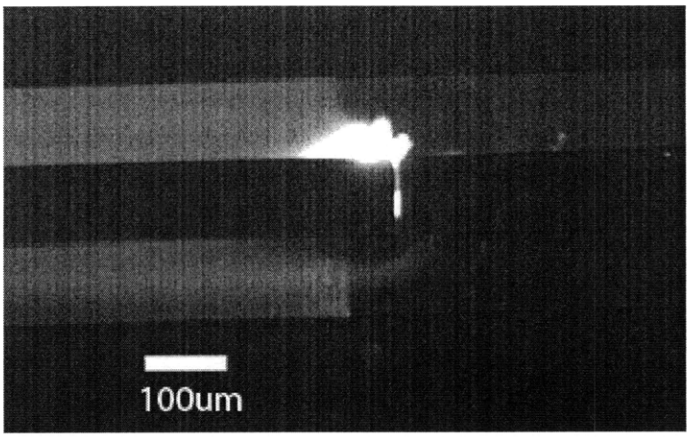


Figure 13. Preconcentration using devices made with 5% Nafion in 1xPBS. The top channel was initially filled with FITC dye and the bottom (ground) channel initially only contained PBS. After the voltage was turned on, FITC dye was observed to migrate through the Nafion junction and into the ground channel.Further voltage application inverts the top channel into an ehnacement mod instead of depletion mode

When positive pressure is used to fill devices or to drive liquid flow, membranes made with 5% Nafion resin solution would sometimes lose its integrity. This causes a device to lose its ability to induce ion depletion and concentration polarization in the sample channel. When the membrane fails, fluorescent molecules can be seen leaking into the ground channel. When voltage is applied at this point, ion depletion effects can be seen in the ground channel instead of the sample channel. Rather than depleting the sample channel, the voltage application causes an enhancement effect and creates a bright "halo" around the junction. This is possibly due to the formation of small cracks and fissures along the junction. When pressure is applied, the imperfect sealing between pdms and Nafion can be forced apart and results in a microchannel in the Nafion membrane itself. This microchannel provides a low resistance path for ions and charged species to flow through (see Figure 14) and disrupts the concentration polarization process. As a result, complicated interactions can happen and an enhancement-like effect can be seen in the sample channel.

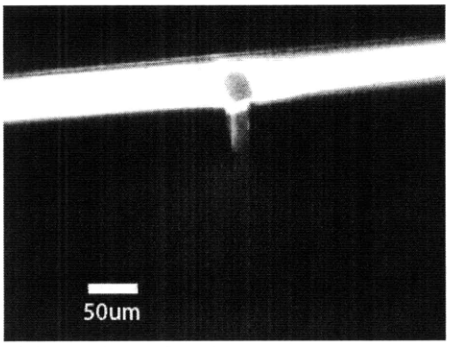
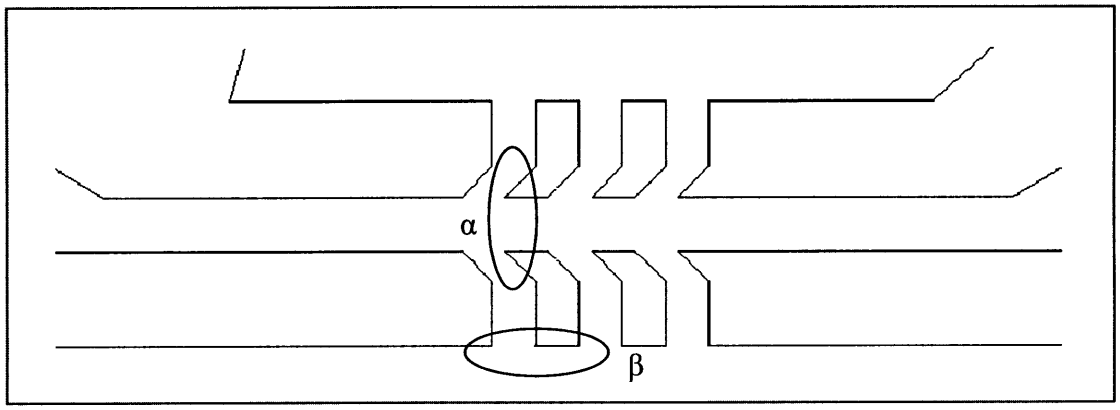


Figure 14. Picture showing possible effect of microchannel after application of high positive pressure. In the picture, it can be seen that fluorescent molecules leak from the sample channel to

the ground channel when voltage is applied. The "halo" shape of the failed concentrator can also be seen in the picture.

The 5% Nafion resin solution also has a high wettability for both glass and PDMS, and therefore requires a surface that's relatively hydrophobic for the capillary valves to work. Due to this reason, PDMS/glass devices that have recently been exposed to plasma cannot be used as their surfaces are hydrophilic. In this state, the Nafion solution would defeat the capillary valves in the junction and leak into the other channels. By using more viscous Nafion however, the solution can be injected immediately after bonding as the increased viscosity helps the capillary valves stop Nafion flow better. Nafion solution increases in viscosity with the weight percent of Nafion in the solution. In order to arrive at the correct viscosity, we mixed roughly equal amounts of 5% and 20% Nafion (both from Sigma-Aldrich) to a solution of about 12% Nafion. The key here is to get the right viscosity so that Nafion would flow pass the first expansion (α in figure 15) and be stopped at the second expansion (β in figure 15). If the viscosity of Nafion is too small for the valve, the valve labeled β would fail to hold back the Nafion, resulting in Nafion leaking into either the main or bypass channel. If the viscosity of Nafion is too high, it would be stopped by the valve labeled α instead of the one labeled β. After bonding, the channels in the device would become hydrophilic. However, we have experimentally observed that additional corona discharges would make it easier to flow Nafion into the channels.

The channel used to deliver Nafion to the capillary valve cannot be too long either. The solvent in Nafion solution evaporates as it flows in microchannels, and the viscosity of Nafion increases with this evaporation. The greater the distance that the solution has to travel, the more difficult it becomes to fill the junctions. Our observation is that the length of the channel would need to be less than 2mm from Nafion injection to the capillary valve for the Nafion to successfully pass through the first expansion.



Figures 15. Schematic drawing of the filling channels illustration of capillary valves.

Another benefit to using high weight percent Nafion solution is that it resists fluidic pressure and high voltages better. Unlike membranes made with 5% Nafion solution, most membranes made with higher weight percent Nafion looks solid after the solution has evaporated. These membranes can resist high ionic strength buffer as well as higher positive pressure applied by the user, making them more versatile and usable than the membranes made with 5% Nafion resin solution. We also found through experimentation that Nafion adheres better to a hydrophilic surface than a hydrophobic one. This maybe caused by the highly charged

nature of Nafion and its subsequent electrostatic interactions with the surfaces. The Nafion composition used in our tests has been fine tuned for use with a hydrophilic PDMS/glass system that has been treated with plasma.

By controlling the hydrophilicity of the material system used, this technique can be expanded to materials beyond pdms/glass; other polymers such as CRC can also be used with this technique when the surface properties are determined.

Differences between multiple and single channels

We experimented with designs that involve having wide Nafion junctions. Since we believed that the current capacity of the junctions is proportional to the cross-sectional area of the junction, a wider Nafion junction should improve the ion-exchange capacity of the system and allow faster flow rates. However, a wide junction is difficult to fill with Nafion, because the capillary valves' ability to hold back liquid inversely depends on the junction width. Therefore, it is more difficult to find a Nafion solution with the correct viscosity to prevent leakage and that would still correctly fill the junction. Thus, a wider junction would be less reliable in producing usable devices. After multiple experiments with mixed results, we decided that the large cross sectional area of wide channels can be replicated with multiple thin channels in parallel. Thinner channels in parallel combine the advantage of a wide channel with the ease of fabrication of thinner channels.

In our experiments, multichannel devices tend to require a lower operating voltage and produce more powerful ion depletion effects. They are also able to withstand higher flow rates before the ion depletion zone fails.

Differences between single and double gates

Concentrators using a double gate design can have better control over the depletion zone and concentration effect [26]. In concentrators made with Nafion and fabricated with capillary valves, we found that single gate devices produce a strong enough depletion zone for slow electrokinetic flow. However, when the flow velocity is increased, or when pressure driven flow is used to drive the liquid, the depletion zone starts to fail, and leakage of concentrated molecules can be seen (Figure 16). Besides decreasing the concentration at the detector zone, the leaked molecules also change the voltage distribution along the channel and contribute to instabilities of the concentration effect. A double gated design provides a much more stable depletion zone and allows for use of a lower voltage.

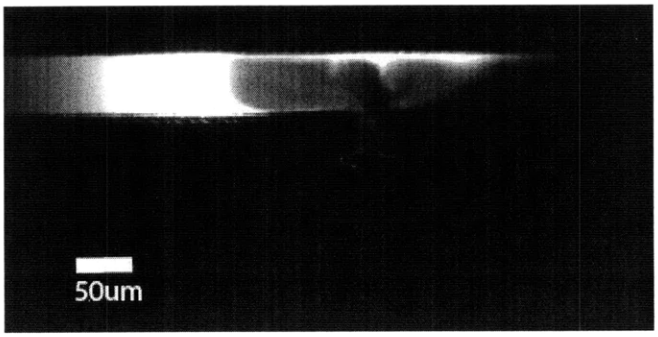
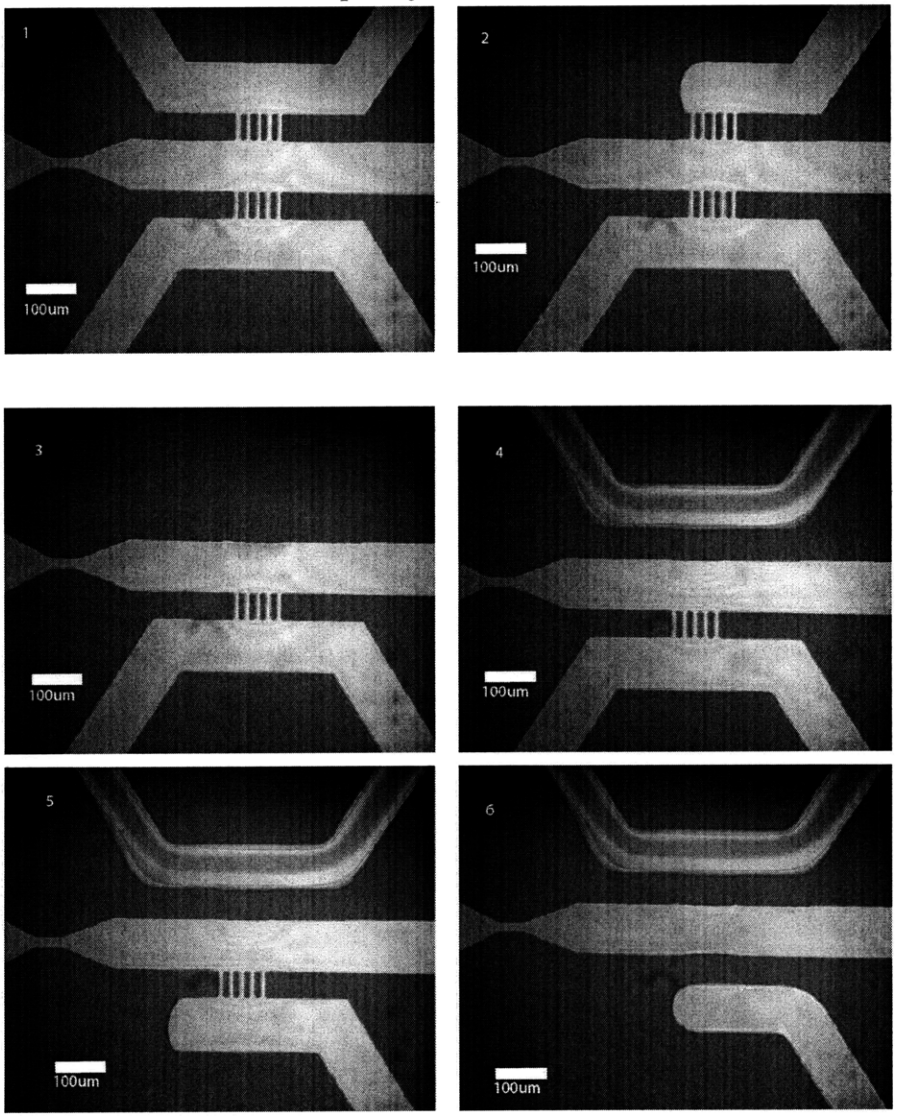


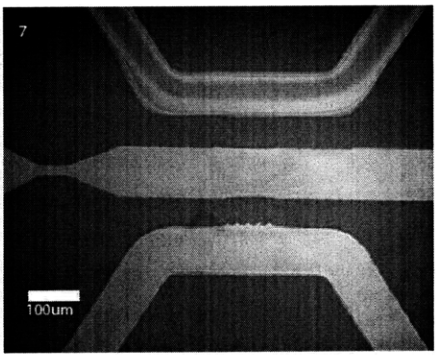
Figure 16. In order to showcase instabilities away from the Nafion junction, an experiment was performed with FITC dye in 1xPBS buffer. The concentrated plug releases molecules into the fluid stream near the channel wall opposite from the Nafion junction.

Using a double gated design would help reduce this problem as additional ion depletion force would be provided from the other side of the device. This provides two benefits: faster flow rate can be used because of the stronger ion depletion forces generated by the duel Nafion membrane system, and more stable preconcentration. As a lower voltage is required to sustain preconcentration under a given flow rate, and the intensity of instabilities scale directly with the applied voltage/electric field, it would be easier to maintain a stable preconcentration.

Device fabrication with capillary valve is illustrated with a series of pictures (Figure 17):



1. Initial device.
2. Nafion is flown into the top ground channel.
3. Nafion has completely filled the top ground channel.
4. Nafion is withdrawn from top channel.
5. Nafion is flown into the bottom ground channel.
6. Nafion is withdrawn from bottom channel.



7. Finished Device. Once nafion in the top and bottom junctions have solidified, the device can be used for preconcentration.

After the formation of these junctions, the devices are placed on a hot plate at 70 degrees for 1 hr to dry the Nafion resin. The devices are then ready for use.

Filling channels

The protocol mentioned in previous paragraphs requires us to withdraw the Nafion that was injected into the grounding channel. While this usually doesn't leave too much residue behind in the channel, the entrance and certain areas can get clogged (see figure 18). These dried Nafion membranes usually don't cause interference with the normal operation of the device. However, bubbles tend to get trapped in these residual Nafion aggregates and be released over time. These bubbles change the resistance of microchannels and can eventually clog the entire channel over time. Once the channel is clogged, the preconcentration fails as current can no longer be delivered to the grounding electrode. Even in cases where the channels are not clogged, the change in channel resistance makes the preconcentration behavior less predictable and less repeatable.

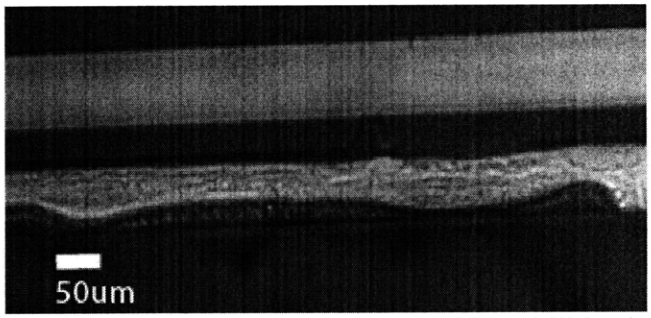


Figure 18. Incomplete removal of Nafion causing clogging and instabilities in the system.

A potential solution to this problem is to have another microchannel dedicated to filling the junctions with Nafion solution. A few points need to be noted when designing this filling channel. If the filling channel is too long, then Nafion can dry out before it reaches the junction. Also, the expansion of the filling channel into the junction is actually more difficult to fill than then the filling system described in the previous section. To solve this problem, we designed a capillary valve that has an angled sidewall at the first expansion; as the capillary pressure required to overcome the valve decreases with the angle between the two, this modification should make it easier to fill the junction. A design similar to that seen in figure 19 is used. In this

design, the angled sidewalls increases the capillary pressure for Nafion to flow into the junction, and the channels on the other side are straight again to prevent Nafion from leaking through to the grounding or sample channels. The devices used in this thesis either have a single 10 microns (wide) by 60 microns (long) channel, or have multiple number of these channels spaced 10 microns apart. Devices with 3, 4, and 5 channels in parallel were tested, and we found that devices with 3 channels in parallel tend to produce the most consistent results in terms of fabrication and reliability. The formations of Nafion junction in these devices are detailed in the next series of pictures:

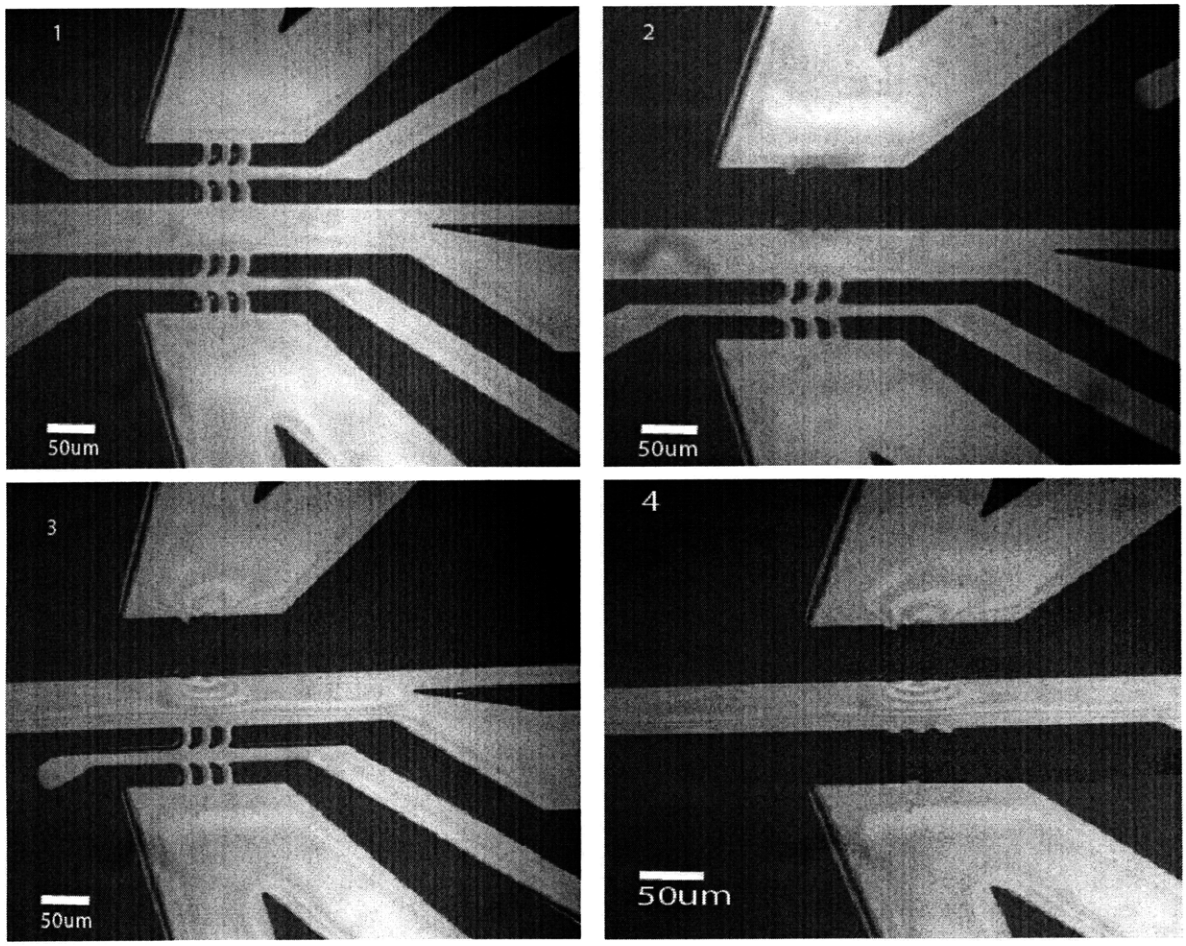
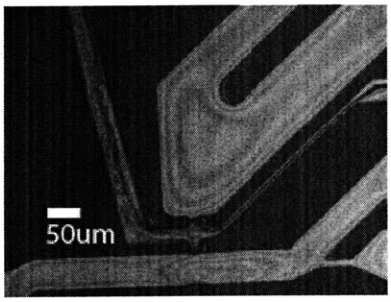


Figure 19. Procedures for using the filling channels to from Nafion junctions:

1. Initial device.
2. Nafion is flown into the top filling channel, until completely filled
3. Nafion is flown into the bottom filling channel, until completely filled.
4. Finished device with functional Nafion junctions. The preconcentrator can be used after the Nafion resin has completely solidified.

The width of the filling channel can also be optimized for easier filling of Nafion. The filling channel expands at the Nafion junction to allow connection between the sample and bypass channels. Because the capillary pressure increases as the width of the filling channel decreases, a lower weight percent Nafion would be required for use in a narrow filling channel. It

is easier to fill the Nafion junctions in devices with wide filling channels. However, drying Nafion tends to stick to the channel sidewalls, and channels that are too wide suffer from having significantly reduced amount of Nafion in the center of the filling channels. When the channels are filled with buffer, we observed buffer replacing the empty spaces in these hollow channels, thus reducing reliability and ion-selctivity. The narrower (10um) filling channels, on the other hand, do not suffer from the same problems. The sidewalls in the narrow channels are close enough to the center so that the dried Nafion would still pack nicely in the channel. Figures 20a and 20b illustrates the differences between the two using the same weight percent Nafion solution.

a) 

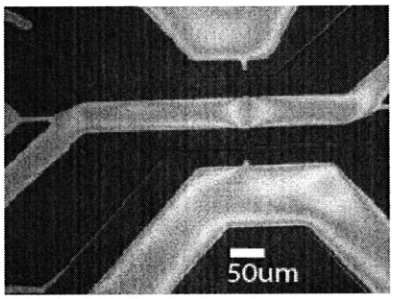
b) 

Figure 20a. Wide filling channel showing dried Nafion sticking to channel walls
Figure 20b. Narrow filling channel showing Nafion junction being successfully filled

Remark: Making double gated multi-channel devices

When functionalizing one of these devices care must be taken so the Nafion does not leak into the main channel or the ground channels. After one of the filling channels is filled, it increases the capillary pressure in the main channel and thus the other filling channel cannot immediately be filled after first one. After the Nafion in the other filling channel is dried, Nafion can be injected into the other filling channel to fill the junction on the other side with Nafion. The mechanism of how this happens is unclear, but we suspect that tiny evaporating Nafion vapor might escape into other areas when the bulk of the liquid is stopped by the valve. These tiny droplets of Nafion partially wets the other sections of the channel, causing the resistance to capillary flow to decrease and inducing leakage when Nafion is injected into the other filling channel.

Characterization – pressure driven flow, 1xPBS

Previous preconcentration systems developed in our lab have primarily been used with electrosmotic flow. Electrosmotic flow mobility is given by the following equation:

$$\mu_{eof} = \frac{\varepsilon\zeta}{4\pi\eta}$$

Where ε is the dielectric constant of the buffer system and η is the viscosity of the buffer system. ζ is the zeta potential and is directly related to the electrosmotic flow mobility.

We see that the electrosmotic flow mobility is affected by a number of factors as implied by the equations above. Changing the pH of the solution affects the zeta potential, as would absorption of molecules to the surface (because the viscosity of the surface charges). Since we

are using surface immobilized antibodies for capturing biomolecules, the electrosmotic mobility would be affected by both the change in zeta potential and also viscosity of the fluid [32]. The ionic strength of the solution would also affect the zeta potential and thus the electrosmotic mobility.

In the previous preconcentrator systems we have primarily used a 10mM dibasic phosphate buffer for our preconcentration experiments [26]. This buffer does not have the same pH or ionic strength as that in most biological fluids. Phosphate buffered saline (PBS) is a biological buffer that has the same ionic strength as the human body and is thus a better buffer to mimic that of real biological fluids. With the high ionic strength of PBS, electrosmotic flow becomes slow except at very high voltages, which causes instabilities in our system. Instead, we can use a pressure driven flow to bring biomolecules close to the depletion region. Since pressure is not affected as much by the buffer choice or constituents of the buffer, we believe that it would be easier for us to apply the same system to a real biological fluid such as serum or amniotic fluid since they have the same ionic strength and ions. Also, our system using Nafion is capable of handling high ionic strength (PBS 1x) solution and thus should be able to use pressure driven flow to preconcentrate molecules in other buffer systems.

We have characterized the performance of pressure driven preconcentration in our devices. By using a Harvard Apparatus syringe pump, we can accurately control the flow rate of the fluid in the devices and approximate an accumulation rate of biomolecules (discounting effects of electrokinetic flow). In figure 14, a graph of fluorescence intensity versus time is shown; we used a solution of 1μg/mL of R-PE in 1xPBS with flow driven by a syringe pump in this experiment. Assuming perfect trapping efficiency, we can calculate the times required for the concentrated zone to reach specific concentrations. The volume of the concentrated plug is approximately $50\mu m \cdot 10\mu m \cdot 100\mu m = 5000\mu m^3 \cdot \frac{m^3}{10^{18}\mu m^3} \cdot \frac{1000L}{m^3} \cdot \frac{10^{12} pL}{L} = 5pL$

With a flow of 0.05 μL/min, we have

$$\frac{0.05\mu L}{\min} \cdot \frac{\min}{60\sec} \cdot \frac{10^6 pL}{\mu L} \approx \frac{83pL}{\sec}$$

Meaning that a10 fold accumulation of molecules can be achieved in 1 second, if all the molecules are trapped in the same zone. By the same token, we would have:

$10 fold \rightarrow \approx 1\sec$

$100 fold \rightarrow \approx 10\sec$

$1000 fold \rightarrow \approx 100\sec$

And so on. However, in reality this strong binding has not been observed. We suspect that a number of molecules leaks pass the preconcentration zone into the areas downstream, especially at when a significantly higher than bulk concentration is reached at the preconcentration zone. Further studies would be required to develop this into a faster assay. If these flow rates can be sustained with significantly less leakage of molecules, the assay speed can be greatly improved and shortened to less than 10 minutes. Further investigation of this is required to provide more accurate results.

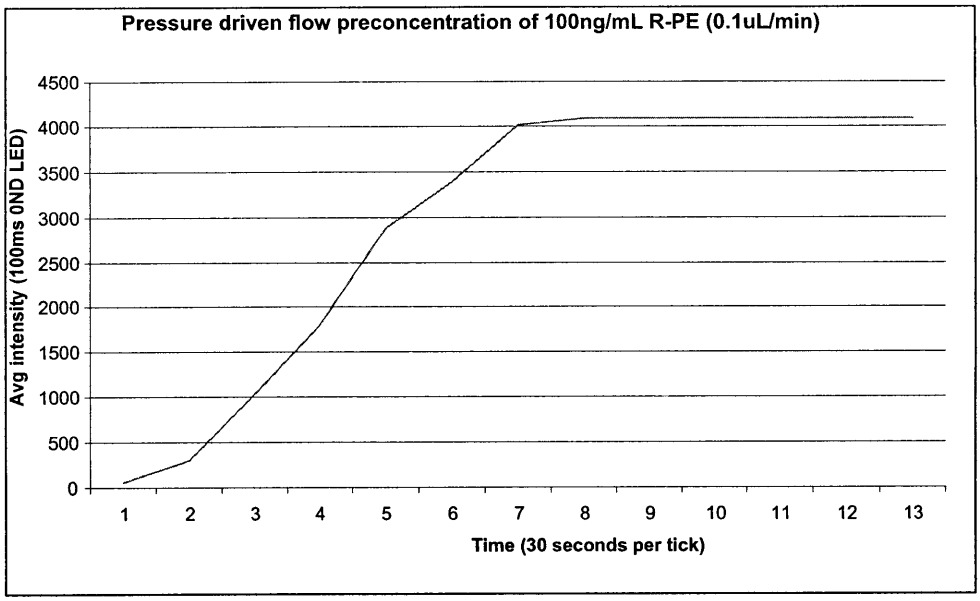


Figure 21. Graph showing preliminary pressure driven flow experiments done on uncoated device. The sample used was a 100ng/mL RPE solution in 1xPBS. In this experiment, flow was driven by a syringe pump at 0.1uL/min; this showed that preconcentration is possible with fast pressure driven flow when a high voltage (>200V) is applied.

Chapter 4

Device characterization and Immunoassay

Biological recognition

The antigen-antibody interaction is one of the most important in modern biological science and engineering. Antibodies are normally produced by the immune system as a natural defense against invading pathogens such as bacteria and viruses. As such, they are specific against each species of pathogen by recognizing their surface markers and attack the pathogen by attaching themselves to these markers. As an integral part of the immune response, a specific type of immune cell, the B cell, would produce antibodies against the foreign object. This process is exploited to produce antibodies for use in an artificial sensing environment. An antibody against a specific antigen is produced in a host organism by inoculating it against the desired antigen. Once the antigen is recognized by the host's immune system, a number of antibodies with varying affinities would be produced by the host's B cells. Since antigens, especially larger proteins and other pathogens would have multiple areas (epitopes) that can be recognized by the antibodies, antibodies produced by different B cells would target different epitopes. A collection of these varying antibodies is called a group of polyclonal antibodies. Since they target different epitopes on the antigen, more than one antibody can bind to a single antigen. Another type of antibodies is designated as monoclonal antibodies. Once the antibody-producing B cells are identified, they can be separated into different clones of cells. Each clone of B cell produces a specific type of antibody that targets a specific epitope on the antigen, and each of these antibodies produced is called a set of monoclonal antibodies. Since antibodies from each clone would target the same epitope on the antigen surface, only one antibody from the same group would be able to bind to the antigen. Antibodies produced by different clones of B cells have different affinities; the affinity of an antibody determines the strength of binding to the antigen, and affects the sensitivity of the sensor. In monoclonal antibody production, it is possible to use an artificial process to pick out the antibodies that have the strongest binding to the antigen to maximize sensor sensitivity. .

The differing properties of monoclonal and polyclonal antibodies have significant impact on the performance of the sensor and the ways they are used. A collection of polyclonal antibodies have the ability to bind more than one epitope on the surface of antigen molecules; this means more than one antibody can bind to the same antigen molecule. If we assume that each binding event produces a set amount of signal, this would result in higher signal intensity. However, since a collection of polyclonal antibodies include antibodies processing low specificity, which can bind to other molecules that process similarly shaped epitopes, the noise level tend to increase and the reliability of the assay tends to be worse. On the other hand,

monoclonal antibodies only recognize one epitope on the antigen molecule. Due to the selection process, monoclonal antibodies can often have a very high affinity and specificity against the specific antigen. This leads to more specific capture of antigen molecules and a lower noise level. Unfortunately, since only one antibody molecule can bind to the antigen, the signal generated by this system tend to be lower than signal produced by a system employing polyclonal antibodies.

In most biological sensing platforms or immunoassays, both types of antibodies are used. Monoclonal antibodies are used for capturing antigen molecules from solution due to its greater specificity. It's critical that non-specific binding, or capture of non-targeted molecules, is minimized in this step, because any signal generated from non-specific binding contributes to additional noise and negatively affects the limit of detection. This is especially true for label-free detection schemes. For molecules that are not natively fluorescent or fluorescently labeled, secondary antibodies that carry a label are often used to generate a signal at the detection stage. The secondary antibody can be either a monoclonal antibody that targets a different epitope on the antigen surface, or it can be a polyclonal antibody. The advantage of using polyclonal antibodies is that it can recognize many different epitopes on the antigen surface and thus would generate higher signal intensity. The problem with non-specific binding is not as severe here because ideally the only immobilized molecules are the targeted ones.

Binding kinetics

Macroscopic immunoassays are often limited by the kinetics of analyte diffusion from the bulk of the solution to the capture agents immobilized on the solid phase. A number of previous studies [35] have indicated mass transport to the solid phase surface as a major limitation for assay speed and consequently sensitivity. By switching to a microfluidic assay format and using the preconcentrator, this problem would be partially solved.

Microfluidic Assays

Traditionally, immunoassays are performed on 96-well plates and other macro-scale plastic support structures. Immunoassays protocols often require many steps that involve manual labor, such as washing and reagent exchange. While many of these tasks have been automated by robotics, these automatic systems often occupy large amounts of space and are costly. The use of these assays usually requires large volumes of sample and reagents as well. Because of these reasons, it would be difficult to use these immunoassays in a point-of-care setting.

Microfluidics-based immunoassays have a number of advantages over conventional assays for a number of reasons. The miniaturization reduces the consumption of reagents, leading to lower costs and the potential to perform tests more often as smaller amount of reagents and samples are consumed [4]. A number of clinical samples and biological samples require invasive procedures to collect, and the ability to minimize the amount of sample required would help make these tests less troublesome to patients. Microfluidics immunoassays are also easier to use and requires less work in preparing the collected samples/reagents, because a number of immunoassay steps can be integrated onto the chip [4]. Fluid flow can be controlled with various valves, pumps, and chemical functionalization on the channel surfaces. Chips that integrate multiple functionalities such as sample preparation, detection, and data analysis have been developed; these chips are useful in a point-of-care or resource-poor settings. Multiplexed

operations are also easier with microfluidic immunoassays, as a high density of channels can be achieved with microfabrication techniques. This example is most prominent in the form of microarrays, where up to tens of thousands of microspots can be formed using inkjet/contact printing.

Another advantage processed by microfluidic devices is a high surface area to volume ratio. Because of this, antigen molecules in the bulk can easily diffuse to the surface of the solid phase, resulting in faster assay speed and potentially more sensitive assays. Another consequence of this increase is that it leads to surface effects playing a more dominant role in the system [4], giving us the ability to fine-tune the properties of the surface and add functionalities to the system.

A number of disadvantages arise from this change in surface area to volume ratio as well. Non-specific absorption tends to be one of the big limiting factors in the implementation of microfluidic diagnostic devices. Since the diffusion distances for all the molecules are shortened in microfluidic devices, high abundance proteins, such as albumin and immunoglobulin, tend to create fouling problems for the sensor over longer incubation periods. This problem is especially troublesome when the detection of low abundance proteins is involved; low abundance proteins typically require longer incubation times to detect, and the problem of non-specific binding is exacerbated.

Fortunately, a number of methods have been developed to reduce non-specific absorption. Most of these involve blocking the channel surfaces with a blocking agent such as BSA. For our experiments, we used a 1% BSA solution to block the channel surfaces, as well as a PBS buffer with Tween-20, a surfactant, to reduce non-specific absorption.

Microchannel dynamics and design

Speed between antibody/antigen binding is governed by a number of different factors. The importance of these factors in turn depends on the transport regime that the system operates under. In a microfluidics channel, the relative importance of convection, diffusion, and reaction, and channel geometry would determine the limiting step in the binding reactions. A thin channel, for example, would have a much shorter diffusion time compared to a thick one, as diffusion times depends on $\frac{h^2}{D}$. A long and thin channel, however, might run into problems with sample depletion because of the fast diffusion of sample molecules onto capture agents compared to convection that brings in more molecules. For our designs, the detector only occupies a small portion of the channel (~200 microns from the Nafion junctions for a total of ~400 microns), and the rest of the channel is coated with protein-repelling coatings. Therefore, sample depletion should not present a problem and the effects of channel height in this case would affect the diffusion distance. However, if we assume that the analyte concentration in the concentrated plug is constant in depth, than the speed at which analytes are transported to the surface would be similar regardless of depth, and changing channel depth would have minimal effects on the performance of the sensor. We decided to etch the channel to around 10 microns because of its relatively low hydraulic resistance and also the ease for Nafion fabrication. When channel height is too large, it becomes more difficult for capillary valves to hold the Nafion in place and to prevent leakage of Nafion resin into other channels. A large channel height also results in more instabilities in the preconcentration zone as the vortexes are scaled with channel height. While a smaller channel height would result in smaller amounts of instabilities, fluid exchange and

pressure driven flow becomes more difficult to control as the hydraulic resistance increases with a thinner channel. We also found that clogging problems happen more frequently with a thinner channel. Due to these concerns, we decided to maintain the channel depth at 10 microns.

The width of the channel is decided by the effects it has on preconcentration. Making the channel narrower makes it easier to deplete the channel. If the channels are too wide, a high voltage would be needed to deplete the entire channel, leading to stronger vortexes and instabilities. There are several reasons for this. The Nafion junctions that generate the depletion forces are only located on the sidewalls, therefore the depletions forces are strongest near the sidewalls and weakest in the center. Incomplete depletion due to a combination of low voltage and wide channel can be seen in Figure 22. This problem is compounded in systems that depend on pressure driven flow. Pressure driven flow has a parabolic flow profile, where fastest flow is located in the center. This leads to the center parts of the channel being the weakest point in the preconcentration system. Decreasing the channel width helps to alleviate the problem as it is easier for the depletion forces generated near the channel walls to hold the molecules in the center back. As the channel width increases, a higher voltage is required to deplete the region, resulting in increased instabilities of the preconcentration zone. These vortexes tend to disrupt concentration polarization and eventually the depletion zone would be flushed away. Narrower channels increase the hydraulic resistance, but this disadvantage can be overcome with the methods detailed in the next section.

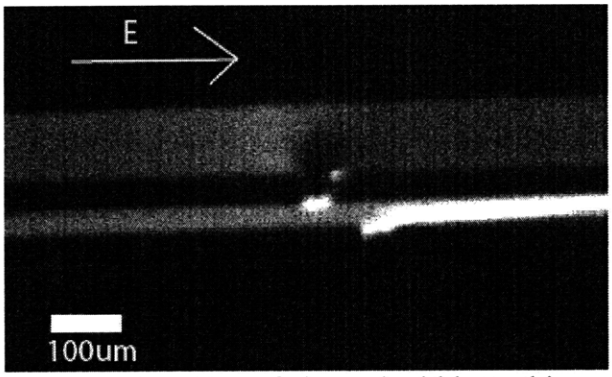


Figure 22. This experiment shows the effect of channel width. In this experiment, a 1xPBS buffer containing FITC dye was used to characterize the system (100μm wide channel), and the voltage was varied from 0 to 100V to test the performance of the system.

Flow rate is another factor we can fine tune to arrive at the correct operational regime. In a microchannel undergoing diffusive, convective, and reactive processes, a boundary layer would be formed near the solid phase of capture agents. This layer is where the concentration of analytes (that is being absorbed at the surface) is equal to that in the bulk. The thickness of this layer depends on the volumetric flow rate. In our system, plastic pipette tips are used as reservoirs for sample and buffer. By putting different amounts of liquid in these reservoirs, a height difference can be created to drive fluid flow in the microchannels. Since we are employing a surface based immunoassay, the volumetric flow rate does not directly dictate the accumulation speed of molecules. Rather, the linear flow speed would be more important because these molecules are being packed into an accumulation front, where the reaction actually occurs with the surface bound capture antibodies. The volume of this area can be related to the volumetric flow rate by the relation $Q = A \cdot v = w \cdot h \cdot v$ where Q is the volumetric flow rate, w

the width of the channel, h the height of channel, and v the linear velocity of flow. If the dimension of the microchannel changes, the linear flow velocity needs to change in order to maintain the same mass/volumetric flow rate. This can be seen in the following mass conservation relation: $w_1 h_1 v_1 = w_2 h_2 v_2$. If the cross-sectional area is halved, the linear flow velocity would double to compensate for the change. The same relation would apply for a change in microchannel height. However, creating microchannels of different height requires the use of two separate mask and lithography steps; therefore, it is much easier to change the width of the channel structure to achieve a faster linear flow rate. Increasing the linear flow rate helps would increase the speed at which molecules arrive at the depletion zone locally, and thus acts as a way to further amplify the preconcentration effects. Some of the concepts discussed here are illustrated in figure 23.

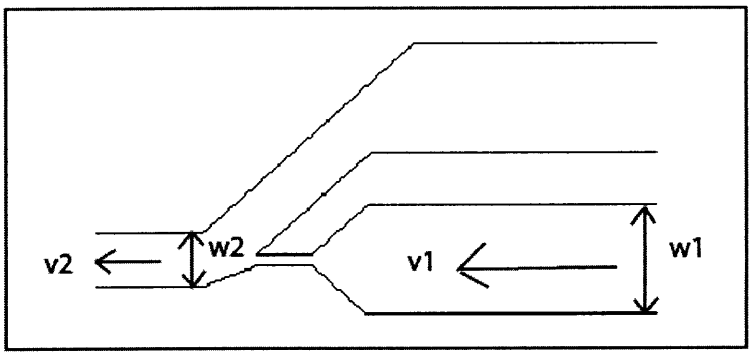


Figure 23. This figure shows the changes in channel geometry detailed in the previous paragraph. Flow entering from the reservoir (v1) sees a change in channel geometry near the Nafion junction (v2). The ratio of the channel widths (w1, w2) determines the ratio of the linear flow velocity (v1, v2).

Designs used to generate the results presented in this thesis have a nominally 100 micron wide microchannel, but the channel width is reduced to 50 microns in the detector region. This results in a 2x increase in local linear flow velocity and thus a two fold increase preconcentration efficiency. We also experimentally observed a lower voltage requirement for preconcentration to start, probably due to the fact that only small volumes need to be depleted of ions. While the width of microchannel can be decreased further, Nafion resin solution tend to leak out into the sample channel if the width is less than 50 microns due to the high capillary pressure present.

Experimental protocols

All experiments described in this subsection of the thesis were carried out using the following protocols.

Reagents

PBS Buffer

Phosphate Buffered Saline (PBS) and phosphate buffered saline with Tween 20 (PBS-T) in a powder form are purchased from Sigma-Aldrich (Sigma-Aldrich, MO) and are used without

further purification. After adding an amount of deionized (DI) water indicated on the package, a PBS 1x or a PBS-T 1x solution is obtained. The solution contains 138mM sodium chloride, 10mM phosphate ions, and 2.7mM potassium chloride with a pH of 7.4 at 25 degree Celsius. In the case of PBS-T, a 0.05% of Tween 20 is also present in the solution. PBS buffers having different ionic strenths are created by diluting these stock solutions further with DI water.

Surface functionalization

Neutravidin molecules are purchased from Invitrogen (Invitrogen, CA) as a lyophilized powder. A 5 mg/mL solution is made by adding 1mL of PBS 1x buffer to the vial. The solution is then further diluted to a concentration of 100ug/mL with PBS 1x buffer. Monoclonal antibodies against R-phycoerythrin are purchased from BD biosciences and are diluted to a concentration of 10ug/mL with PBS 1x buffer. R-phycoerythrin molecules are purchased from BD Biosciences. Various concentrations of this molecule are created by diluting the stock solution with an appropriate amount of PBS-T.

Remark: Non-specific absorption

Tween-20 is a polysorbate surfactant commonly used in molecular biology research. The main purpose for the addition of Tween-20 to PBS in our systems is for the prevention of non-specific absorption of R-phycoerythrin molecules on uncoated PDMS surfaces. The influence of Tween-20 on the behavior of the preconcentrator has been tested and we have concluded that it causes minimal interference with the concentrator devices.

Equipment

Voltage

A power supply (Keithley, OH) is used for generating voltage in these experiments. The output of the power supply is connected to four voltage dividers. Each of these voltage dividers consists of a fixed value resistor and a potentiometer. Each of these can be independently controlled to give a different voltage output for the various electrodes of the concentrator.

The outputs of the voltage dividers are connected to the preconcentrator device via electrical cables and platinum wires that are dipped in the reservoirs.

Illumination

An Olympus mercury lamp is used to provide large scale illumination for pictures taken under bright field. A Cairn Research (Cairn Research, UK) P150 OptoLED power supply and light source. Two different Light Emitting Diodes (LEDs) are installed in our system, providing illumination at 470nm and 530nm. The LEDs are controlled by a digital power supply and provides an adjustable power setting. A power setting is chosen by comparing its intensity with that produced by a new mercury arc lamp. Natural density filters are used to decrease the intensity of illumination and to prevent photobleaching from occurring.

Remark: Methods of illumination

Light in a mercury lamp is generated by an electric arc through the high pressure gases contained within the bulb. A high voltage is used to ionize the gases in the lamp, which in turn conducts electricity in an arc form, similar to a lightning arc. The performance of a mercury lamp degrades with time, and this effect is especially evident after the rated life of the bulbs. A LED-based system uses a solid state device for generating light. Besides having uniform intensity over time and the field of view, LED systems are also more durable compared to a mercury lamp. The power of the LED light system can also be measured in real time, resulting in more accurate and repeatable experiments.

Microscope

An Olympus IX-71 inverted microscope was used to make observations and measurements. For immunoassay quantification and measuring various levels of R-phycoerythrin bound the surface, an excitation light with 530nm wavelength is used. R-phycoerythrin emits strongly at 575 nm, and the appropriate filters and dichroic mirror have been chosen for these parameters. For our experiments, an objective lens with 20x magnification was used.

Data acquisition and processing

We used a PCO CCD SensiCam camera to capture images for analysis. Images are captured using Scanalytics's IPLab software, with various exposure times. The LED illumination usually has a smaller field of view than a full frame window (under 2x2 binning); in this case an Region of Interest (ROI) would be selected to limit captured pictures to areas with uniform illumination. Scripts that are programmed into IPLab (BD Biosciences, MD) are used to generate data for immunoassay readouts; time lapse data used for the immunoassay are taken every 5 seconds for 30 minutes.

Immunoassay with R-phycoerythrin

R-phycoerythrin (R-PE) is a red protein that is present in red algae and cyanobacteria. It is a brightly fluorescent protein with a strong absorption peak at 545/565 nm and an emission peak at 575 ± 10nm. The protein has a molecular weight of 240kDa and an isoelectric point (PI) value at around 4.5. When used in a PBS buffer, the protein carries a negative charge but has the tendency to stick to the sidewalls/glass microchannel surfaces. PBS-T is used to reduce non-specific absorption. In our experiments, we observed that PBS-T has effectively prevented non-specific binding in the uncoated areas of the microchannel; this would prevent sample from being depleted upstream and increase signal intensity in the detector region around the Nafion junctions. In later experiments, the rest of the channel is incubated with a 1 v/v% Bovine Serum Albumin (BSA) solution to prevent non-specific binding.

The immunoassay calibration curves are generated to measure the response of our surface immunoassay to different levels of R-PE. In order to perform the calibration task, a number of devices without the concentrator are made (but otherwise have the same feature sizes) and a

flow-through assay is performed on these devices. The protocol established in chapter 2 is used to functionalize the surface before injection of samples into the channel.

A fast flow rate is necessary to ensure that the assay would not be limited by convective transport effects. A height difference between different reservoirs can be used to generate a pressure, but can be inaccurate because of meniscus formation in the plastic reservoirs. A better way to induce pressure driven flow is to use a syringe pump. In this case, a Harvard Apparatus PHD 2000 syringe pump is used to inject liquid into the pdms chip directly. A flow rate of 1uL/min is used to deliver sample solution into the microchannels for these immunoassay experiments.

Remark: Syringes

When tubing is first inserted into a PDMS chip, a large pressure is generated due to the air in the PDMS reservoir being compressed in the reservoirs. Since the tubing and the plunger in the syringe are not perfectly rigid, the generated pressure would in turn compress the tubing. This initial spike in back pressure creates a flow rate that takes a long time to stabilize, resulting in results that are less accurate. Glass syringes made by Hamilton contain a plunger that doesn't compress as much as the ones found in BD-made plastic syringes, and these are used in the experiments. After the tubing is inserted, a pressure equilibration step is inserted to ensure accurate flow rate. Typically, we would wait around 10 minutes before starting the image acquisition processes.

Immunoassay results

Data points for the R-PE Immunoassays for are collected at different concentrations in 10x increments. After pressure equilibration, PBS buffer containing different concentrations of R-PE is flowed into the channel, and the results are recoded at a rate of 1 frame per 5 seconds. After 30minutes, the flow is stopped, and PBS-T is used to flush the channel for 5 minutes at 1uL/min to remove molecules that are loosely bound to the surface. The detector region is then quantified using exposure methods mentioned above. All the experiments are performed in triplicate to ensure repeatability and reliability of the results.

Figure 24 below contains calibration curves for the immunoassay at an exposure time of 500ms. From the graph, we can see that some of the more concentrated samples, such as 1ug/mL and 10ug/mL samples, produce the same readings at this exposure setting. Multiple exposure settings are used in order to increase the dynamic range of the assay. If only one exposure time, e.g. 1000ms, is used, the sensor would be saturated for both 1ug and 10ug. If an exposure time of 100ms is used, only 10ug would saturate the camera. However, at the 100ms exposure setting, signal resulting from a sample with 1ng/mL concentration is difficult to read is thus prone to interference from noise signals. Thus, the dynamic range is expanded by ten fold with the use of different exposure times. These results can be presented in a three dimensional graph, as shown in figure 4. From this figure, we can see that the fluorescence intensity increases about linearly with exponentially increased exposure times. For each exposure setting, we can see that the dynamic range shifts with the shift of the region where the immunoassay produces a linear response. Table 3 shows the average intensities of the immunoassay under different exposure settings.

A time-lapse graph can be useful to illustrate the binding mechanics of the immunoassay. Figure 25 shows a series of time lapse plots taken at a 500ms exposure time. We can see that the samples with high concentrations of R-PE, such as the 10ug/mL and the 1ug/mL samples, reach steady state fairly quickly (within first 10 minutes). At moderate concentrations such as 100ng/mL, the increase in intensity is approximately linear with time. At low concentrations, we can see that the increase in intensity is still linear, but a steady-state is not reached within the experimental time frame. At these low concentrations, binding happen very slowly and can be shown to take many hours to complete [33]. The lower limit of detection for the R-PE assay is about 1ng/mL.

Concentration	Average intensity (100ms)	Average intensity (250ms)	Average intensity (500ms)	Average intensity (1000ms)
Blank (control)	59	69	88	126
1ng/mL	78	122	190	326
10ng/mL	126	242	431	752
100ng/mL	216	514	955	1774
1µg/mL	1084	3054	4095	4095
10ug/mL	3689	4095	4095	4095

Table 3. Immunoassay results using surface functionalization detailed in chapter 2

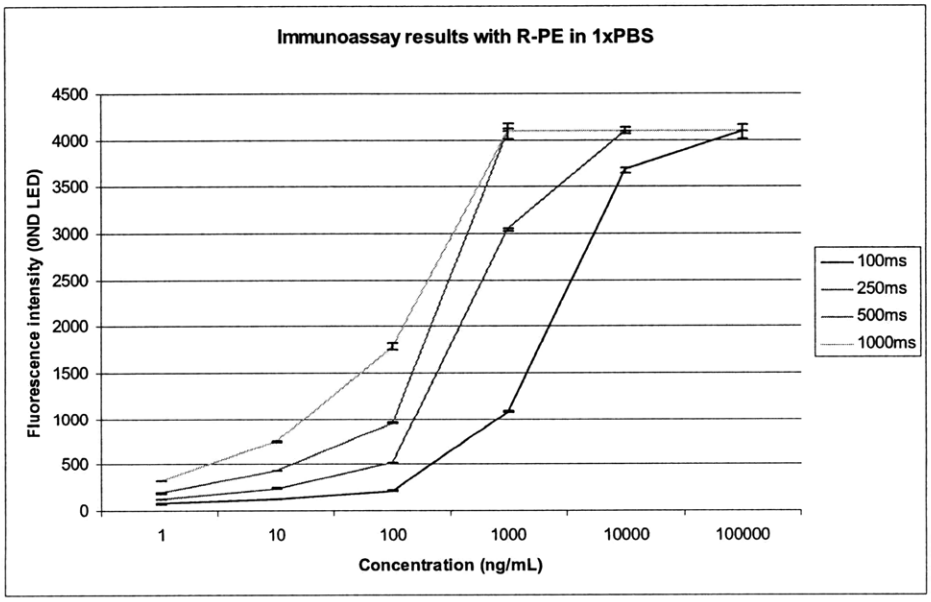


Figure 24 Immunoassay results

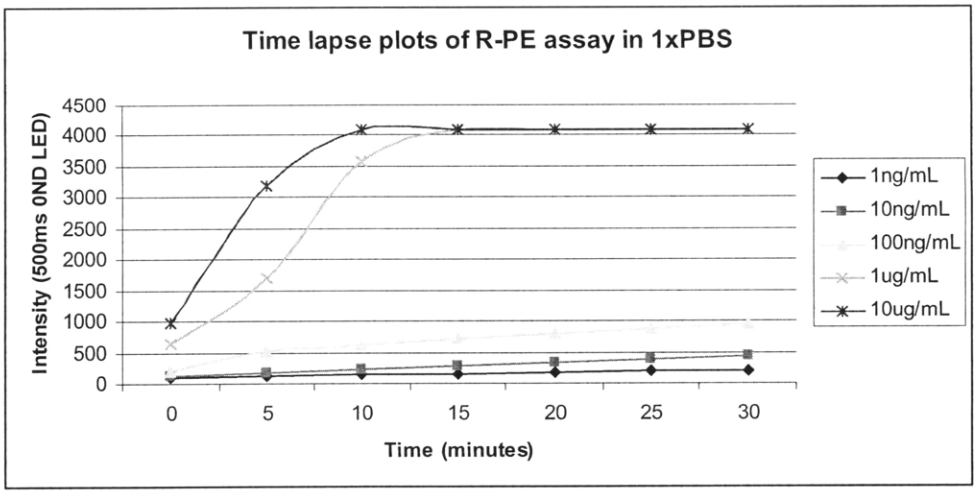


Figure 25 Time-lapse plots of immunoassay results. Differences in kinetics can be seen between high and low concentration samples.

Since the capture antibodies are patterned using non-specific binding, it's possible that the patterned antibodies lack uniformity. If true, this non-uniformity can create uncertainty in the accuracy and repeatability of the assay. However, the images obtained in the experiments (Figure 26) did not show significant variations in fluorescence intensity across the detector area. In order to further minimize the effects of pattern non-uniformity, an averaging method is used to compute the fluorescence intensity at each concentration. A large section within the patterned area of the microchannel is selected based on the intensity, and IPLab is used to compute the average intensity across this area. The max/min values at each concentration are also recorded, and these do not deviate from the average values too much. One explanation is that the nanoscopic non-uniformities do not result in significant differences when viewed at a microscopic magnification, which is what's being done in our experiments.

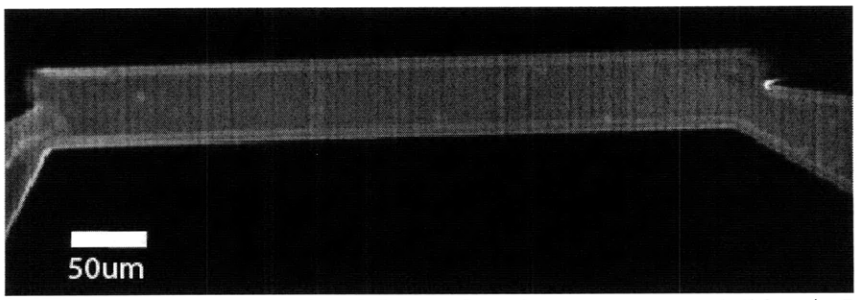


Figure 26. This image shows an example of the surface immunoassay. A 10ng/mL solution of RPE in 1xPBS is being shown captured by patterned antibodies, and the surface is relatively uniform despite the presence of contaminants.

Preconcentration experiments

Preconcentration experiments are started in the same way as the regular immunoassays. First, the detector region is coated with antibodies, and then the bypass channels are filled with buffer. In place of a syringe pump, a height difference in the reservoirs is used to generate a pressure difference to drive fluid flow across the channel. After pressure equilibration, a small

voltage would be applied and subsequently increased until ion-depletion effects are seen. After ion-depletion has started, the experiment would be run for 30 minutes for molecules to accumulate and bind to the capture antibodies. The voltage is subsequently turned off, and the sample channel is flushed with PBS-T for five minutes. The area where molecules were concentrating on is then quantified using the procedures detailed above.

Since pressure driven flow is used to drive accumulation of biomolecules, it might be possible for the concentrator to be used with only two electrodes instead of the typical three. Figure 27 illustrates the usage of two electrodes. The third electrode is rendered unnecessary because electrokinetically driven flow no longer plays a dominate role in the preconcentration experiments. In place of the third electrode, which is used to control the tangential field in electrokinectically driven flow, pressure is used to generate the flow instead. This brings several advantages as well as disadvantages. First of all, the coupling of tangential field and normal field decreases because now that there are no additional electric field across the depletion region other than the ones that are applied across the Nafion junction. The amount of instabilities tends to decrease with this reduction of coupling. This decrease in instabilities also allows higher voltages to be applied across the Nafion junction without the concentration polarization regions being disrupted. With higher voltages, higher flow rates can be sustained and can result in faster accumulation of molecules. The system becomes easier to use as well, as the tangential field is now set to be the same as the normal field, and a separate factor for fine tuning preconcentration is eliminated. In order to maintain a stable preconcentration zone for a set pressure, we now only need to vary the voltage applied across the Nafion membrane. Once the pressure/voltage relationship has been set, the system is quite stable and requires fewer human inputs to maintain. In fact, it is possible to run the system for more than 30 minutes without monitoring the voltage levels once ion-depletion has started. This increase in stability has the potential to make the preconcentration procedure to become more amenable to automation and repeatable.

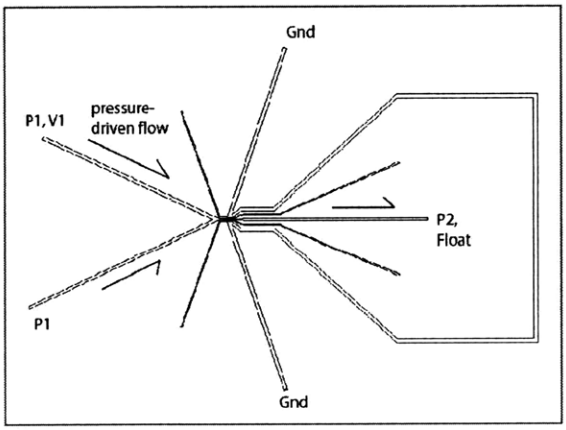


Figure 27. This schematic illustrates the use of two electrodes instead of three. The flow velocity can be controlled by varying the pressure difference, P1-P2, and the voltage V1. Since a tangential field is no longer required to drive fluid flow, flow rate can be more controllable and results more reliable.

"Dot" based preconcentration

The preconcentrators developed previously in our lab produces a concentration region in the shape of a plug. The shape and location of the plug depends on a number of factors – flow

rate, voltage, the analyte being concentrated, the ionic strength of the buffer used, and the amount of time the experiment has been running. The method used in making Nafion membranes is an important factor in deciding on the properties of the concentration effects as well.

Using R-PE as an analyte with PBS buffer, we observed different preconcentration behavior in devices made with capillary valves that are different from those made with surface patterned Nafion. Under similar flow rates and voltages, devices made with surface patterned Nafion produces a plug that is similar in shape to that seen in concentrators made in the silicon/glass system (Figure 28a). When the same experiment is performed in a device with capillary valve patterned Nafion, a "dot" shaped preconcentration zone is produced instead (Figure 28b). This dot shaped area usually forms close to the Nafion junction, and then slowly becomes larger and extends to the center of the channel over time (Figure 29a). When fluorescence intensity is measured across the width of the channel near the Nafion junction, we find that the basic "dot" shape of the concentrated molecules actually remained, and the brightest areas are those areas around the Nafion junctions (Figure 29b).

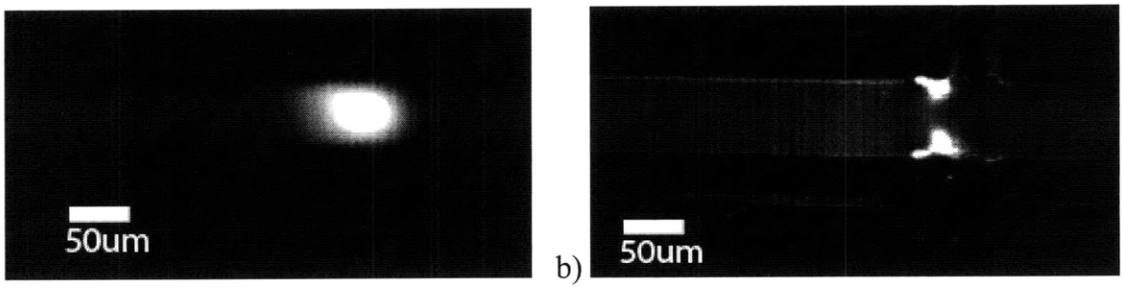


Figure 28. Comparison between dot and plug based preconcentration. A "plug" based preconcentration zone is shown in Figure 28a, and a "dot" based preconcentration zone is shown in Figure 28b. The experiments are performed in 1xPBS with 100ng/mL RPE samples.

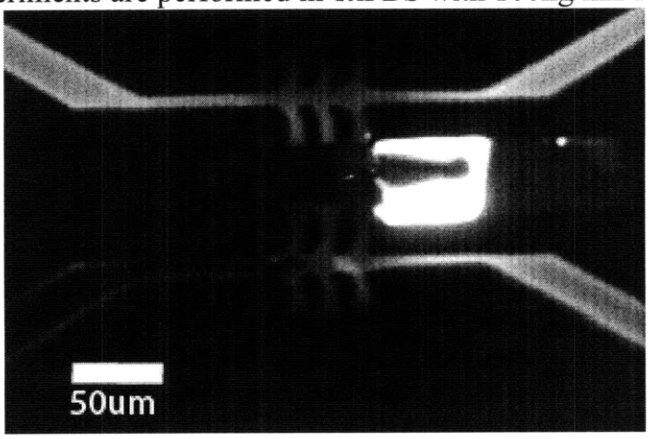


Figure 29a. Appearance of a "dot" based preconcentration zone. Because of the high localized intensity, the "dot" seems to extend into the center parts of the channel into a "plug". However, as the intensity plot in Figure 29b shows, the areas with the highest intensity are still those near the Nafion junctions.

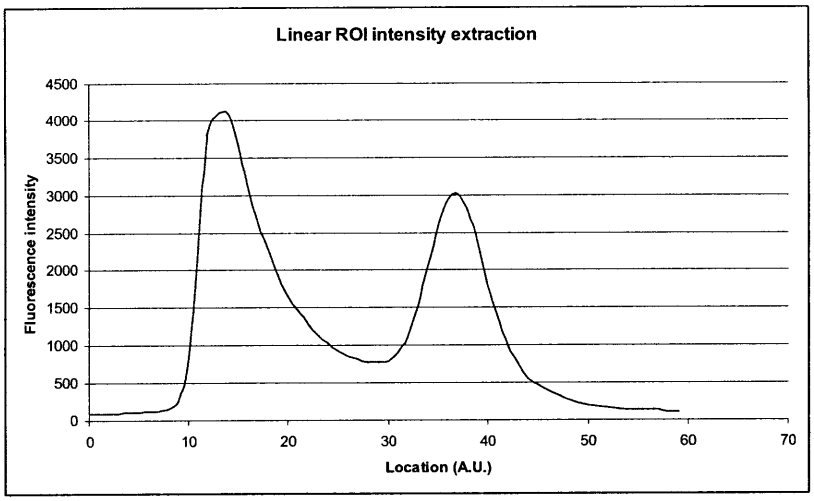


Figure 29b. Intensity profile of the plug across channel width in fig. 29a

By using a highly concentrated R-PE solution (1ug/mL) we can use a low exposure time (10ms) to find the reason for the differences with high speed capture sequences. We found that these "dot" areas are actually vortexes generated by the second kind of electrosmotic flow [36].

When ion-depletion starts, it seems that the charged molecules are forced into these fast moving/circulating vortexes and accumulated there. One way to explain the phenomenon is that the shape of the depletion zone changes with the fabrication method of the Nafion membrane. These Nafion junctions create depletion zones of different shape and this and results in the shape of the concentration zone. These concentration zones are quite stable and can reach preconcentration factors of ~100 times in a PBS buffer system. The shape of these concentration zone changes from a "dot" to a plug when the flow rate is decreased. This change showed that the shape of the preconcentration zone depends on the flow rate and the magnitude of electric field applied. If the applied field is high (to sustain a higher flow rate), then the vortexes are faster and thus the molecules are attracted into these vortexes easily; if the applied field is low, the vortexes are slower and thus some of the molecules are released into the areas outside.

The type of molecule used also changes the behavior of the concentrator. In our experiments with the capillary valve based devices, Fluorescein isothiocyanate (FITC) molecules are more tend to form a plug shaped concentration zone rather than a "dot" shape one, whereas R-PE molecules tend to form a dot shaped concentration zone. There are several possible reasons for this. FITC molecules have significantly less mass than a protein like R-PE, and therefore a higher charge-to mass ratio. It is possible that the higher charge-to-mass ratio allows FITC molecules to escape into the center part of the channel and form a differently shaped concentration region. The isoelectric point (PI) of these molecules also seems to contribute to the different behavior with the concentrator. Further study of the behavior of the concentrator would be very beneficial towards expanding its functionalities with different molecules.

Preconcentration results

The samples are analyzed in the same way as the experiments performed with regular immunoassays. The areas around where the molecules concentrated at are selected, and their average, max, and min fluorescence intensities are extracted with IPLab. Since the shape of the preconcentration zone does not span the entire channel, only these are as are selected for

evaluation. When preconcentration is done for 30minutes, we found that the limit of detection for R-PE has shifted from 1ng/mL to 0.01ng/mL (figure 30 and table 4).

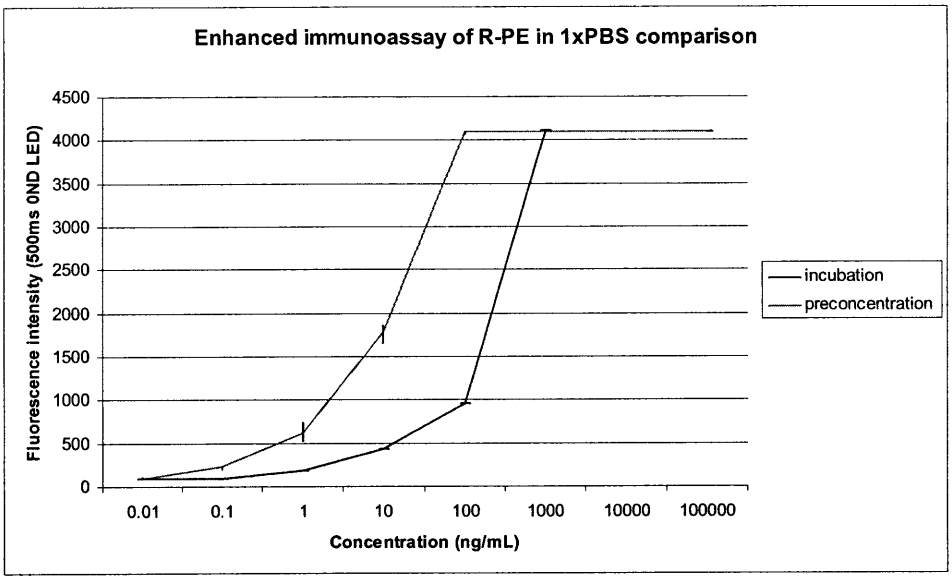


Figure 30. Enhanced immunoassay with enhanced binding curves superimposed on the original ones. Exposure time chosen here is 500ms. Preconcentrator was operated for 30 minutes, and the results were recorded after a PBS flush.

Concentration (R-PE)	Incubation (30 minutes)	Preconcentration (30minutes)
1pg/mL	88	88
10pg/mL	88	**98**
100pg/mL	88	**225**
1ng/mL	**190**	**612**
10ng/mL	**431**	**1778**
100ng/mL	**955**	**4095**
1μg/mL	**4095**	**4095**
10μg/mL	**4095**	**4095**

Table 4. Comparison of fluorescence intensity between incubation and preconcentration for the same time period. The experiments are performed in 1xPBS with R-PE molecules, and the exposure time is 500ms. The bolded numbers indicate the concentrations detectable with the assay.

Tracer for low concentration and non-fluorescent molecules

Tracers, or fluorescent markers, need to be used in a number of our experiements. High concentrations ($\geq$ 100ng/mL) of R-PE molecules can be preconcetrated relatively easily. At these concentrations, the fluorescence signal from unconcentrated R-PE molecules can be easily detected, enabling the observation of ion depletion and concentration behavior. At lower concentrations however, it becomes difficult to detect the fluorescence signal and determine if ion depletion has started. Another reason is that most molecules useful for biological or medical

applications are not natively fluorescent. These molecules require an indicator that would indicate the process of ion-depletion and preconcentration

Two different fluorescent molecules were tested for use as tracers in our experiments. We first tried using an Immunoglobulin G (IgG) labeled with FITC as a tracer. IgG was picked because of its high molecular weight (150kDa), which is close to the molecular weight of R-PE. A 100ng/mL concentration of IgG-FITC is used to visualize the concentration effect and ensure that leakage of concentrated plug did not occur. While IgG-FITC works well for visualizing the behavior of the concentrator, it causes problems with non-specific binding when a high concentration of the molecules is reached. Since IgG molecules are large proteins, they tend to have a greater tendency to stick to hydrophobic surfaces or other proteins. Also, since it's labeled with FITC and not natively fluorescent, a high concentration of the molecule is needed for visualization. Another molecule used is the Green Fluorescent Molecule (GFP). This molecular has a much lower molecular weight than R-PE (~50kDA). GFP is considerably brighter than IgG-FITC and can be visualized at concentrations down to 10ng/mL. Because of this, GFP does not pose as big a problem with non-specific binding, due to lower concentration of GFP being used compared to experiments based on IgG-FITC.

Challenges for non-fluorescent molecules

Non-fluorescent molecules present another set of challenges. As different molecules exhibit different behavior with the concentrator, we cannot be sure if these molecules all concentrate at the same rate or location. Some of these molecules indeed concentrate at different locations; for example, when a solution of FITC and R-PE molecules, two distinct bands can clearly be seen (Figure 12). In this experiment, the shape of the concentrated plugs are quite different – RPE forms a tightly packed, rectangular concentration zone while FITC molecules forms a more diffuse concentration zone. The locations that the molecules concentrate at are quite different as well, and can possibly be explained by the differing molecular characteristics of the two molecules. It's possible that the longer plug formed by FITC molecules is due to the higher diffusion coefficient of the molecules; the higher diffusion coefficient would lead to a more "stretched" preconcentration zone as the diffusive forces tend to move the molecules away from the preconcentrator. The location of the concentrated plug might be related to the charge carried by the molecule, or the charge to mass ratio of the molecules. A number of forces are acting on the molecules at any given time – pressure driven flow, electrokinetic flow, depletion force, and electrophoretic force. Our of these, pressure driven flow and electrokinetic flow push the molecules toward the depletion zone, the depletion force would push them away from the depletion zone, and the electrophoretic force does either depending on the charge of the molecule. A balance of these forces results in a stable stacking boundary. Besides pressure driven flow, flow due to all the other forces depend on the charge carried on the molecules. Therefore, we hypothesize that the mobility, by the extension the diffusion constant, would help us distinguish the location at which molecules would concentrate at. Further investigations into the behavior of molecules with different PI and molecular weight should yield insight into how molecular characteristics influence preconcentration behavior. These would be useful for picking the appropriate tracers for non-fluorescent molecules and eventual multiplexed operation of the concentrator.

Challenges for using serum/biological fluids

While PBS 1x buffer has the same ionic strength and types of the ions as human serum, it differs from serum in many ways. Serum or other biological fluids contain many high abundance proteins, which complicate detection of lower abundance proteins with non-specific absorption. These high abundance proteins also cause problems with the preconcentration process. When proteins reach a very high concentration, they might aggregate to each other and precipitate out of solution. Indeed, high abundance proteins such as human serum albumin (HAS) would cause increasing amounts of proteins to stick to the microchannel surface. These precipitating proteins also interfere with the operation of the preconcentrator, necessitating a step to remove these proteins,

One way we employed to solve this problem is to remove most of the high abundance proteins. We used a high abundance protein removal kit from Pall life sciences to remove about 90 percent of the HAS and Igs from the sample. We have experimentally observed that the HAS and Ig depleted serum can be used for preconcentration experiments and a significant increase in instability has not been seen.

Chapter 5

Conclusion and Future Work

In this thesis, a novel way to fabricate preconcentrators and infiltrate Nafion into microfluidic devices was developed. These devices were then coupled to a new surface-based immunoassay for characterization. We learned that these devices, with high aspect ration Nafion membranes, can perform preconcentration at much higher rate than previously developed devices. They also work well with high ionic strength buffers, such as PBS 1x, that more closely mimic physiological fluids. We also found that these devices exhibit different characteristics in operation: these new devices confine trapped molecules into a "dot" shape rather than a "plug" shape, implicating a different electric field distribution and opening doors for further scientific inquiry.

A 100x improvement for the assay of R-PE molecules have been achieved with a 30 minute preconcentration period, with the fluid driven by a height difference in reservoirs. With more accurate pressure control, it is foreseeable that a much higher performance can be achieved (down to a 100x improvement in 10 minutes). Further work would also involve the preconcentration of non-fluorescent molecules, testing out some of the strategies detailed in the previous chapter, and multiplexed detection of different biological molecules. Ultimately, the device would integrate functionalities to purify native biological fluids, including high abundance protein separation, cell separation, and secondary labels for detecting useful biological markers directly from whole blood.

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