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Total Integrated Sample Preparation for Microfluidic Immunoassays in Complex Biological Matrices

by

Akwasi Asare Apori

A dissertation submitted in partial satisfaction of the requirements for the degree of Joint Doctor of Philosophy with University of California, San Francisco in Bioengineering in the Graduate Division of the University of California, Berkeley

Committee in Charge:

Professor Amy E. Herr, Chair
Professor Adam R. Abate
Professor John S. Newman

Fall 2011
Total Integrated Sample Preparation for Microfluidic Immunoassays in Complex Biological Matrices

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by Akwasi Asare Apori
Abstract

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Joint Doctor of Philosophy with University of California, San Francisco in Bioengineering

University of California, Berkeley

Professor Amy E. Herr, Chair

A high-throughput protein analysis platform with integrated sample preparation is developed to address the identified technology gaps in biomarker validation, clinical and point-of-care diagnostics. The goals of the technology are to automate and integrate protein sample preparation with electrokinetic separations, implement immunoassays capable of processing raw biological fluids, and perform high-throughput protein assays targeted for disease diagnosis.

Integration of multiple functions is a hallmark of microfluidic tools. Efforts to fully incorporate required sample preparation steps within electrokinetic assays would benefit from a surge in technology development. On-chip fluorescence labeling, analyte enrichment, buffer exchange, reagent-sample mixing and matrix protein depletion are essential to realize the “sample-to-answer” paradigm of lab-on-a-chip systems for targeted proteomics—which hold the key to rigorous validation of potentially high impact biomarkers relevant for drug discovery, clinical diagnostics, and patient therapy and monitoring. Integration of sample preparation techniques would also expand the protein analysis application space to point-of-care assays in low resource settings.

Presently, introduction of in-situ fabrication methods has enabled advances in integration of multiple reaction zones into microfluidic geometries. Fabrication is performed at the microscale using chemically and physical functionalized polyacrylamide gels photo-patterned on microfluidic chips. These zones facilitate precise control of assay performance parameters including sample resolution, background noise, dynamic range, binding kinetics, and dispersion
among others while taking advantage of microscale interactions to reduce assay time, cost, and reagent consumption while increasing sensitivity, and portability.

In this dissertation several novel techniques for on-chip proteomics and immunoassays with integrated sample preparation are introduced for the analysis of serum or proximal fluids. These methods are applied to develop laboratory medicine and point-of-care assays for the following applications: 1) immunosubtraction polyacrylamide gel electrophoresis for detection of protein mobility and binding specificity, 2) on-chip fluorogenic labeling integrated with native electrophoresis for mass isoform detection, 3) low molecular weight compound sandwich fragment antibody assays for detecting drugs of abuse, 4) integrated enrichment and labeling of proteins facilitated with size-exclusion membranes, 5) cerebrospinal fluid rhinorrhea detection for emergency room screening of traumatic brain injury, and 6) rapid single-step electrophoretic and isotachophoretic immunoassays for serum Hepatitis C confirmatory diagnostics. The unifying theme of all techniques presented herein is the precise electrokinetic control and manipulation of sample and reagents on-chip to automate and expedite time consuming and manual labor intensive benchtop immunoassays based on diffusion driven interaction.
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1 Introduction

1.1 Motivation

The onset of personalized medicine has generated a broad need for advances in the field of proteomics. As doctors seek to identify the appropriate drug for a given patient that treats the proper ailment, the identification and validation of biomarkers will be imperative. Validated biomarkers can be used in clinical diagnostics, drug discovery, and patient monitoring among other fields. Currently, adequate technology does not exist to handle the complexity of identifying useful biomarkers in biological fluids at high throughput rates. In addition, the biomarkers that will be of the greatest benefit will neither be abundant nor easy to locate. For these reasons, there has recently been much research in the field of high-throughput proteomics to address the validation stage of the biomarker pipeline.

Microfluidic technology can provide many benefits for validating potential biomarkers. The benefits of scaling microfluidic proteomic platforms are numerous. Electrophoretic separation devices scaled to the micron level benefit from a rapid decrease in diffusion time and flow rate which scales with \((\text{distance})^3\) as described by Janasek et al. This results in over \(1000\times\) reduction in separation times and required sample volume as well as increased resolution. Other benefits of scaling proteomic devices include decreased cost per assay and increased application space, including portable and point-of-care diagnostics.

Potentially high-impact biomarkers relevant for drug discovery, clinical diagnostics, and patient therapy and monitoring have yet to be rigorously validated. Presently a technology gap limits translation of potential biomarkers from the discovery phase to clinical use (Figure 1-1). Suspect biomarkers, or proteins useful for identification of a disease state, must be analyzed with an accurate, repeatable, and quantitative technology. Furthermore, if the tool is to be used for validation studies that require 1000s of samples and multiple markers, the assays and associated sample preparation necessitate minimal manual intervention and the capability for fast analysis. Protein sizing (i.e., sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS-PAGE) with silver staining, a key analytical technology supporting biomarker validation, is low throughput (>1 day for analysis) and lacks automation potential (e.g., in streamlined sample preparation and sizing). Consequently, currently available analytical technology constitutes a critical bottleneck in the biomarker pipeline from discovery to clinical utility.

While integration of multiple functions is a hallmark of microfluidic tools, efforts to incorporate sample preparation fully into on-chip PAGE would benefit from a surge in technology development. On-chip noncovalent protein labeling with fluorescent dye is essential to realize the “sample-to-answer” paradigm of lab-on-a-chip systems for targeted proteomics. Integration of additional sample preparation techniques including sample enrichment, buffer exchange, matrix protein depletion, and reagent-sample mixing would also benefit benchtop proteomic tools as well as point-of-care assays for low resource settings. A handful of notable efforts in on-chip labeling have been reported; nevertheless, simplified and automated on-chip sample preparation techniques promise to expand the protein analysis application space.
Figure 1-1. Biomarker validation pipeline. As candidate biomarkers move from discovery to validation and clinical assay development, an increasing number of samples must be tested for fewer analytes at each step. (Image reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology, Copyright 2006).

Current benchtop based sample preparation techniques increase significantly in duration and cost with each step (Figure 1-2). Once the patient sample is collected it must be physically transferred to various different apparatuses for sample preparation each requiring physical removal of the sample and setup for the following preparation stage by a trained technician. The various preparation steps can include the following: the use of a centrifuge or vacuum pump for analyte enrichment, fluorescent sample labeling using chromatography spin columns to remove excess dye, incubation and mixing of the sample with a target antibody using a tube rocker or shaker, and finally detection of the target analyte via ELISA or slab gel electrophoresis. Each transfer step between a new apparatus adds time and cost due to technician time and labor, equipment setup and maintenance, biohazard waste production, etc. Additionally, because the majority of benchtop sample preparation and detection techniques rely on slow diffusion based techniques there is little active control over the required sample/reagent mixing needed at step. Thus, benchtop sample preparation techniques are expensive since each assay step adds cost while increasing the assay time to get from the raw sample to target analyte detection. However, integrated on-chip sample preparation techniques allow all required steps to be performed in a single device thus obviating the need for additional transfer steps after the initial sample load. Additionally, by using actively driven electrokinetic transport modes (electrophoresis, isotachophoresis, electro-osmosis, etc.) for sample/reagent mixing the time for various on-chip
sample preparation steps is significantly reduced over benchtop methods with automated and programmable control. Therefore, microfluidic techniques have potential for use in rapid and low cost sample-in-answer-out systems that quickly provide target analyte detection in a single step after loading the raw patient sample.

**Figure 1-2.** Benchtop sample preparation and detection protocol compared to integrated on-chip techniques. Benchtop sample preparation methods require several transfer steps between different apparatuses and these techniques rely on diffusion based sample/reagent mixing thus increasing assay cost and duration at each step and requiring high amounts of manual intervention. On-chip methods developed in this dissertation take advantage of active and programmable electrokinetic sample/reagent transport and mixing as well as integration of multiple processes on a single device to significantly decrease the cost, assay time, and user complexity for diagnostic devices.

### 1.2 Electrokinetic Microscale Transport

The advantages of using electrokinetically driven transport at the microscale over diffusion based methods include increased programmability and automation while decreasing assay times. As shown in Figure 1-3, using electrokinetic transport such as electrophoresis allows active control of particle motion (i.e., electrophoretic mobility $\mu_e$) by manipulation of the applied electric field. During electrophoresis, charged particles (e.g., proteins, nucleic acids, etc.) migrate due to the electrostatic force generated by the applied field which is counterbalanced by friction as the
particles move through some medium—separating into different zones based on the charge-to-mass ratio of the various species in the sample. Electrophoretic transport time over fixed distances within the microfluidic geometry is regulated by varying applied electric field, which can be precisely programmed using a software controlled power supply. For diffusion, particle motion is driven by random fluctuations as they migrate down a concentration gradient. Diffusion is a passive transport process; therefore, to decrease diffusion times for a reaction or mixing the physical length scales over which the reaction occurs must be decreased. For most biological assays it is more difficult to modify the geometry of the reaction chamber in a programmable fashion, making diffusion based assays less versatile for integration of many rapid assay steps than electrokinetically driven assays.

**Figure 1-3.** Electrophoretic transport allows active control while diffusion based transport is passive. Transport times over a fixed distance can be controlled during electrophoresis by manipulation of the applied electric field; however, transport times are constant over a fixed distance in a diffusion based device. Manipulating diffusion based transport times requires altering the reactor geometry or the distance over which the reaction occurs.

### 1.3 Bis/Acrylamide Size-Exclusion Membranes for Proteomic Devices

On-chip electrophoretic protein separation has been demonstrated as a viable means for identifying proteins in a complex solution. Commercial devices that exploit microfluidic
technology for protein separation are currently available including the BioRad Experion System and the Agilent 2100 Bioanalyzer. These automated technologies enable highly repeatable and efficient separations over a large dynamic range in a fraction of the time required for benchtop SDS-PAGE techniques. The Experion commercial system can perform chip based separations of complex samples in microfluidic channels in approximately one minute per separation (30 min total assay time)\(^6\) which compares favorably to slab gel SDS-PAGE which can take over an hour.

Polyacrylamide gels polymerized on-chip are a common electrophoretic separation medium\(^4\). The properties of the separation medium can be used to control separation parameters including assay time, peak resolution, background noise, separation efficiency, protein adsorption, etc. A novel method for low-concentration analyte enrichment and detection was developed by Song et al. of the Biosystems Research group at Sandia National Laboratories.\(^7\) Laser patterning was used to create dense nanoporous membrane regions in an acrylamide gel that permitted buffer and small molecules to pass through but excluded proteins based on the molecular weight cutoff of the membrane (> 5700 Da).\(^8\)

This size-exclusion membrane was further evaluated and integrated with electrophoretic sizing by Hatch et al. (Figure 1-4). A 50 µm size exclusion membrane was patterned with a size cutoff of 10 kDa and shown to concentrate proteins rapidly (< 5 min) 1000-fold with subsequent high-resolution separation in a larger pore acrylamide sieving gel.\(^9\) Protein concentrations as low as 50 fM were detected after 30 minutes of enrichment at the membrane, pointing to the application of this method for point-of-care diagnostics as well as proteomic research using proximal fluids (e.g. saliva, urine). Herr et al. exploited the enrichment capabilities of the size-exclusion membrane to detect low concentration biomarkers for periodontal disease in saliva samples.\(^10\)

**Figure 1-4.** A-C. Size-exclusion membrane for enrichment and detection of proteins allows rapid sample enrichment with subsequent separation in a single device\(^9\)(Image reprinted by permission from the American Chemical Society: Analytical Chemistry, Copyright 2006). D. Point-of-care diagnostic application of size-exclusion membrane shows successful periodontal disease biomarker detection\(^10\) (Image reprinted by permission from PNAS, Copyright 2007).
1.4 Dissertation Work Overview

This dissertation work herein focuses on integration of multiple analyte processing and detection methods into a microfluidic format and translation of the newly developed technologies into clinically relevant assays (Figure 1-5). Thus there is equal focus on description and characterization of sample preparation methodology, target assay development, and clinical applications with disease specific data interpretation.

**Figure 1-5.** Techniques covered in the dissertation are focused on development integration and translation of complex microfluidic assay techniques into novel integrated assays translatable to clinical use. Several sample preparation techniques were integrated with various target assay detection methods and applied to diagnosis of numerous diagnostic biomarkers.

In this dissertation several novel techniques for on-chip immunoassays with integrated sample preparation are introduced. These include immunosubtraction polyacrylamide gel electrophoresis for detection of protein mobility and binding specificity, on-chip fluorogenic labeling integrated with native electrophoresis, low molecular weight compound sandwich fragment antibody detection for drugs of abuse, integrated enrichment and labeling of proteins facilitated with size-exclusion membranes, cerebrospinal fluid rhinorrhea detection for emergency room screening of traumatic brain injury, and finally pore-limit electrophoresis and isotachophoresis for rapid single-step Hepatitis C confirmatory assays. The unifying theme of all techniques presented herein is the precise electrophoretic control and manipulation of sample and reagents on-chip to automate and speed up time consuming and manual labor intensive benchtop immunoassays based on diffusion-driven interactions. Additionally, all tools are fabricated at the microscale using chemically and physical functionalized zones patterned within polyacrylamide gels on microfluidic chips—which permits novel separation techniques taking advantage of microscale interactions to reduce assay time, cost, and reagent consumption while increasing sensitivity, and portability.
2 Theory and Modeling of Discrete Photopatterned Polyacrylamide Gels

Chapter Abstract: This chapter summarizes the theory behind design and operation of a microfluidic device that incorporates the use of a porous polymer membrane (bis/acrylamide) to allow sample processing (i.e., enrichment, labeling, analyte capture, binding) of low-concentration proteins prior to electrophoretic separation. Several properties relevant to controlling the desired pore size and thus size-exclusion properties of the membrane are analyzed and modeled. These include the effects of monomer to cross-linker ratio, initiator concentration, photo-initiation duration and power, and polymer material selection on the membrane pore size. Techniques for improved fabrication including the use of a thin-film polymer coating to control pore size and fouling properties of the membrane are also discussed. Finally, characterization techniques including hydrogel swelling experiments and epifluorescent imaging to determine pore size and incorporation of a membrane interpenetrating network into the separation gel are also considered.

2.1 Design and Characterization of Bis/Acrylamide Polymer Size-Exclusion Membranes for On-Chip Proteomic Applications

Although the efficacy of the polymer size-exclusion membrane for protein enrichment in microfluidic devices has been demonstrated, models of the polymer synthesis parameters affecting the molecular weight cutoff (or size-exclusion) properties has not been published for this application. This chapter summarizes the important polymer-synthesis parameters for controlling pore size of a size-exclusion membrane. In addition, pore-size characterization methods are discussed as well as new materials to deal with membrane fouling, which is known to alter both the molecular weight cutoff and separation capabilities (due to injection dispersion) at the size-exclusion membrane interface.

2.1.1 Calculating Sample Protein Volume

Determination of the desired pore size of a size-exclusion membrane begins with selection of the minimum protein size which must be excluded from passing through the membrane. All molecules below this chosen pore size will flow through the membrane, including but not limited to buffer molecules, unbound fluorescent labels, background contaminants, etc. Proteins impinging upon the membrane can be modeled as individual polymer chains surrounded by fluid elements. These molecules occupy a space defined by their hydrodynamic volume, which is the volume of a polymer coil in a given solution. Approximating the polymer chain with the freely-rotating chain model, we can take the volume of a single protein as the radius of gyration, \( R_g \), defined as the average distance from an element in the chain to the chain center of mass:

\[
\langle R_g^2 \rangle = \frac{\int r^2 \rho(r) dl}{\int \rho(r) dl} = \frac{\langle R^2 \rangle}{6} = \frac{C_\infty l^2 n}{6}, \tag{2.1}
\]

where \( r \) is a position vector, \( \rho \) is a mass element, \( R \) is the end-to-end distance, \( n \) is the number of chain segments, and \( l \) is the length of a chain segment. \( C_\infty = \langle r^2 \rangle_o / \langle r^2 \rangle_f \) is a measure of the
stiffness of the polymer chain measured by the ratio of the end-to-end distance between a
discrete rotation-angle model to the freely-rotating chain model. \( C_\infty \) values are typically
calculated experimentally and can be taken from the literature.

An alternative method for characterizing the polymer volume is by experimentally determining
hydrodynamic radius \( R_H \) from light-scattering measurements.

\[
R_H = \frac{k_B T}{6\pi \eta D_o} \tag{2.2}
\]

where \( k_B \) is the Boltzmann constant, \( \eta \) is viscosity of solvent, and \( D_o \) is the diffusion coefficient.
\( R_H \) gives the dimension of a sphere that diffuses at the same rate as the hydrated polymer in the
given solvent, and equation (2.2) approximates the radius theoretically (i.e., Stokes radius).
Calculating \( R_H \) via an experimental method would be preferred for native proteins due to the
influence of the secondary and tertiary structure on \( R_H \). Equation (2.1) however can be used as
an approximation in the case when working with a reduced and denatured protein, given that \( C_\infty \)
is available. Light-scattering values for some common proteins used in molecular weight ladders
are shown in Table 2-1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>( R_H ) (nm)</th>
<th>MW (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme</td>
<td>1.9</td>
<td>14.7</td>
</tr>
<tr>
<td>Chymotrypsinogen</td>
<td>2.4</td>
<td>25</td>
</tr>
<tr>
<td>Human insulin (pH 7)</td>
<td>2.7</td>
<td>34.2</td>
</tr>
<tr>
<td>Ovalbumin</td>
<td>3.0</td>
<td>43</td>
</tr>
<tr>
<td>Hemoglobin</td>
<td>3.5</td>
<td>65</td>
</tr>
<tr>
<td>BSA</td>
<td>3.6</td>
<td>67</td>
</tr>
<tr>
<td>Hexokinase</td>
<td>4.3</td>
<td>102</td>
</tr>
<tr>
<td>Urease (^{12})</td>
<td>7</td>
<td>545</td>
</tr>
<tr>
<td>Apoferritin</td>
<td>8.2</td>
<td>443</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>10.1</td>
<td>669</td>
</tr>
</tbody>
</table>

Due to the unavailability of experimental data on the hydrodynamic radius or characteristic ratio
for many proteins and potential analytes of interest, a simplified model can be developed to
determine the size at which a protein will be excluded by a porous membrane. Figure 2-1 shows
one approximation method in which pore size is determined by molecular weight between cross-
links in the sieving matrix network (e.g., polyacrylamide gel) while Figure 2-2 shows an image
of polyacrylamide and the nonuniform pore sizes observed with a scanning electron microscope
(SEM). A protein chain and a cross-linked gel chain are assumed to have a similar size and
conformation if they are the same molecular weight. Protein chain contraction or expansion in a
given solvent is also assumed to be proportional to that of the network chain.
Figure 2-1. Protein passing through a pore of a cross-linked network. Proteins take on a larger hydrodynamic volume in a good solvent (A) than in a poor solvent (B), yet the pore size is assumed to remain proportional to hydrodynamic volume when modeling size exclusion by \( M_c \).

Figure 2-2. SEM of freeze-dried polyacrylamide gel.\(^{13} \) Structure representative of gels with greater than 4% T acrylamide and 0.5-2% C. (Image reprinted by permission from Elsevier: Analytical Biochemistry, Copyright 1975.)

2.1.2 Calculating Membrane Pore Size

A porous polymer size-exclusion membrane can be fabricated using acrylamide cross-linked with bis-acrylamide. This bis/acrylamide gel will have a pore size controlled by several synthesis parameters including monomer to cross-linker ratio, starting initiator concentration, polymerization duration, and temperature.
The monomer to cross-linker ratio determines the molecular weight between cross-links in the gel. As acrylamide monomer units (functionality 2) polymerize they encounter bis-acrylamide monomer units (functionality 4) and create a cross-linked network after reaching the gel point. The frequency with which a bis-acrylamide cross-linker molecule is inserted into the growing chain depends upon the concentration of the cross-linker molecules in the solution. The insertion of monomer (A) to cross-linker (B) can be described by the copolymer composition equation:

\[ F_1 = \frac{r_1 f_1^2 + f_1 f_2}{r_1 f_1^2 + 2f_1 f_2 + r_2 f_2^2} \]  

(2.3)

where \( F_1 \) is the mole fraction of A-type repeat units in the copolymer formed at a given temperature, \( f_1 \) and \( f_2 \) are mole fractions of monomer A and monomer B in the solution, and \( r_1 \) and \( r_2 \) are reactivity ratios of monomer A and monomer B.

In general, radical polymerization is not very selective \( (r_1 \approx r_2) \), thus the copolymer composition depends mostly upon the comonomer composition. Studies have shown however that the acrylamide (AA) and N,N’-methylene-bis-acrylamide (BA) comonomer system has reaction ratios that change after reaching the gel point (Table 2-2). The larger reaction ratio of BA before gelation indicates the tendency for preferential BA insertion, which causes BA to link to other BA units forming aggregates of many BA units separated by AA chains. After gelation starts to occur BA is less likely to insert itself preferentially, forming multi-unit sequences and begins to behave closer to the ideal case of random-copolymer formation \( (r_1 r_2 = 1) \).

<table>
<thead>
<tr>
<th></th>
<th>( r_1 ) (AA)</th>
<th>( r_2 ) (BA)</th>
<th>( T(\degree C) )</th>
<th>Solvent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before gelation</td>
<td>0.57</td>
<td>3.36</td>
<td>22</td>
<td>H(_2)O</td>
</tr>
<tr>
<td>During gelation</td>
<td>0.64</td>
<td>1.77</td>
<td>22</td>
<td>H(_2)O</td>
</tr>
</tbody>
</table>

In Figure 2-3, a simulation shows the copolymerization equation plotted for instantaneous AA and BA as a function of monomer composition. Increasing the crosslinker concentration in the initial feed will increase incorporation into the copolymer; however the relationship is not linear, demonstrating copolymer drift. For example, Figure 2-5B shows that when making an acrylamide gel from a monomer solution with 6% total acrylamide (%T) and 0.66% cross-linker bis/acrylamide (%C) yielding a molar ratio of 0.11, the actual copolymer composition will incorporate cross-linker at a higher rate (yielding a molar ratio of 0.19 before gelation and 0.15 during gelation). A typical membrane gel concentration of 22%T 6%C (0.27 molar ratio) will incorporate cross-linker at a ratio of 0.47 prior to gelation and 0.37 during gelation. This result clearly shows the importance of knowing precise monomer/cross-linker ratios during polymerization. Small changes in the initial ratio can increase copolymer drift, causing membrane and gel fabrication with significant differences in monomer/cross-linker ratio.
Figure 2-3. Simulation showing molar fraction of instantaneous copolymer composition \( F \) as a function of monomer composition \( f \) for acrylamide (AA) and bis-acrylamide (BA) at 22°C in water.
The theoretical molecular weight between crosslinks ($M_C$) can be calculated by determining the average sequence length of AA monomers. A sequence of AA corresponds to a chain of monomers that is uninterrupted by a BA crosslinker molecule. This average sequence length can be defined by the probability of inserting an AA monomer given in the following equation:\textsuperscript{15}

\[
l_{AA} = 1 + r_{AA} \frac{f_{AA}}{f_{BA}}
\] (2.4)

where $l_{AA}$ is the average AA monomer sequence length, $r_{AA}$ is AA reactivity ratio, and $f$ is the instantaneous monomer molar fraction for AA or BA.

The average molecular weight between cross-links can be calculated from the average sequence length given that the molecular weight per repeat unit for acrylamide can be obtained from the chemical formula (Figure 2-4).

Figure 2-4. Acrylamide chemical structure.

The average molecular weight between cross-links can be defined by the following formula:

\[
\bar{M}_c = \bar{M}_o l_{AA}
\] (2.5)

Figure 2-5 shows the theoretical molecular weight between cross-links as a function of comonomer composition. This model gives an approximation for size-exclusion properties of a membrane neglecting the contribution of BA units to the distance between cross-links. The simulation in Figure 2-5 shows that the theoretical size-exclusion cutoff for a 22%T 6%C membrane is less than 200 Da or 0.5 nm.

The molecular weight cutoff size tends to increase rapidly once acrylamide monomer molar ratio is increased over 0.9. In order to polymerize a very precise cutoff weight over 500 Daltons, it is necessary to control the comonomer ratio very accurately given the sensitivity of the pore size to comonomer concentrations in this range where many biologically relevant proteins are found. This extreme sensitivity of pore size to initial cross-linker concentration suggests that alternative materials could be used to linearize the relationship and more easily control large pore sizes.
Figure 2-5. Simulation showing molecular weight between cross-links and hydrodynamic pore size as a function of instantaneous acrylamide molar ratio.

An important feature apparent in Figure 2-5 is the role of the proportionality factor in calculating pore size based on molecular weight between cross-links. This factor $\alpha$ can be defined as:

$$MW_{crosslink} = \alpha \cdot MW_{protein} \quad (2.6)$$

This proportionality or shape factor results in the theoretical $M_c$ along the y-axis predicting much smaller pore sizes than expected and must be scaled by $\alpha$ in order to align with the experimentally determined protein hydrodynamic radii. Figure 2-6 shows that $\alpha$ can be calculated from the slope when plotting protein MW vs. $M_c$ ($M_c$ determined from where Table 1 experimental $R_H$ protein values fall on Figure 2-5). The first order linear approximation gives $\alpha = 0.004$. This means that a protein should be assumed to be approximately 0.4% of its MW when using molecular weight between cross-links as a size cutoff parameter. Calculating membrane size exclusion properties based on protein experimental $R_H$ values is most accurate when available given the difficulty in determining the most appropriate shape factor. This is particularly noticeable given the comparison between the linear fit for $\alpha$ ($y = 0.004x + 846.1$, $R^2=0.975$) versus the power law fit which appears to be more accurate at larger MW values ($y = 9.631x^{0.438}$, $R^2 = 0.999$).
Other synthesis factors that can affect the size-exclusion property of membranes polymerized by a photo-initiator are temperature, initiator concentration, and light intensity. Both rate ($R_p$) and degree ($\bar{x}_n$) of polymerization are affected by incident light intensity as follows:\textsuperscript{15}

\[
R_p = k_p \left( \frac{\phi \varepsilon I_0}{k_t} \right)^{1/2} [M][I]^{1/2} \tag{2.7}
\]

\[
\bar{x}_n = \frac{k_p[M]}{(1 + q)(\Phi \varepsilon I_0 k_t)^{1/2}[I]^{1/2}} \tag{2.7a}
\]

where $k_p$ and $k_t$ are rate constants for polymerization or termination, $M$ and $I$ are monomer and initiator concentrations at a given time, $I_0$ is incident light intensity, $\phi$ is quantum yield, $\varepsilon$ is initiator molar absorptivity, and $q$ is the fraction of termination reactions that occur by disproportionation. Increasing monomer, initiator, or light intensity will cause an increase in polymerization rate, although $R_p$ is more sensitive to changes in monomer concentration than initiator or light intensity. Increasing monomer concentration will also increase $x_n$; however increasing initiator concentration or light intensity will decrease $x_n$.

For UV lamp photo-initiated polymerization, the variables most often changed during membrane formation with a photo-mask are distance from the light source and duration of polymerization. Since $I_0$ drops off as the square of distance from the source, moving the UV source farther from
the gel decreases \( R_p \) and requires longer polymerization times. Since \( R_p \) decreases with \( I_o^{1/2} \), membrane polymerization time depends linearly on distance to the lamp: \( t_{poly} \propto d_{lamp} \).

The effect of temperature on \( R_p \) and \( x_n \) comes from the dependence of the rate constants on temperature as follows:\(^{16}\)

\[
k_p = A_p e^{(-E_p/RT)} \quad (2.8)
\]

\[
k_t = A_t e^{(-E_t/RT)} \quad (2.8a)
\]

where \( R \) is the gas constant, \( T \) is temperature, \( A \) is the collision factor for a particular reaction (polymerization or termination), and \( E \) is the activation energy. Analysis of these equations shows that increasing temperature serves to increase \( R_p \) but decrease \( x_n \).

In general, monomer to cross-linker ratio is the major synthesis factor affecting pore size. Increasing UV intensity will allow for more rapid polymerization of the membrane. The same is true of increasing initiator concentration or temperature; however these parameters should not have a large impact on pore size if the polymerization continues to completion. Although increasing these parameters also decreases \( x_n \) for a linear polymer, when making a cross-linked network polymer, \( x_n \) always increases rapidly towards infinity and gel formation.

Increasing light intensity \( (I_o) \) is the best alternative for speeding up photo-initiated membrane polymerization. Increasing temperature or initiator concentration to sufficient levels can make the monomer outside of the desired photo-patterned region extremely reactive and susceptible to unwanted polymerization. Optimal operating ranges for \( T \), \( [I] \), and \( I_o \) should be established experimentally.

### 2.1.3 Model Limitations

Limitations of theoretical modeling for polyacrylamide gel pore sizes lie primarily in the distribution of the pore-sizes near gel/monomer interfaces. In this region studies have shown that pore sizes are typically nonuniform with smaller pores present at any discrete interface compared to pores further away from the interface. The basic mechanism responsible for the smaller pore size present at the interface is the high rate of diffusion of new monomer molecules to the interface from the monomer free solution compared to the relatively immobile monomer particles being polymerized into the gel far from the interface. Additionally, cross-linked polymer network chains are shorter when forming in free solution compared to a gel due to increased reactivity of bisacrylamide (see Table 2-2), thus potentially further reducing the average pore size at a discrete photopatterned polymer interface.

Hou et al. developed techniques to mitigate this nonuniformity in pore size by lowering UV intensity during the polymerization process, thus preventing formation of a denser network of polymer chains at the discrete photopatterned interface compared to the center of the gel.\(^{17}\)
Other major limitations to the accuracy of the model include the 2-D modeling of the pores which in 3-D may have a smaller effective exclusion radius due to the fact that other pore chains can pass orthogonally through pores in a given plane. Finally, considerations of proteins that become entangled, or foul the membrane network, were excluded in the modeling although this phenomenon is observed experimentally and known to reduce further the effective membrane pore size. Detailed consideration of the effect of fouling proteins is discussed later in this chapter and in the immunosubtraction experimental results (Chapter 4). Both the effects of the 3-D mesh network and fouling can be incorporated into the proportionality factor given by equation 2.6 to further improve further the pore-size theoretical model.

2.2 Experimental Characterization of Pore Size

Various synthesis parameters ranging from comonomer ratios and polymerization time to the microchip optical transmission properties and channel dimension can cause the actual pore size to vary somewhat from the theoretically determined size. Thus, the membrane should be characterized experimentally to verify membrane pore size most accurately.

Swelling of a cross-linked gel to equilibrium provides direct information on the molecular weight between cross-links in a gel. Thermodynamic considerations at equilibrium require that the potential free energy of solvent in the gel equal that of the pure solvent. This leads to the Flory-Rener swelling equation:16

$$\ln(1 - \nu^2) + \nu^2 + \chi_1 \nu^2 = -\frac{\rho_2 V_1}{M_C} (\nu_2 - \frac{\nu_2}{2})$$ (2.9)

where \(\nu_2\) is the volume fraction of polymer in the swollen gel:

$$\nu_2 = \frac{1}{q} = \frac{V_o}{V} \quad (2.9a)$$

\(M_C\) is the molecular weight between cross-links, \(\chi_1\) is the polymer-solvent interaction parameter, \(\rho_2\) is the polymer density, \(V_1\) is the molar volume of solvent, \(V_0\) is the volume of the unswollen gel, \(V\) is the volume of the swollen gel, and \(q\) is the volume swelling ratio.

Conducting a swelling experiment to find \(M_C\) of the size–exclusion membrane would require taking a gel sample from the membrane and immersing it in a solvent of known \(\chi_1\). The weight of the initial sample compared to the weight of the final swollen gel sample at equilibrium can be used to calculate the mass swelling ratio \(q_m\):

$$q_m = \frac{m_s}{m_u} \quad (2.9b)$$

$$q = q_m \left(\frac{\rho_p}{\rho_s}\right) + 1 \quad (2.9c)$$
where \( m_s \) and \( m_u \) are the swollen and unswollen gel masses and \( \rho_p \) and \( \rho_s \) are the polymer and solvent density. Measuring \( q_m \) experimentally and solving equations 2.9c will provide an accurate measure of gel pore size. Table 2-3 provides the necessary constants from published literature to solve equations 2.9c for the bis/acrylamide system.\(^\text{18}\) Figure 2-7 shows data for a swelling experiment reflecting the decrease in swelling ratio with the increase of crosslinker on a poly(acrylamide-co-acrylic acid) gel.\(^\text{19}\) As cross-linker concentration is increased, pore size is expected to decrease exponentially although the swelling ratio decreases more linearly.

**Table 2-3: Swelling equation parameters for bis/acrylamide gel in water.\(^\text{18}\)**

<table>
<thead>
<tr>
<th>( \chi_1 )</th>
<th>( \rho_2 )</th>
<th>( \rho_{\text{water}} )</th>
<th>( V_1 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.48</td>
<td>1.35,g/mL</td>
<td>1,g/mL</td>
<td>18,mL/mol</td>
</tr>
</tbody>
</table>

**Figure 2-7.** Polymer swelling as a function of cross-linker concentration.\(^\text{19}\) The effect of crosslinker (methylen bisacrylamide, MBA) composition in gel interpenetrating networks on swelling ratio in (○) calcium and (●) phosphate solutions shows the decrease in swelling ratio with the increase of crosslinker. \([\text{PVA}] = 3.0\, \text{g}, \ [\text{AM}] = 14.0\, \text{mM}, \ [\text{AA}] = 14.5\, \text{mM}, \text{ and } T = 27^\circ\text{C} \pm 0.2^\circ\text{C}.\) (Image reprinted by permission from Taylor & Francis: Journal of Macromolecular Science Part A -- Pure and Applied Chemistry, Copyright 2004.)
2.2.1 Membrane Fouling

Membrane fouling was postulated to be caused by the nonspecific adsorption of proteins to the membrane interface after being concentrated against the membrane via electrophoresis. Molecules in solution adsorb to an interface to minimize the interfacial free energy. During experiments, protein molecules are introduced to the membrane at increasing concentration as they are enriched prior to separation. After applying an electric field to elute the protein band from the membrane edge, some molecules do not elute either because they have adsorbed to the membrane boundary, have become entangled in the larger pores among the interface pore distribution, or have become entangled in a lower density gel gradient formed near the boundary.

![Figure 2-8](image)

**Figure 2-8.** Membrane boundary fouling made visible with fluorescently labeled proteins. Images are taken during separation after proteins are eluted from membrane in the arrow direction. The same membrane is observed after < 10 assays (A) and after > 50 assays (B). All membranes formed by UV lamp photopolymerization.

Proteins typically adsorb on nontreated surfaces by physiosorption, which is characterized by weak Van der Waal’s forces such as dipole-dipole or dipole-induced-dipole forces between segments of the protein and the surface. Treated surfaces such as silanized glass channel sidewalls can adsorb molecules via chemiosorption, which is characterized by stronger forces including covalent bonding or electrostatic interactions of ions. However, acrylamide gels have been shown not to significantly adsorb proteins from a free solution over a 24h period. Given the weak forces caused by physiosorption, it is likely that the membrane fouling as observed in Figure 2-8 is not due to adsorption but rather a result of forcing proteins into a gradient gel at the border of the membrane that has a larger pore size. This fouling at the front of the membrane is capable of altering the size-exclusion characteristic as proteins build up and entanglement at the edge, effectively shrinking the pore size after several uses. Therefore, if membrane fouling is not minimized, data collected using the membrane for protein enrichment and separation will vary over the chip lifetime as the effective pore size shrinks.

In addition to gradient-gel formation, another cause of fouling may be nonlinearity in the pore size of bis/acrylamide gels. Naghash et al. suggest micro-gel formation prior to the onset of macro-gelation due to the high reactivity of bis crosslinkers causing cyclization reactions. This could also result in large statistical variations in pore size homogeneity in the bis/acrylamide gel.
2.3 Alternative Membrane Design: Fabrication Methods

Carefully controlling membrane fouling at the interface is crucial for repeatable use of the on-chip enrichment device. Two methods that can be employed to control this are employing more precise photolithography techniques to polymerize the membrane or utilizing a cross-linked polymer with more precisely controlled interface characteristics.

The first alternative for membrane fabrication to reduce gradient-gel formation at mask interfaces is by using a high-powered laser for precise control of membrane patterning. This increased UV intensity of the laser increases the rate of polymerization resulting in substantially shorter exposure periods to reach the desired degree of polymerization (see dependence of $R_p$ and $x_n$ on light intensity $I_o$, equations 2.7 and 2.7a). Given that $R_p \alpha I_o^{1/2}$, a 4 kW Nd:YAG (neodymium-doped yttrium aluminum garnet; Nd:Y$_3$Al$_5$O$_{12}$) laser would polymerize a membrane in 16% of the time required for a 100 W UV lamp. Unlike lasers, UV lamp intensity falls off as $1/\text{distance}^2$ from the source, further reducing times for laser photo-initiated polymerization compared to lamps at the same distance from the monomer. Herr et al. were able to pattern membranes with UV laser photo-initiation in 15 to 30 seconds as opposed to the 8 to 15 minutes required for a UV lamp$^{10}$ (a $32 \times$ reduction in fabrication time).

By reducing polymerization time, laser photo-initiated polymerization reduces chain formation at the boundaries from growing chains. Reducing polymerization time also reduces activation of radical-forming initiator near the boundary exposed for long periods of time during UV lamp photo-initiation. Gradient-gel formation at the membrane boundaries decreases when using the precise patterning geometry of the laser beam which has a Gaussian intensity distribution in the radial direction. Patterning with an incandescent UV lamp source may result in nonuniformity on the membrane edges as light refracts through the mask at various angles, leading to further gradient-gel formation (Figure 2-9).
2.4 Alternative Membrane Design: Novel Polymers

Using alternative monomers, other than polyacrylamide, to synthesize the size-exclusion membrane can provide more control over pore size as well as reduce membrane fouling. Interpenetrating polymer networks (IPN) have been shown to allow control over gel pore size. Bearinger et al. fabricated a poly(ethylene glycol) (PEG) IPN at the boundary of a cross-linked polyacrylamide gel.\textsuperscript{20} The PEG IPN prevented any appreciable protein adsorption from an aqueous solution over a 24h period. In addition, the shear strength of cells attached to the
PEG/acrylamide IPN were 25% less than those attached to the polyacrylamide gel without the IPN.

Improving the polyacrylamide gel fouling properties and pore-size uniformity can potentially be achieved using an interpenetrating polymer network by grafting a thin PEG/di-acrylate (PEGDA) layer to a bis/acrylamide gel. The IPN would be photo-polymerized on the edge of the bis/acrylamide membrane. This new material on the membrane boundary would be effective for controlling pore size, reducing the gradient-gel at the boundary, and reducing fouling. PEGDA has been shown to have beneficial properties when used in a blend to create polymer electrolytes. The electrical conductivity of this polymer may also provide benefits to reduce concentration polarization encountered across the membrane after repeated use and fouling.

Figure 2-10. Schematic of PEGDA interpenetrating polymer network comonomer structure. Schematic of proteins loaded against IPN thin film attached to a gradient gel on the edge of the membrane.
PEGDA can make an ideal material for an interpenetrating network because of the nonfouling properties of PEG and the controllability of pore size allowed by using diacrylate as the cross-linker. Diacrylate is much larger than bis-acrylamide, meaning that the pore size would be larger if substituted in place of bis-acrylamide. This would linearize the exponential region of the cross-linker concentration to \( M_c \) ratio curve where the cutoffs for many proteins of interest are located (see Figure 2-5). This diminished linear sensitivity would enable more accurate pore-size fabrication by reducing errors associated with minor inaccuracies in gel reagent measurement.

2.4.1 Characterization and Performance Testing of Novel Membrane Designs

The key performance metric for any new membrane material is reproducibility of desired pore size. This is characterizeable by swelling experiments as discussed earlier in the chapter. Additional characterization techniques unique to thin IPN films that would be useful include performing ellipsometry to measure the PEGDA film thickness. In order to characterize the composition of the IPN, X-ray photoelectron spectroscopy (XPS) can be performed at the surface and at penetrating depths in order to determine the elemental composition of the membrane IPN. Both ellipsometry and XPS measurements would need to be performed on a model system with a gel fabricated off-chip on a substrate (e.g., glass slide).

Epifluorescent microscopy can be used for on-chip membrane characterization. Fluorescent tagging of PEG and DA molecules allows detection of the incorporation throughout the membrane and subsequently formed separation gel. Although unpolymerized PEG and DA monomers are removed from the chip prior to polymerization of a separation gel, incorporation of unevacuated PEG and DA monomer via diffusion into the bis/acrylamide separation gel is possible. The quantity of incorporated PEGDA gel and the distribution must be assessed for this protocol because of the effect of a PEGDA/bis/acrylamide separation gel blend on electrophoretic separation (band dispersion, protein mobility rate, etc.).

Performance testing of PEGDA interpenetrating networks can be performed to evaluate membrane penetration by using proteins of known sizes. The molecular weight cutoff limit can be assessed by electrophoresing a series of fluorescently labeled ladder proteins against the membrane and measuring penetration distance into the membrane boundary. The percentage of proteins remaining in the membrane post-elution can also be quantified to assess repeatability of the membrane-cutoff location and elution efficiency (testing results shown in chapter 4).

Fouling performance tests can similarly be performed by loading proteins against the membrane while varying loading time and current to establish the parameters at which fouling occurs. In particular, testing the reusability (fatigue resistance) of the membrane after repetitive loading of protein against the membrane within the expected loading current operating range is useful. Calculating the E-field or current damage threshold (membrane yield strength) is also an informative test for setting operating limits for the membrane. This can be performed by loading labeled proteins against the membrane at increasing electric field strengths until observing forced penetration of proteins into the membrane (testing results shown in chapter 4).
2.5 Conclusion

In this chapter the polymer synthesis parameters that affect the pore size of a bis/acrylamide size-exclusion membrane have been evaluated. The most important factor in determining pore size was found to be the monomer to cross-linker ratio. The membrane pore size in the biologically relevant protein range varied exponentially with cross-linker ratio suggesting the potential difficulty in fabricating pore sizes to filter similarly sized proteins. Potential copolymer drift was also shown to originate from the preferential insertion of bis-acrylamide over acrylamide, particularly at the onset of polymerization.

Membrane fouling was observed that would lead to a reduction of the effective pore size due to entangled proteins stuck at the membrane edge. The likely origin of the membrane fouling was shown to be formation of a gradient or nonuniform gel at the membrane boundary as opposed to nonspecific protein adsorption. Improvements to enable accurate control over pore size during membrane synthesis include grafting of a PEGDA interpenetrating polymer network on the edge of the membrane used for size-exclusion or high power UV laser membrane fabrication. UV laser membrane patterning would eliminate gradient-gel formation while the PEGDA IPN would coat possible membrane edge gradients with a thin polymer layer of controllable and uniform pore size. Both suggested design alterations would be expected to reduce membrane fouling, and results of the laser fabrication method are discussed further in chapter 5.
Chapter Abstract: Microfluidic immunoassay techniques offer advantages in speed, automation, and portability over bench-top gold standard counterparts. In particular, on-chip immunoassubtraction is a rapid homogeneous immunoassay used for reporting both protein native mobility and binding specificity. Immunoassubtraction is performed by removing antibody-bound target proteins from electrophoretic detection via a size-based exclusion filter, while unbound nontarget proteins are able to pass through the filter for downstream detection. Immunoassubtraction is achieved on-chip via the use of discrete patterned polyacrylamide gel regions. Additionally, polyacrylamide (PA) gel regions are used to define sample preparation functions on-chip for protein enrichment, fluorescent labeling, and antibody-target binding prior to immunoassubtraction. Chapter 3 provides detailed step-by-step protocols for the immunoassubtraction and sample preparation membrane device fabrication techniques as well as the electrophoretic assay protocol for determining target protein mobility and binding within complex biological samples including cerebrospinal fluid. Details specific to fabrication of pore-limit gradient gels are contained in Chapter 7.

3.1 Introduction

The field of microfluidic chip based immunoassays has shown the potential to greatly advance clinical chemistry and diagnostics on inexpensive and disposable platforms amenable to use at the point-of-care (POC). Microfluidic chips afford advantages in surface-to-volume ratios that improve antibody-antigen reactions and reduce sample and reagent usage, which both significantly benefit immunoassays. Simplicity, ease of use, and low power consumption are all crucial to POC diagnostics which must accurately quantitate chemical compounds within complex biological fluids (e.g., blood, saliva, urine, nasal fluid) while typically requiring processing steps ranging from removal of unwanted compounds to analyte enrichment, labeling, and detection. In particular, microfluidic immunoassays with integrated sample preparation for biomarker detection have been shown to overcome bottlenecks associated with bench-top slab-gel and capillary electrophoresis methods. Recent work has shown that homogeneous mobility-shift assays employing electrophoretic sample transport also benefit from increased automation, rapid assay times, portability, and ability to integrate sample preparation functions while obviating the need for complex device components including valves and pumps. Immunoassubtraction, the gold standard for laboratory assessment of monoclonal gammopathies, is a special limiting case of the homogeneous mobility shift assay in which a target antibody is used to remove the signal of binding antigen from the downstream detection signal.

Immunosubtraction is a powerful laboratory medicine tool used for quantifying proteins via both binding selectivity and analyte mobility within biological fluids. Benchtop immunosubtraction analysis is typically performed on a heterogeneous platform using capillary or slab-gel electrophoresis coupled with diffusion based analyte binding and extraction (e.g., bead immobilized antibodies). The current bench-top immunosubtraction protocols have minimal capability for automation and integration with sample preparation steps and does not benefit from reduced sample volumes and rapid assay times achievable with microfluidic assays. These issues are addressed with on-chip homogeneous immunoassubtraction, which is achieved by photo-
fabricating precise features within a discontinuous polyacrylamide gel to create a step decrease in pore-size at the start of a polyacrylamide gel electrophoresis (PAGE) separation channel. The discrete pore-size decrease serves as a size-based ‘immunofilter’ by excluding any target analyte which binds to the capture antibody to form large antibody-antigen complexes from subsequent PAGE in the microchannels. The on-chip protocol requires performing a subsequent pair of electrophoretic separations on a sample; the control separation without the presence of target antibody and the immunosubtraction separation with the presence of target antibody. The resulting electropherograms are compared to determine extraction (and thus binding) of sample proteins with the target antibody. The on-chip immunosubtraction assay completes in 5 to 10 min with integrated sample preparation compared to 4 to 12 h for the off-chip method, which requires diffusion based labeling and binding sample preparation steps followed by standard bench-top separation techniques including capillary electrophoresis and bead-based target extraction.

Advances in discretely patterned in-situ PA gel fabrication have allowed functionalization of unique gel regions within a single microdevice capable of separating proteins with respect to biochemical properties useful for immunoassays ranging from on-chip zymography and western blotting to ultra-short portable CE and immunosubtraction. Critical to fabrication of the on-chip immunosubtraction assay is the discrete PA sieving matrix with a discontinuity from large pore-size loading gels (3%T) to small pore-size separation gels (12%T) establishing size-based filtration without chemical immobilization or other permanent fixation of the target subtraction antibody. In this chapter we describe the technique for fabrication of discrete PA gels for performing on-chip immunosubtraction assays. Fabrication of a small pore-size (40%T, 6%C) integrated upstream sample preparation membrane to allow electrophoretic colocalization of reagents for enrichment, labeling, and binding of proteins is also detailed.

3.2 Materials

3.2.1 Microdevice Fabrication

1. Glass microfluidic chips: Chips were designed in-house and fabricated using standard glass microchannel wet etching, drilling, and thermal bonding. Commercial fabrication of glass chips provides ready access to both standard and custom geometries. Several foundries offer standard or custom glass chips including Caliper Life Sciences (vendor used in this work), Micronit, Micronics, Aline, Micralyne, and Microfluidic Chip Shop among others. Figure 3-1 shows the chip geometries chosen for both the immunosubtraction assay and the integrated sample preparation/immunosubtraction assay. The former geometry consists of a loading channel that intersects a long separation channel (4.56 cm, 80 µm wide by 20 µm deep), while the latter consists of seven loading wells connected through a network of channels to a long separation channel (2.05 cm, 80 µm wide by 15 µm deep).

2. Chip manifold: An optional custom made Delrin chip manifold was fabricated in-house and mated to the chip with 60 µL sample reservoirs for loading sample and buffer reagents. Alternatively, 200 µL pipette tips can be cut to fit securely into the microfluidic chip wells, acting as sample reservoirs.
Figure 3-1. Microfluidic chip images for A) simple t-junction chip used for immunosubtraction and B) multi-channel offset junction chip used for sample preparation integrated with immunosubtraction

3.2.2 Fabrication and Data Acquisition Equipment

1. UV laser (membrane fabrication): Photo-polymerization was achieved with a 2 kW 355 nm Nd:YAG laser (Teem Photonics, Lafayette CO) with cylindrical focusing optics (Melles-Griot) and chrome on glass mask with 100 µm slit (Photo Sciences, Torrance CA).

2. Inverted microscope (immunofilter fabrication): Photo-polymerization was achieved on an Olympus IX-50 microscope (Melville, NY) via illumination with a 100 W mercury arc lamp using a 4× objective with UV transmission (UPLANS-APO, NA 0.18 Olympus) and 500 µm by 4 mm film mask (Fineline Imaging, Colorado Springs CO). Data acquisition was performed using the IX-50 epi-fluorescence microscope with a 10× objective as shown in Figure 3-2.
Figure 3-2. Olympus IX-50 epi-fluorescent data acquisition system. A computer controlled CCD camera (CoolSNAP HQ2) and high voltage power supply (LabSmith) used to perform on-chip PAGE experiments. Chips were mounted in custom manifolds for sample loading and electrode positioning.

3. UV flood illumination lamp (loading gel fabrication): Photo-polymerization of the loading gel was performed with a 100 W UV flood illumination lamp (UVP B100-AP, Upland CA).
4. High voltage power supply: A custom programmable HVPS with platinum wires used for electrodes was used to perform electrophoretic loading and separations on chip. The power supply contained 8 independently controllable channels and was capable of voltage output between 0 to 3000 V with 10 nA current resolution.

5. Imaging: A 1392 × 1040 Peltier-cooled interline charge-coupled device (CCD) camera (CoolSNAP HQ2, Roper Scientific, Trenton NJ) was used to capture images of protein migration.

3.2.3 Chip Surface Treatment Solutions

1. NaOH channel cleaning solution: 1 M NaOH. Dissolve 4 g NaOH into 100 mL deionized water. Vortex as necessary to ensure complete dissolution of NaOH.

2. Channel functionalization solution: 2:3:5 ratio of 3-(trimethoxysilyl)propyl methacrylate: glacial acetic acid: deionized water. Mix 200 µL 3-(trimethoxysilyl)propyl methacrylate with 300 µL glacial acetic acid. Vortex as needed to ensure complete dissolution. Add 500 µL of deionized water, mixing thoroughly. Solution should turn from opaque to transparent when properly mixed (see Note 1—section 3.4).

3. Channel wash solution: 30% acetic acid or methanol. 30% acetic acid—mix 3 mL glacial acetic acid with 7 mL deionized water. Alternatively, methanol can be used for the wash solution (see Note 2).

3.2.4 Gel Fabrication Solutions

1. Sample Preparation Membrane Precursor Solution: 40%T 6%C acrylamide/bisacrylamide solution (%T—total percentage w/v, %C—cross-linker percentage w/v). Dissolve 132 mg bisacrylamide into 10 mL of 40% acrylamide stock in a microcentrifuge tube (1.7 or 2 mL tube). Vortex as needed to ensure complete dissolution (see Note 3).

2. Loading Gel Precursor Solution: 3%T 3.3%C acrylamide/bisacrylamide solution. Add 50 µL 30% acrylamide/bisacrylamide stock solution (29:1), 400 µL deionized water, and 50 µL 10× tris/glycine buffer to result in 500 µL of the 3%T loading gel solution.

3. Immunofilter Gel Precursor Solution: For the desired empirically determined pore size, fabricate the immunofilter gels with the following reagents (see Note 4):

<table>
<thead>
<tr>
<th>Gel %T</th>
<th>30%T Acrylamide/bis Stock Solution (29:1)</th>
<th>Deionized Water</th>
<th>10× Tris/Glycine Buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>166 µL</td>
<td>284 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>12</td>
<td>200 µL</td>
<td>250 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>13.5</td>
<td>225 µL</td>
<td>225 µL</td>
<td>50 µL</td>
</tr>
<tr>
<td>15</td>
<td>250 µL</td>
<td>200 µL</td>
<td>50 µL</td>
</tr>
</tbody>
</table>
4. UV photoinitiator: The UV photoinitiator 2,2’-azobis[2-methyl-N-(2-hydroxyethyl) propionamide] (VA-086) is available from Wako Chemicals (Richmond, VA). This water soluble photoinitiator (VA-086) should be added to all gel solutions (1-3) in the ratio of 1 mg per 500 µL solution to make them photo-active prior to on-chip polymerization (see Note 3).

3.2.5 Samples and Running Buffers

1. Electrophoresis Buffer: 1× tris/glycine native buffer. Dilute 10× tris/glycine native electrophoresis buffer (25mM Tris, pH 8.3, 192 mM glycine) into deionized water.

2. Sample Buffer: Dilute all purified proteins and clinical samples to the desired concentration in 1× tris/glycine native electrophoresis buffer.

3. Antibodies: Purified antibodies for the targets of interest were purchased from Abcam (Cambridge, MA) and diluted to the desired concentrations in 1× tris/glycine native buffer (i.e., mouse monoclonal antibody to S100, rabbit polyclonal to tau, rabbit polyclonal to prostaglandin D synthase, goat polyclonal to prealbumin, mouse monoclonal antibody to C-reactive protein).

4. Sample Proteins: Purified proteins for model protein ladders were purchased from Abcam (Cambridge, MA) and Invitrogen (Carlsbad, CA). Biological samples including cerebrospinal fluid were purchased from Biological Specialty Corp (Colmar, PA). All sample proteins were labeled using the Alexa Fluor 488 protein labeling kit (Life Technologies, Carlsbad, CA) and purified from excess dye with P-6 and P-30 Bio-Gel columns (Bio-Rad, Hercules, CA). Samples were diluted to the appropriate concentration in 1× tris/glycine native buffer (see Note 5).

5. On-Chip Labeling Dye: Reagents from the Quant-iT Protein Assay Kit (Invitrogen Molecular Probes, Eugene, OR) were used for on-chip labeling. Quant-iT dye reagent was mixed with 1× tris/glycine native buffer at a ratio of 1:4 to form the on-chip dye solution.

3.3 Methods

3.3.1 Chip Preparation and Functionalization

Figure 3-3 outlines the overall fabrication process and assay workflow. The first step requires surface modification of the glass chips followed by in-situ photopatterning of the discrete functionalized gels as described in this section.

1. Vacuum load NaOH wash solution onto each chip well ensuring the solution loads into all channels without bubbles. Incubate 5 minutes.

2. Remove NaOH wash solution via vacuum.

3. Wash channels by loading deionized water into all channels and removing by vacuum. Repeat once.
Figure 3-3. Summary of protocols for A) fabrication of photo-patterned PA gels for the on-chip sample preparation and/or immunosubtraction assay and B) the assay workflow for detecting target proteins using microfluidic immunosubtraction
4. Prepare 500 µL silane solution with photoinitiator in micro-centrifuge tube. Degas while sonicating for 1 min.

5. Load silane solution into all channels via capillary action. Load large drops on wells sufficient to cover entire well but not touching adjacent wells. Incubate 30 minutes (see Note 6).

6. Remove silane solution via vacuum.

7. Wash out silane solution by loading 30% acetic acid solution into all channels via vacuum. Use excess 30% acetic acid to wash silane off surface of chips then dry thoroughly using vacuum. Repeat once.

8. Wash channels by loading deionized water into all channels and removing by vacuum. Repeat once (see Note 7).

3.3.2 In-situ Gel Patterning

1. Prepare approximately 500 µL of membrane precursor solution with photoinitiator in a micro-centrifuge tube (40%T 6%C acrylamide/bisacrylamide with 1 mg VA-086 per 500 µL precursor solution). Skip steps 1-5 for the t-junction chip.

2. Degas the membrane precursor solution while sonicating for at least 3 minutes. Agitate the solution aggressively during the first minute of degassing by tapping tube against the bottom of the sonicator.

3. Load 3 µL membrane precursor solution into each well of the chip, being sure to allow the solution from the first well to wick into all channels, and removing any air before loading onto subsequent wells (see Note 8).

4. Turn on the laser to align the 100 µm mask onto the desired position on the laser system stage with respect to the center of the incident collimated laser sheet and tape into place (Figure 3). Use tape to mark on the monitor where the center of the laser aligns with the mask. After closing the laser shutter, manually align the chip so that the desired membrane location within the channel is aligned with the collimated laser location as marked on the monitor. Open the shutter for 60 to 75 seconds to polymerize the membrane region (see Note 9).

5. Remove membrane precursor solution by loading large drops of 1× tris/glycine native buffer onto each well and vacuuming buffer through all channels fore and aft of the membrane. Do not allow any air bubbles to be vacuumed onto the chip during this step.

6. Prepare approximately 500 µL of immunofilter precursor solution with photoinitiator in a microcentrifuge tube (10 to 15 %T 3.3%C acrylamide/bisacrylamide with 1 mg VA-086 per 500 µL precursor solution).
7. Degas the immunofilter precursor solution while sonicating for at least 3 minutes. Agitate the solution aggressively during the first minute of degassing by tapping tube against the bottom of the sonicator.

8. Vacuum load large drops of the immunofilter precursor solution into the 5 wells to the right of the polymerized membrane and the connecting channels. Vacuum load large drops of 1× tris/glycine native buffer into the two wells to the left of the membrane. Vacuum load large drops of immunofilter precursor solution into all channels for t-junction chip.

9. Turn on the microscope mercury lamp and align to the UV pass filter cube. Allow a minimum of 5 minutes for the UV intensity to reach equilibrium.

10. Measure UV intensity with a UV meter. Modify UV intensity with neutral density filters or by decreasing the intensity of a tunable light source until reaching an intensity of approximately 5.8 mW/cm².

11. Close the UV shutter then align the 500 µm by 4 mm film mask on the epi-fluorescence microscope stage. Use the halogen light source to align the mask in the center of the viewing area and tape the mask to the stage. Align the microfluidic chip so that the immunofilter channel is centered within the mask opening.

12. Open the UV shutter and expose the immunofilter for 6 minutes (see Note 9).

13. Remove immunofilter precursor solution by loading large drops of 1× tris/glycine native buffer onto the 4 loading wells and vacuuming buffer through all loading channels. Do not allow any air bubbles to be vacuumed onto the chip during this step. Vacuum buffer drops through the 3 loading wells for the t-junction chip.

14. Prepare approximately 500 µL of loading gel precursor solution with photoinitiator in a microcentrifuge tube (3%T 3.3%C acrylamide/bisacrylamide with 1 mg VA-086 per 500 µL precursor solution).

15. Degas the loading gel precursor solution while sonicating for at least 3 minutes. Agitate the solution aggressively during the first minute of degassing by tapping tube against the bottom of the sonicator.

16. Vacuum load large drops of loading gel precursor solution into the 4 loading wells (S, B2, L, A) and connected channels. Place large drops of excess 3%T loading gel precursor on the 4 loading wells; place a large drop of immunofilter precursor solution on the well the end of the immunofilter (BW) while putting large drops of 1× tris/glycine native buffer on the buffer wells (B1, SW). Vacuum 3%T loading gel precursor through the 3 loading wells for the t-junction chip.

17. Turn on the 100 W UV lamp warming up for 5 minutes prior to use. Measure UV intensity with a meter and raise or lower the height of the lamp to achieve a proximate UV intensity of 10 mW/ cm² at the stage.
Figure 3-4. Schematic for in-situ discrete PA gel fabrication in multi-channel offset junction chip. A) Exposure of 40% T precursor with a photo-mask and UV Nd:YAG laser to polymerize sample preparation membrane. B) Exposure of 12% T precursor with a photo-mask and microscope mercury lamp source to pattern immunofilter. C) Exposure of entire chip with UV flood lamp to polymerize 3% T precursor solution for loading gel (color added to differentiate PA regions).
18. Place the chip on the stage and expose to UV for 10 min. Remove chip and inspect excess gel drops over wells to ensure polymerization. Store chip in 20 mL tube with 1× tris/glycine native buffer until used.

3.3.3 On-chip Immunosubtraction

1. For either the t-junction chip (Figure 3-1A) or multi-channel offset junction chip (Figure 3-1B), load a minimum of 5 µL of the sample into the sample well reservoir (S). For the immunosubtraction assay, incubate the target analyte antibody (or antibodies for multiplexing) with the sample for 1 hour prior to loading into the sample well (see Note 10). Fill all other wells with approximately 60 µL 1× tris/glycine native buffer.

2. Prime the sample into the loading channel by applying an electric field of 300 V/cm (1.5 µA) across the S-SW2 channel for at least 2 min (see Note 11). Priming the channel is necessary prior to each assay to ensure repeatable concentration of sample in the device. *Skip this step for the t-junction chip.*

3. Electrophoretically load the sample across the S-SW channel to enrich protein at the 40%T 6%C enrichment membrane by applying an electric field of 300 V/cm (1.5 µA). Load sample across the membrane for a minimum of 5 s or longer durations to enrich the sample concentration further (see Note 12). *Load sample across S-SW junction for 2 min for the t-junction chip with no membrane.*

4. Apply a field of 300 V/cm across BW-SW to concentrate the protein at the membrane (~ 1 min). *Skip this step for the t-junction chip.*

5. Elute the sample from the membrane by applying 300 V/cm across B1-BW until the sample band passes the first cross junction (~ 5 s). *Skip this step for the t-junction chip.*

6. Inject and separate the band across the immunofilter by applying 300 V/cm across B2-BW (~ 2 min). Image the channel at a fixed location downstream of the immunofilter using the 10× objective to generate electropherograms as shown in Figure 4 (see Note 13). *Inject and separate sample by applying 300 V/cm across B-BW for the t-junction chip.*

3.3.4 On-chip Sample Preparation

1. For the multichannel offset junction chip, load a minimum of 5 µL of the sample into the sample well reservoir (S) and 5 µL of sample reagent (either labeling dye solution or target antibodies solution) into the sample preparation reagent well (Ab/L). Fill all other wells with approximately 60 µL of 1× tris/glycine native buffer (see Note 14).

2. Prime the sample and sample preparation reagent (dye or Ab) into the loading channel by applying an electric field of 300 V/cm (1.5 µA) across both the S-SW2 channel and Ab/L-SW2 channel (see Note 15). Sample preparation reagent will diffuse and react with the sample while priming and when concentrated at the sample enrichment membrane.
3. Simultaneously electrophoretically load the sample across the S-SW channel and the sample preparation reagent across the Ab/L-SW channel to enrich at the 40%T 6%C enrichment membrane by applying an electric field of 300 V/cm (1.5 µA) across each channel. Load sample across the membrane for a minimum of 5 s, loading for longer durations to enrich the sample concentration further.

4. Follow steps 4 to 6 for on-chip immunosubtraction (section 3.3).

**Figure 3-5.** Immunosubtraction assay data collection. A) Time sequence of CCD images for control and immunosubtraction of S100B in presence of ovalbumin (OVA). Control: target protein electrophoresis through the filter; Immunosubtraction: antibody is added and S100B-antibody complexes are excluded from the separation channel by the filter, resulting in ‘subtraction’ of the target analyte. B) Measuring fluorescence intensity within a chosen region of interest at 1.5 mm downstream of the filter is used to generate control and immunosubtraction electropherograms showing subtraction of the S100B target peak (-).
3.3.5 Data Analysis and Interpretation

1. Process captured CCD images to analyze differences in protein migration between control and immunosubtraction runs using image analysis software (Image J software from National Institutes of Health was used in this work).

2. Generate electropherograms by measuring fluorescence intensity over time at the chosen single point detection location. Select an appropriate region of interest (ROI) that lies completely within the separation channel (160 µm by 80 µm ROI used for analysis in Figure 3-5). Select a second ROI outside of the channel and subtract the detected fluorescence from the first ROI in order to exclude noise sources from the signal including lamp fluctuations (see Note 16).

3. Confirm detection of a target analyte by measuring the decrease in peak area between the control (no Ab) and immunosubtraction (+ Ab) runs for a given protein (i.e., protein with the same mobility in both runs). Successful immunosubtraction, and thus detection of selectivity between the target protein and antibody, will result in a significant decrease in electropherogram peak area for the target band. Peak area can be measured using custom Gaussian curve-fitting algorithms or those within data analysis software (e.g., MATLAB, OriginLab, etc). Determine thresholds for unambiguous peak detection (S/N > 3) at a significant level over run-to-run peak area variability empirically for a given assay and data acquisition system.

4. Quantify analyte concentration by performing a dose-response of extracted peak area between control and immunosubtraction runs over a given analyte range (or biological sample dilution) with samples of known concentration. Compare extracted peak area of the unknown sample to the dose-response curve for quantitation. Ensure that samples used to generate the dose-response curve and the unknown both have antibody at or in excess of the optimal antibody-antigen binding ratio to achieve complete target analyte peak subtraction.

3.4 Notes

1. 3-(trimethoxysilyl)propyl methacrylate is immiscible in water; thus following the proper mixing order with glacial acetic acid first followed by water is required. The mixture of 3-(trimethoxysilyl)propyl methacrylate, glacial acetic acid, and water is referred to as silane solution.

2. Methanol can be substituted for the channel wash solution instead of 30% acetic acid; however do not substitute methanol for acetic acid in the silane wall treatment solution. Also note that methanol has a significantly lower viscosity than acetic acid or water and thus can be used to remove unwanted bubbles at any gel/liquid interfaces when loaded via capillary action.

3. Measuring precisely 1 mg of photoinitiator is difficult. The author recommends measuring ~1 mg into a micro-centrifuge tube before adding the appropriate volume of precursor solution to maintain the proper w/v ratio (1 mg / 500 µL). For example, add 450 µL of precursor solution to 0.9 mg VA-086 or 550 µL of precursor solution to 1.1 mg VA-086, etc.
4. Cut-off filter pore size is a function of assay separation conditions (e.g., electric field, buffer concentration, sample concentration) as well as polyacrylamide concentration (%T, %C). The author typically used 12%T 3.3%C for the immunofilter concentration when attempting to extract the S100B-antibody complex at the immunofilter (161 kDa) under an applied electric field of 300 V/cm. This was sufficient in preventing complex detection from downstream electrophoresis during the 2 min separation time. Using larger pore sizes for the immunofilter that are insufficient to size-exclude the target protein-antibody complex migration will result in a homogeneous mobility shift assay across the PA gel discontinuity. Molecular weight cut-off properties of the immunofilter can be determined empirically by separating a ladder of known proteins across the filter and observing which proteins are excluded under the specified assay conditions (e.g., electric field, buffer concentration, sample concentration).

5. For experiments measuring exogenous clinical S100B levels in CSF, CSF and S100B were labeled separately with Alexa Fluor 488 and purified prior to adding the desired S100B concentration into the CSF sample.

6. Do not incubate silane solution greater than 30 minutes. Longer incubations will cause the silane to become more viscous as the solvent evaporates, making it difficult to wash the silane from the chip.

7. Chips may be stored after functionalization of the channels with the silane coating and prior to in-situ gel polymerization. The author has had success storing chips for several days in air tight containers at room temperature or for several weeks in vacuum chambers with desiccators prior to photo-polymerizing gels.

8. Loading an identical concentration of gel into each channel prevents pressure driven flow during photo-polymerization. This can also be achieved by putting large drops of a viscous polymer (e.g., hydroxyethylcellulose) onto the wells after loading the gel precursor solution. Failure to equilibrate the pressure head between wells will lead to non-repeatable polymerization conditions and possible dispersion of desired discrete photo-patterned regions.

9. Longer exposure times for the immunofilter can result in nonuniform filter pore size at the interface that causes protein band dispersion during electrophoresis.

10. The optimum molar ratio of target antibody to antigen must be determined empirically with the goal of maximizing specific target extraction while minimizing non-specific blockage at the immunofilter interface. The author found that an antibody/antigen ratio of 4-to-1 was optimal for achieving specific extraction of S100B from CSF.

11. Required potentials for loading are a function of channel length, gel pore size, and buffer conditions. Thus the author has listed the required electric field in V/cm with the associated current for the specified Caliper chip geometry shown in figure 1 (Caliper 7A chip) using a 3%T loading gel and 12%T immunofilter with 1× tris/glycine native buffer for electrophoresis. These values should be adjusted as necessary for different gel concentrations and buffer conditions.
12. Sample enrichment increases linearly with load time until reaching limits due to concentration polarization across the membrane interface from enriched ions. The author found that TI, OVA, and CRP could each be enriched linearly for at least 120 s enrichment time. If sample enrichment and other preparation functions are not necessary, the assay can be run without the 40%T 6%C sample enrichment membrane following the same electrophoretic loading and separation protocol on the multi-channel offset chip (or alternatively on the simple t-junction chip).

13. Optimize imaging conditions by choosing an imaging location down the immunofilter sufficient to resolve the target species of interest within the minimum desired assay time. Camera exposure times should be minimized to provide adequate unambiguous sampling of the fastest moving protein peaks (i.e., 5 time points minimum); however, note that decreasing sampling time will decrease sensitivity of fluorescence detection for the assay. Magnifications larger than 10× can also be used to increase fluorescence sensitivity, but captured images should always include sufficient area outside of the channels to allow for subtraction of background fluorescence.

14. This protocol is for performing either on-chip antibody binding or fluorescent dye labeling prior to immunosubtraction in a 7 well chip. Modifying the chip geometry to add an 8th well can provide individual wells for both dye and target antibody reagents permitting integrated on-chip enrichment, labeling, and antibody binding using a similar loading protocol.

15. Load sample and reagent proportionally to the desired ratio for dilution. For example to dilute the labeling dye into the sample at a 1:5 ratio, modify the electric fields to load dye at 100 V/cm and sample at 500 V/cm if both are in solutions of similar conductivity.

16. In order to improve peak separation resolution, an ROI should be chosen that is narrow enough to contain only one migrating peak at a time, but large enough to sample each peak of interest multiple times as it migrates through the ROI. Inclusion of an internal standard, for example free dye or a high-mobility protein peak that does not interfere with analytes of interest, is also customary to allow normalization of run-to-run variability due to sample loading.
4 On-Chip Immunosubtraction for Determination of Protein Mobility and Affinity: Application to Traumatic Brain Injury

Chapter Abstract: This chapter details an automated immunosubtraction assay on a reuseable microfluidic platform to rapidly quantitate mobility and binding specificity of low abundance protein markers in complex biological fluids. A polyacrylamide sieving matrix (3.5%T/12%T) is polymerized in-situ providing size-based exclusion of target analyte-antibody complex from subsequent in-line electrophoresis, while permitting passage of unbound proteins. Comparison of control (no antibody) and immunosubtraction (antibody present) electropherograms provides assessment of target analyte identity, mobility, and concentration. The immunosubtraction assay developed here does not rely on chemical immobilization of subtraction antibody-antigen in the microchannel network. Instead the polymer filter reversibly excludes antibody-antigen complex, making a single channel reusable and, thus, useful to both the control and immunosubtraction assays. Optimization of the assay (e.g., antibody-antigen ratio and driving electrical current) resulted in ~ 90% extraction of target. On-chip immunosubtraction provides an advantage in detection speed, automation, and results interpretation over bench-top PAGE and CE technology. Results demonstrate the ability to simultaneously detect S100B and C-reactive protein, putative protein biomarkers for traumatic brain injury (TBI), from raw human cerebrospinal fluid (CSF) in ~ 2 min. Demonstration of the assay as a clinical diagnostic for S100B detection (65 nM) indicates a lower limit of detection of ~ 3.25 nM for S100B in CSF, which is well within the clinically relevant concentration range.

4.1 Introduction

Immunosubtraction is a widely used technique to quantify serum or urine proteins by extraction (subtraction) of a target protein prior to native polyacrylamide gel electrophoresis (PAGE). Antibodies specific to the target protein are introduced into the sample and form antibody-antigen complexes after binding the target protein. Antibodies are typically fixed to an immobile affinity substrate, such as Sepharose beads, which permits antibody-antigen complex removal from the sample prior to reaching the sieving matrix for separation via PAGE. Subsequent comparison of subtracted electropherograms to control (no target extraction) is performed to identify the target protein of interest. Immunosubtraction has benefits over protein electrophoresis, ELISA, and immunostaining techniques since immunosubtraction resolves sample proteins by mobility while also providing information on the specificity of antibody binding. Extraction of specific protein peaks as determined during PAGE results in straightforward interpretation of the final assay readout over other electrophoretic methods, although visual inspection of complex electropherograms is typically required. Immunosubtraction is the gold standard for clinical assessment of monoclonal gammopathies within complex biomarker screening algorithms. Recently, commercial technology for capillary electrophoresis has exploited the benefits of immunosubtraction in an automated format for the clinical laboratory using a limited selection of monoclonal proteins. However, current bench-top immunosubtraction technology is slow, labor intensive, requires
Recently, the application of immunosubtraction as a generalized detection platform for non-serum based assays has been undertaken as clinical laboratories face demands to create assays with improved accuracy, precision, speed, and affordability. Treatment and monitoring of numerous malignancies and trauma for which low abundance biomarker detection is difficult would benefit from the capability to expand the use of immunosubtraction as a tool for routine proximal fluid biomarker detection in the clinical setting. Proximal fluids often harbor protein biomarkers enriched as compared to systemic fluid levels (e.g., serum), thus allowing sensitive and targeted analyte detection.

In particular, analysis of cerebrospinal fluid (CSF), the interstitial fluid surrounding the brain and spinal cord, would benefit from increased biomarker assay automation and throughput owing to the numerous CSF protein candidates for a traumatic brain injury (TBI) detection panel. In the U.S. alone, nearly 1.7 million people annually suffer brain injuries from sports, automobile accidents, assault, and military combat. Rapid TBI clinical diagnostics for first responders is critical to improve treatment and prognostic outcome. A biomarker of particular interest in CSF is S100B (beta subunit S100, S100β), a member of the S100 family of calcium-modulated proteins involved with the regulation of cellular functions and associated with development, function, and disease of the nervous system. Detection of elevated S100B levels in CSF has shown promise as a diagnostic for TBI and spinal cord injury in several studies.

In this chapter, a development of a microfluidic immunosubtraction assay is presented along with results for detection of TBI biomarkers. On-chip adaptation of the technique provides advantages over macroscale electrophoretic assays including increased automation, rapid analysis times, low sample volume, reduced cost, and increased sensitivity. Previous work by our group and others using nonuniform in-situ fabricated PA gels for both on-chip immunoblotting and ultra-short separations have shown promise for the technology needed to integrate specific protein immobilization with subsequent native separations. This chapter presents the design of a microfluidic immunosubtraction device using a discontinuous PA gel (3.5%T/12%T) to establish size-based filtration that requires no chemical immobilization or permanent fixation of the target extraction antibody. The device is characterized to establish optimal assay conditions with respect to antibody-antigen ratio and electrophoretic separation conditions for proteins present in physiologically relevant ranges. Subsequently, the new technique is used for detecting putative TBI protein markers from raw human cerebrospinal fluid.

4.2 Materials and Methods

4.2.1 Reagents and Proteins

Solutions for 30% (29:1) acrylamide/bis-acrylamide, 3-(trimethoxysilyl)propyl methacrylate (98%), and glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO). The water-soluble photoinitiator 2,2’-azobis[2-methyl-N-(2-hydroxyethyl)propionamide] (VA-086) was purchased from Wako Chemicals (Richmond, VA). Premixed 10× tris/glycine native
electrophoresis buffer (25 mM tris, pH 8.3, 192 mM glycine) was purchased from Bio-Rad (Hercules, CA). Recombinant S100 beta protein (S100B), mouse monoclonal antibody to S100 (S100 antibody) and mouse monoclonal antibody to C-reactive protein (CRP antibody) were purchased from Abcam (Cambridge, MA). Native CRP purified from human serum was purchased from CalBiochem/EMD (Gibbstown, NJ). Ovalbumin (AlexaFluor 488 conjugated) was purchased from Invitrogen (Carlsbad, CA). Human cerebrospinal fluid from pooled samples was purchased from Biological Specialty Corp. (Colmar, PA). Unlabeled proteins were fluorescently labeled in-house using the Alexa Fluor 488 protein labeling kit (Life Technologies, Carlsbad, CA). Purification of labeled proteins was performed with P-6 and P-30 Bio-Gel columns (Bio-Rad, Hercules, CA). Post-labeling, proteins were diluted with 1× tris/glycine native buffer to attain desired concentrations. Proteins were stored at 4°C in the dark until use. Protein-antibody complexes were formed by incubating the target protein with the relevant antibody for at least 1 h at room temperature. Sizing assays of biological samples were performed with the Bioanalyzer 2100 (Agilent, Santa Clara, CA).

4.2.2 Chip Fabrication and Surface Preparation

Quartz microfluidic chips were purchased from Caliper Life Sciences (Hopkinton, MA). A standard t-chip channel layout fabricated through wet etch processing was used for all experiments (Figure 4-1). The two intersecting channels have fluid wells at each terminus denoted as sample (S), sample waste (SW), buffer (B), and buffer waste (BW). The separation channel is 2.5 mm in length, ~ 80 µm wide, and 15 µm deep. Channels were prepared for in-situ gel polymerization by first washing with 1 M NaOH for 5 min to remove debris from the channels followed by a wash with deionized water to remove the NaOH. This was followed by channel wall functionalization to enable covalent linkage to PA with a 2:3:5 ratio solution of 3-(trimethoxysilyl)propyl methacrylate, glacial acetic acid, and deionized water loaded via capillary action. After 30 min the surface preparation solution was vacuum purged, and channels were flushed twice with 30% glacial acetic acid followed by two washes with deionized water.

4.2.3 Exclusion Filter Fabrication & Function

Filters were fabricated from in-situ photo-patterned PA gels. PA precursor solutions were made by diluting the total volume of the 30%T (w/v) acrylamide/bisacrylamide solution to the desired ratio and adding 0.2% (w/v) VA-086 photoinitiator. All PA gel precursor solutions were sonicated and degassed 5 to 10 min prior to loading into channels for photo-polymerization. The molecular weight cut-off filter consisted of a 12%T (total acrylamide %T, with 3.3% cross-linker) PA region. 12%T filters were empirically determined to have a pore-size cutoff near 150 kDa, making this filter composition relevant to extraction of S100B-antibody complexes. Adjacent to the filter, a 3%T loading gel was polymerized, creating a step discontinuity in the gel from large to small pore-size between the loading gel and filter region (Figure 4-1). Filters were fabricated via photolithography using a film transparency mask with a 500 µm by 4 mm opening (Fineline Imaging, Colorado Springs, CO). The open region of the mask was aligned with the desired region of the separation channel containing 12%T precursor, and then exposed to UV power at ~ 5.8 mW/cm² for 4 to 5 min. UV illumination projection was accomplished via a 100 W mercury arc lamp and a 4× UV objective on an Olympus IX-50 microscope (Melville, NY) in conjunction with neutral density filters to achieve the desired power. After photo-patterning of
the filter, the unpolymerized 12%T precursor in the loading channels was exchanged with 3%T PA precursor solution through a vacuum purge. The entire unmasked chip was then subjected to flood illumination for 10 minutes at a distance of 18 cm from the 100 W UV lamp (UVP B100-AP, Upland, CA) giving ~10 mW/cm².

Figure 4-1. Immunosubtraction microfluidic chip schematic. A) A standard t-chip channel layout fabricated through wet-etch processing was used for all experiments. The discrete 3%T to 12%T interface visible in the brightfield image was fabricated in a polyacrylamide gel serving as a size-exclusion immunofilter. B) The two intersecting channels have fluid wells at each terminus denoted as sample (S), sample waste (SW), buffer (B), and buffer waste (BW). The assay was run using a standard T-chip injection by applying a current across (S) to (SW) to load analytes followed by a current across buffer (B) to (BW) to inject a plug and separate the analytes at the downstream single point fluorescence detector. The separation channel is 2.5 mm in length, ~ 80 µm wide, and 15 µm deep.

4.2.4 Immunosubtraction assay

An immunosubtraction assay consists of two electrophoretic separations: 1) a control separation (no immunosubtraction antibody present) and 2) a subsequent immunosubtraction run (with antibody present, Figure 4-2). Results are compared, and absence of a specific protein peak(s) in
the immunosubtraction electropherogram indicates the mobility of the immunosubtracted protein target(s). All assays are conducted under native (nonreducing, non-denaturing) conditions, to preserve the affinity of target protein to antibody. Importantly, the immunosubtraction assay does not employ chemical immobilization of subtraction antibody in the microchannel network. Instead the PA filter reversibly excludes the antigen-antibody complex, making a single channel reusable and, thus, useful to both the control and immunosubtraction assays.

Sample was loaded onto the chip by applying a 1.5 µA current between S and SW, while B and BW were maintained at 0 µA current. For sample injection to the PA filter, 3 µA was applied between B and BW, while S and SW were maintained at 0 µA. For control cases in which no antibody was present, sample proteins were mixed just prior to performing separations. For the immunosubtraction cases, target protein was incubated for one hour with the relevant antibody prior to loading. Three replicate runs for each sample were run in immediate succession for all cases. After each assay, protein complexes excluded at the filter were removed by reversing the applied electrical potential (i.e., applying 3 µA from BW to SW, while B and S were maintained at 0 µA). Channels were electrophoretically flushed with 1× tris/glycine buffer prior to each new assay. To generate electropherograms, the separation channel was imaged at 1.5 mm downstream of the separation junction. The channel was also imaged at the filter interface (250 µm downstream of the separation junction) to yield measurements of total excluded complex fluorescence.

4.2.5 Apparatus and Imaging

The chip was seated in a custom Delrin manifold to provide large sample reservoirs for each well. 5 µL of sample were loaded into the sample well with all other wells initially filled with 60 µL 1× tris/glycine native buffer. Electrophoretic loading of sample was performed with a programmable high-voltage power supply (Caliper Life Sciences, Hopkington MA). Platinum wire was used as electrodes. Imaging was performed with inverted epi-fluorescence microscopes (IX-70, Olympus, Melville, NY and Diaphot 200, Nikon Instruments, Melville, NY). Images were collected with a 10× objective (NA 0.3), using a filter cube optimized for GFP detection and a 0.63× demagnifier (Diagnostic Instruments Inc., Sterling Heights, MI). A 1392 × 1040 Peltier-cooled interline CCD camera (CoolSNAP HQ2, Roper Scientific, Trenton NJ) was used to record images of the protein migration. An image exposure time of 250 ms was used with 2× pixel binning applied in the y-direction (transverse to separation). Image analysis was performed using Image J software (National Institutes of Health, http://rsbweb.nih.gov/ij/). All images were background subtracted to account for variability in background signal. Electropherograms were generated by measuring fluorescence intensity in a region of interest (ROI) occupying the width of the channel and a small fraction of the length (ROI ≈ 160 µm by 80 µm) at the end of the separation channel ~ 1.5 mm downstream from the filter interface. Variations in signal intensity due to CCD noise or external light fluctuation were accounted for by normalizing the recorded signal to a free-dye internal standard or total fluorescence of the injected sample in the separation channel.
Figure 4-2. Immunosubtraction protocol via molecular weight cutoff filters. The immunosubtraction protocol consists of two PAGE separations across a discrete filter. Control separation without the presence of target antibody allows passage of all proteins through the filter. The immunosubtraction case requires addition of unlabeled target antibody to the sample, which ‘subtracts’ out target analyte via size exclusion during PAGE separation across the filter.

4.3 Results and Discussion

4.3.1 Immunosubtraction of S100B

Immunosubtraction integrates antibody based extraction of protein target with subsequent native PAGE. Comparison of PAGE electropherograms from systems with and without the subtracting antibody present yields immuno-affinity information correlated with the electrophoretic mobility of the target protein, as well as levels of the target species with calibration. Figure 4-3 presents comparative control and immunosubtraction data for analysis of S100B protein (208 nM) with matrix surrogate ovalbumin present (49 nM). The series of CCD images in Figure 4-3 shows that when S100 antibody (693 nM) is added to the sample, size-based exclusion of the large S100B-antibody complexes at the 12%T filter interface reduces the peak area of free S100B in subsequent PAGE. The 12%T filter pore size prevents passage of proteins >150 kDa such as the S100B-antibody complex (161 kDa) while permitting passage of proteins smaller than the pore size cutoff including the unbound S100B (11 kDa) and ovalbumin (45 kDa), resulting in unambiguous immunosubtraction of the target protein. Figure 4-4 presents electropherograms collected using a single point detector 1.5 mm downstream of the filter interface for the corresponding time-sequence CCD image montage shown in Figure 4-3. While specific and
significant reduction of the target is required to identify the target, full subtraction is not necessary. Consequently, on-chip immunosubtraction can be optimized to maximize specificity of subtraction (as discussed in later sections).

**Figure 4-3.** Time sequence of CCD images for control and immunosubtraction of S100B in the presence of ovalbumin (OVA). A) Control: target protein electrophoresis through the filter shows S100 peak resolved from OVA peak. B) Immunosubtraction: antibody is added, and S100B-antibody complexes are excluded from the separation channel by the filter, resulting in ‘subtraction’ of the target S100 analyte as shown by (-). Images show inverted fluorescence.
From the PAGE analysis, the apparent electrophoretic mobilities of the S100B protein and ovalbumin are $1.1 \times 10^{-8}$ m$^2$/Vs and $3.5 \times 10^{-9}$ m$^2$/Vs respectively, within the 12%T PA gel. Under the PAGE conditions employed, baseline separation of the two peaks is achieved at 1.5 s and in a separation length of 0.4 mm. Figure 4-4 also reveals the minimal effect of immunosubtraction on the ovalbumin peak area or mobility, as ovalbumin is able to traverse the filter interface. As the 12%T filter is present in both the control and immunosubtraction assays, comparison of the control to the assay is effective in identifying specific proteins smaller than ~150 kDa that are subtracted via antibody interaction. The filter pore size can be adjusted to accommodate a wide range of size cut-off points.

**Figure 4-4.** Comparison between control and immunosubtraction electropherograms collected at 1.5 mm downstream of filter shows subtraction of the S100B target peak (-) while the on-target protein is unaffected (OVA).

### 4.3.2 Filter Function

Size exclusion at the filter is used to subtract large antigen-antibody complexes from the samples studied. For a sample containing S100B (208 nM) and ovalbumin (48.6 nM), the fluorescence signal at the filter increases linearly with increasing S100 antibody concentration (Figure 4-5). The increase in fluorescence results from enrichment of size-excluded species at the filter interface, here S100B-antibody complexes. Figure 4-5 shows increased intensity of the excluded fluorescence initially due to increased complex formation for all starting S100B concentrations. The increase in fluorescence reaches a maximum at an antibody-S100B ratio near 2.5-to-1 after
which there is little change with the addition of further antibody due to saturation of the CCD imaging system; antibody saturation also contributes to signal saturation for antibody-S100B ratios above 3-to-1. Average filter fluorescence measurements for excluded S100B-antibody complexes are plotted in Figure 4-6. Lower limit of detection (LLOD) for S100B via fluorescence measurements at the filter interface is 193 pM extracted with 1.67 nM antibody.

**Figure 4-5.** Detection of subtracted S100B-antibody complex at the filter. A) Inverted greyscale images of S100B-antibody complexes excluded at the filter where they are detected by single point imaging. Net fluorescence exclusion at the interface increases as a result of increasing the antibody-S100B ratio or increasing the starting concentration of S100B. S100B concentrations for the image columns from left to right are 42 nM, 208 nM, 330 nM, and 495 nM.
As previously mentioned, no irreversible or chemical immobilization of the antibody is employed (i.e., no biotin-streptavidin linkage of antibody to the PA filter), making reuse of the filter for both the control and immunosubtraction assays facile and feasible. Furthermore, multiple replicate controls and subtraction assays are all readily performed in one microchannel (Figure 4-7). Replicate runs did not result in significant permanent protein immobilization, allowing some devices to be used for more than 100 runs before eventual rapid onset of degradation of the sieving matrix where permanent fouling of the filter interface was observed.

**Figure 4-6.** Normalized excluded fluorescence relative to maximum fluorescence as a function of antibody-S100B ratio for several S100B concentrations measured at the filter interface.

**Figure 4-7.** Multiple immunosubtraction assays are performed in a single reusable microchannel that has minimal permanent immobilization of target analyte at the filter interface after 55 assay runs. Repeated use shows filter degradation after 105 runs due to nonspecific protein fouling. Dashes indicate channel wall; arrows indicate filter location.
4.3.3 S100B Immunosubtraction Assay Optimization

Optimization of immunosubtraction assays requires both minimization of nonspecific extraction and maximization of protein target subtraction. To maximize target subtraction, the ratio of target analyte-to-subtraction antibody concentration was varied. The S100B peak subtraction was quantified to determine the antibody level that facilitated electropherogram target identification. Increasing concentrations of S100 antibody were introduced while holding the S100B and ovalbumin concentrations fixed. In a model system with S100B and ovalbumin used to determine device operating limits, increasing the antibody concentration results in a nearly linear decrease of free S100B peak area over a large operating range for initial S100B concentration ranging from 42 to 495 nM (see Figure 4-8 and Figure 4-9).

**Figure 4-8.** Specific subtraction of target analyte was optimized with several model systems containing S100B and ovalbumin at different initial concentrations. Addition of increasing concentrations of S100 antibody to the sample resulted in specific reduction of the S100B peak area up to 85% measured 1.5 mm downstream of the filter interface.
Figure 4-9. Extraction of nonspecific protein ovalbumin (corresponding to the samples shown in Figure 4-8). Peak area measurements taken 1.5 mm downstream of the filter show that nonspecific ovalbumin extraction was initially minimal with the addition of S100 antibody before decreasing to a lower limit of 40% extraction for several initial protein concentrations of the model system.

To characterize peak extraction further, a model sample was constituted to investigate the impact on immunosubtraction of high levels of background protein (i.e., human albumin) which makes up ~50% of total CSF protein constituents. The model sample consisted of buffer with 2.75 µM ovalbumin and 104 nM S100B; the ovalbumin was employed at a concentration 26× greater than the target analyte. Repeatable detection of the S100B peak area is achieved over an S100 antibody concentration range from 104 nM to 1665 nM (Figure 4-10). The S100B peak area initially decreases linearly until reaching a limit of ~95% extraction from the control case (0 antibody) peak area. At this point further addition of antibody does not improve subtraction. This plateau in subtraction at the lower limit is governed by binding kinetics when approaching saturation of the bound antigen. Maximum subtraction is initially achieved at an antibody-
S100B ratio of 8-to-1 in contrast to 2.5-to-1 found for the model system where S100B and ovalbumin are present in the sample in the same concentration range. The variation in maximum subtraction conditions likely reflects expected variation in the antigen-antibody agglutination strength which decreases proportional to the dilution of the antigen-antibody concentration in the sample.57

Figure 4-10. Optimization of S100B immunosubtraction. Physiologically relevant levels of S100B (104 nM) in the presence of ovalbumin (2.75 µM), a high abundance background protein (1-to-26 ratio of S100B to ovalbumin). Results suggest S100B is extracted in a specific manner decreasing with addition of antibody up to ~95% while ovalbumin peak area decreases 40% due to nonspecific blockage.

To characterize nonspecific extraction of nontarget species from the PAGE analysis, ovalbumin peak area is assessed in the S100B model sample for a range of S100 antibody concentrations (Figure 4-10). The ovalbumin peak area initially remains constant before decreasing to 65% of the control case with the addition of S100 antibody above 833 nM, which differs from the immediate linear decrease in S100B observed due to antibody binding and complex exclusion. Nonspecific extraction is attributed to blockage of the filter interface by the S100B-antibody complex whose presence at the interface increases with increasing antibody concentration. Since little species resolution occurs in the 3%T loading gel, the majority of nonspecific blockage occurs from the portion of complexed proteins in the injected plug that reach the filter interface prior to uncomplexed proteins. Unlike specific immunosubtraction due to antigen-antibody complex formation and exclusion, the nonspecific blockage does not decrease below 35% of the initial control case for the S100B-ovalbumin system and the onset of this reduction occurs at a
higher antibody concentration (556 nM). Figure 4-6 demonstrates that there is no net signal when no antibody is present, suggesting that the filter itself is not responsible for (pore-sized based) physical exclusion of the target proteins assayed in this work.

![Figure 4-11: Non-Specific Blockage of S-100 Protein](image)

![Figure 4-11: Non-Specific Blockage of Ovalbumin](image)

**Figure 4-11.** Effect of protein concentration on nonspecific immunofilter blockage. Nonspecific blockage of S-100 protein able to pass through the filter interface occurs at over 10 pg of IgG (A) and over 1000 pg BSA (B).

Additionally, studies to assess specificity of S100B subtraction were performed. In particular, investigating potential nonspecific interactions with IgG is important given the abundance of IgG in biological matrices such as serum. Figure 4-11A shows that extraction of S100B by non-target antibody IgG of similar size to the S100 antibody does not show significant reduction of peak area until much larger concentrations are introduced. The S100B (234 nM) peak area is reduced 50% with addition of 5 µM IgG, a concentration of nonspecific antibody 26× the target
protein concentration. Whereas 50% specific subtraction of S100B peak area can be achieved with an S100 antibody concentration only 2× greater than the target antibody as shown in Figure 4-10, further demonstrating the specificity of subtraction due to binding of S100B protein to S100 antibody.

The addition of non-IgG proteins, such as BSA, were also shown capable of causing filter blockage when large enough quantities were added (over 1000 pg). As expected, BSA (66 kDa) could be added in ~10× larger quantities than IgG (150 kDa) before blocking the protein because of the smaller size, which requires more protein molecules to block a filter pore. The onset of filter blockage was likely to be a function of both the size and concentration of the blocking protein as well as the target protein. However results shown in Figure 4-11 demonstrated that the effects of blockage were nearly identical on S100B (11 kDa) and OVA (45 kDa), indicating that their difference in molecular weight did not significantly impact their nonspecific blockage at the filter interface.

The effect of increasing S100 antibody on nonspecific ovalbumin protein extraction is not significantly altered between the physiologically relevant sample and the higher concentration model system results. Thus, the detection capabilities of the on-chip immunosubtraction protocol are not considerably affected by high concentrations of endogenous nonspecific proteins expected in physiological samples; specific target analyte subtraction is anticipated with minimal nonspecific extraction by increasing the antibody-S100B ratio. The extraction efficiency studies demonstrate that S100 antibody in excess of 833 nM should be used for optimal detection of endogenous levels of target S100B. LLOD for S100B is 1.1 nM with the epi-fluorescent microscope and CCD imaging system used. The LLOD is well within the clinical range of S100B which can reach levels up to 65 nM (26 µM S100B mean concentration) 48 hours after spinal cord injury.58

4.3.4 Multiplexed Target Detection of S100B and CRP

On-chip immunosubtraction also enables rapid multi-analyte detection which was characterized by simultaneously assessing two putative TBI markers (S100B and CRP).43, 59 Samples containing S100B (208 nM), ovalbumin (48.6 nM), and CRP (98.6 nM) are analyzed for subtraction of both S100B and CRP. Multiplexed immunosubtraction is achieved by introducing multiple antibodies, each with an affinity towards the desired target. PAGE analysis after immunofiltration and subsequent comparison of the resulting electrophoretic profile to the control profile completes the assay. Targets in samples comprised of the multiple proteins in buffer are incubated with their respective antibodies (1.11 µm S100B, 725 nM CRP) and assayed via a 3.5%T/12%T loading gel-filter system with subsequent PAGE (Figure 4-12). In ~2 minutes, the control sample is baseline resolved into three distinct peaks: S100B, ovalbumin, and CRP corresponding to the mobility of the sample constituents. The addition of either S100 or CRP antibody results in extraction of the respective target protein at the filter, altering the electropherogram peak profile. The addition of both S100 and CRP antibody results in the simultaneous extraction of both target analytes from the electropherogram. Quantitation for multiplexed assays is realized via peak area measurements which do not require spectral resolution of multiple labeling fluorophores (Table 4-1).
Figure 4-12. Rapid multi-analyte detection of two biomarkers is achieved with the filter by addition of the desired target antibody. Both CRP and S100B can be detected simultaneously or independently on the same device resulting in unique and simple to interpret electropherograms.

Table 4-1. Peak Area (% of control) for multi-analyte detection.

<table>
<thead>
<tr>
<th>Extraction Antibody</th>
<th>S100B</th>
<th>OVA</th>
<th>CRP</th>
</tr>
</thead>
<tbody>
<tr>
<td>S100</td>
<td>39±6</td>
<td>(117 ±13)</td>
<td>48±4</td>
</tr>
<tr>
<td>CRP</td>
<td>58±14</td>
<td>75±18</td>
<td>0**</td>
</tr>
<tr>
<td>S100 and CRP</td>
<td>12±0.5</td>
<td>52±10</td>
<td>0**</td>
</tr>
</tbody>
</table>

*a net increase, **no detectable signal, n = 3, ± standard deviation

The multiplexed analysis has significant implications for rapid assessment of biomarker panels, which requires simultaneous detection of several analytes. In particular, changes in levels of up to 59 proteins may result from traumatic brain injury adding to the complexity of biomarker detection.43 The current multiplexing results and chip reusability studies indicate the ability to assay complete TBI biomarker panels within a single immunosubtraction device.
4.3.5 **Comparison of Filter Exclusion and Peak Area Quantitation**

Increasing S100B concentrations were detected with a fixed antibody-S100B ratio in order to characterize the dose-response of the physiologically representative sample with respect to quantifying total target-analyte concentration (Figure 4-13). Both peak area detection and fluorescence intensity measurements vary linearly ($y = 0.007x + 0.072, R^2 = 0.998$) when S100B concentration is increased from 3 to 1085 nM (antibody-S100B ratio held constant at 2.7-to-1). For clinical applications, this would allow the use of on-chip immunosubtraction for quantitation of endogenous concentration or a simple binary (positive/negative) assay for the presence of target analyte above a threshold. Nonspecific blockage of ovalbumin at the filter interface does not bias the linearity of filter fluorescence measurements of S100B; both methods provide accurate quantitation of the target-analyte concentration over the physiologically relevant range. However, multi-analyte detection and confirmation of target-protein mobility require detection via peak area measurements of separated proteins.

![Figure 4-13](image.png)

**Figure 4-13.** Comparison of the peak area and filter complex exclusion quantitation methods reveals that both techniques have a linear detection range for physiologically relevant concentrations of S100B.
4.3.6 Separation Current Optimization

The effect of varying the separation current on detection signal is also characterized. Since the polyacrylamide sieving matrix is malleable, sufficiently high separation currents or sufficiently long separation durations can drive proteins previously excluded by the filter through (and into) the deformable filter interface. Therefore, the fluorescence intensity measured at the interface is transient. Furthermore, antibodies and antigen-antibody complexes can become irreversibly entangled in the filter, making reuse of the channel problematic. The separation current must be optimized for exclusion of target protein-antibody complex over a sufficient time to measure intensity, prevent complex peak migration to the detector, and baseline resolve protein bands (i.e. separation resolution ≥ 1). To assess the duration for excluded fluorescence signal remaining at the interface, samples were assayed with increasing separation currents varying from 1 to 6 µA (Figures 4-14 and 4-15).

![Figure 4-14.](image)

Figure 4-14. Separation current can be used to optimize the duration of the transient excluded fluorescent signal at the filter interface. A decrease in separation current results in an exponential increase of signal duration.
The signal decay time decreases exponentially with increasing separation current which forces larger proteins through the filter. For a sample containing S100B (312 nM), ovalbumin (2.75 µM), and S100 antibody (832 nM); the decay time of the signal exiting the imaging ROI decreases exponentially with increasing current. This decay is caused by proteins migrating out of the imaging capture region, not from photo-bleaching. Complexed proteins remain within the fixed region of interest 7× longer with the application of a 1 µA separation current than with the application of a 6 µA separation current.

The effect of separation current on the extraction efficiency of peak area measurements is minimal, as shown in Figure 4-16. Based on these results, a 3 µA separation was implemented for all assays presented in the study. The 3 µA current meets the operating parameters for detecting S100B including baseline separation resolution from albumin and other abundant matrix proteins as well as sufficient duration of the fluorescent signal at the filter.

4.3.7 S100B Detection in Human Cerebrospinal Fluid

S100B was detected in a spiked human CSF sample to determine the efficacy of the on-chip immunosubtraction protocol in a minimally processed human diagnostic fluid. Pooled raw human CSF samples from healthy individuals were labeled with AlexaFluor 488 and analyzed on-chip diluted 4:1 in buffer. Electrophoretic separation (200 V/cm) of an S100B spiked CSF sample results in several peaks, with free dye as well as high-abundance CSF proteins clearly visible in the electropherogram (Figure 4-17). Apparent electrophoretic mobilities are: S100B at $8.5 \times 10^{-9}$ m²/Vs, Peak 1 at $4.1 \times 10^{-9}$ m²/Vs, and Peak 2 at $2.8 \times 10^{-9}$ m²/Vs. The S100B peak (65 nM) migrates past the detector at 12 s.
Figure 4-16. The effect of separation current on the extraction efficiency of peak area measurements is minimal. 3 µA, 4 µA, and 5 µA separations all result in maximum subtraction of S100B target (A) and 40% nonspecific extraction of ovalbumin (B) at the same antibody-S100B ratio. Peak areas are both normalized to the control (no antibody) S100B peak area.
Incubating S100 antibody (333 nM) with S100B (65 nM) results in specific subtraction of the S100B peak compared to the control with minimum nonspecific extraction of Peak 1 and Peak 2. After addition of antibody, three replicate assays were performed showing that subtracted S100B peak area reduces to 31% ± 14 from the control while Peak 1 is 106% ± 6 and Peak 2 is 97% ± 8 of the control. Results show that nonspecific extraction of nontarget protein bands in CSF is almost negligible. The large concentration of confounding matrix proteins (0.15 to 0.45 mg/ml) within CSF does not impede the specificity of detecting S100B (0.715 µg/ml) at an elevated concentration indicative of spinal cord injury.\textsuperscript{51}

Figure 4-17. Quantitation of TBI biomarker S100B from raw human cerebrospinal fluid. S100B is spiked into pooled human CSF at clinically relevant concentrations (65nM).

The knowledge of S100B mobility allows rapid, specific detection via quantifying peak area without the need to identify all CSF matrix proteins; however sizing analysis performed with the Bioanalyzer indicates a Peak 2 molecular weight of ~62 kDa. Thus Peak 2 is presumably albumin, which typically constitutes 40 to 60% of CSF protein.\textsuperscript{60} Peak 1 concentration (~19 µg/ml) is too low for sizing characterization with the Bioanalyzer; however, the more sensitive on-chip immunosubtraction method shows the Peak 1 relative concentration compared to albumin, which indicates that the peak is probably transferrin which has a molecular weight of 55 kDa and can comprise up to 25% of total CSF protein.\textsuperscript{61} The native CSF electropherograms
show the difficulty in visually interpreting extraction and identity of a minute quantity of target analyte in the presence of large concentrations of confounding proteins, further illustrating the need for automated and quantitative on-chip peak area analysis.

4.4 Conclusion

The on-chip immunosubtraction protocol demonstrates accurate detection of target analyte via rapid PAGE to provide data on mobility and specificity of target molecules. The target analyte forms a complex with the extraction antibody preventing passage through the filter and altering the subsequent control and immunosubtraction electropherograms. LLOD for determining specificity and mobility of proteins was 1.1 nM, with specificity-only based detection of excluded proteins at the filter interface, reaching a limit of 193 pM. Optimization of antibody-antigen ratio and applied separation current resulted in the ability to immunosubtract up to 95% of target analyte while extraction of nontarget proteins did not reach below 35% for single analyte detection in a model system. While specific and significant reduction of the target is required to identify the analyte, full subtraction is not necessary. Consequently, on-chip immunosubtraction can be optimized to maximize specificity of subtraction.

The on-chip immunosubtraction device was used for detection of S100B (65 nM) spiked into a pooled sample of human cerebrospinal fluid in under 2 minutes using 5 µL of sample. This suggests potential capability as a rapid means to assess putative protein markers of traumatic brain injury. Detection in the biological sample also proved to be more specific since extraction of nontarget proteins was negligible when detecting S100B in CSF. Performing immunosubtraction on-chip in an automated and programmable manner on a uniform platform opens up the application space to detecting numerous biomarkers for various proximal fluid diagnostics, as well as for serum and urine analyses. Automated immunosubtraction also facilitates integrated on-chip sample preparation including on-chip antibody binding, sample fluorescence labeling, and in-line protein enrichment to improve detection sensitivity as covered in chapter 6.
Chapter Abstract: On-chip immunosubtraction provides an advantage in detection speed, automation, and interpretation of results over bench-top PAGE, CE, gas chromatography (GC), and mass spectrometry (MS) technology while also expanding the application space for immunosubtraction assays beyond serum and urine monoclonal protein detection to areas including low molecular weight compound detection. CE and GC/MS are commonly used to detect small molecules including drugs, vitamins, and hormones in the laboratory setting. In this chapter the versatility of the on-chip immunosubtraction technology is demonstrated with the on-chip detection of low molecular weight compounds. Customized fragment (Fab) antibodies developed for use in rapid, portable biosensors for drugs of abuse were developed via phage display by collaborators at VTT Biotechnology (Espoo, Finland). Folic acid (FA), tetrahydrocannabinol (THC), and testosterone (TES)—low molecular weight compounds typically not detectable with electrophoretic mobility-shift assays—were detected in less than 2 min using the on-chip immunosubtraction assay validating the potential for use in portable biosensors.

5.1 Introduction

Immunosubtraction has been previously validated for uses ranging from cancer detection from serum to trauma detection in cerebrospinal fluid. Another major area of interest for proximal fluid detection includes testing of saliva for disease biomarkers, health monitoring, or illicit use of drugs of abuse. Simple and portable biomarker diagnostics would be of particular importance for detecting low molecular weight compounds, such as opiates or steroids which are typically detected via gas chromatography or mass spectrometry (GC/MS) of urine or via ELISA blood testing. In the past, rapid detection of low molecular weight compounds such as testosterone (288 Da) or THC (314 Da) proved difficult with electrophoretic methods given the long capillary length required to resolve such small differences in molecular weight. Recently, researchers at VTT Finland successfully developed antibody fragments via phage display useful for biosensors that bind low molecular weight compounds including morphine, testosterone, THC, and folic acid. These Fab fragment antibodies undergo a conformational change after binding small molecules allowing them to then bind secondary antibodies specific to the epitope conformational change. This technology has provided a means to enhance mobility shift (via antibody binding) for small compound detection in electrophoretic assays. Small compound detection makes the immunosubtraction tool useful for future portable deployment of rapid drug diagnostics for use by law enforcement, anti-doping agencies, the private sector, etc.—applications that are not easily achieved with benchtop immunoassay formats such as ELISA and FRET plate reader assays.

In this chapter, development of a microfluidic immunosubtraction assay for low molecular weight compound detection is presented. On-chip adaptation of the technique provides advantages over macroscale electrophoretic assays or GC/MS including increased automation, rapid analysis times, low sample volume, reduced cost, and increased sensitivity. Subsequently, experimental results are reviewed for using immunosubtraction to detect folic acid, tetrahydrocannabinol, and testosterone.
5.2 Materials and Methods

5.2.1 Reagents and Proteins

Testosterone, THC, folic acid, and their respective antibodies were obtained directly from collaborators (Dr. Tarja Nevanen and Prof. Kristiina Takkinen) in the Protein Engineering group of VTT Biotechnology (Espoo, Finland). The properties of these proteins and reagents are shown in Table 5-1.

Table 5-1: VTT low molecular weight compounds and antibodies

<table>
<thead>
<tr>
<th>Sample</th>
<th>MW* (Da)</th>
<th>Label</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA</td>
<td>441</td>
<td>none</td>
<td>Folic acid—a vitamin essential in production or red blood cells and other biological functions</td>
</tr>
<tr>
<td>FA-AP</td>
<td>90,441</td>
<td>AF488</td>
<td>Folic acid-alkaline phosphatase conjugate</td>
</tr>
<tr>
<td>FA-HA</td>
<td>66,441</td>
<td>AF488</td>
<td>Folic acid-human serum albumin conjugate</td>
</tr>
<tr>
<td>Anti-FA</td>
<td>50,000</td>
<td>none</td>
<td>Antibody Fab fragment binding either FA-AP conjugate or FA-HA conjugate</td>
</tr>
<tr>
<td>THC</td>
<td>314</td>
<td>none</td>
<td>Tetrahydrocannabinol—psychoactive chemical in cannabis</td>
</tr>
<tr>
<td>T3</td>
<td>50,000</td>
<td>AF488</td>
<td>Antibody Fab fragment binding THC</td>
</tr>
<tr>
<td>T3-104</td>
<td>50,000</td>
<td>AF568</td>
<td>Antibody Fab-fragment binding THC to T3-104 immunocomplex</td>
</tr>
<tr>
<td>TES</td>
<td>288</td>
<td>none</td>
<td>Testosterone—a natural steroid hormone</td>
</tr>
<tr>
<td>TES220-A568</td>
<td>50,000</td>
<td>AF568</td>
<td>Antibody Fab fragment binding testosterone</td>
</tr>
<tr>
<td>IC B1-A488</td>
<td>50,000</td>
<td>AF488</td>
<td>Antibody Fab fragment (B1 phage display source) binding the immunocomplex of testosterone and anti-testosterone Fab fragment (TES220)</td>
</tr>
<tr>
<td>IC B12-A488</td>
<td>50,000</td>
<td>AF488</td>
<td>Antibody Fab fragment (B12 phage display source) binding the immunocomplex of testosterone and anti-testosterone Fab fragment (TES220)</td>
</tr>
</tbody>
</table>

*Listed MW values do not account for labeling by dye molecules since degree of labeling per protein is variable. (AF568~ 792 Da, AF488~643 Da)
Testosterone samples were made soluble in 1× tris/glycine by addition of up to 40% ethanol. For off-chip preparation of immune-complex binding for IS positive runs, anti-hapten AB was incubated simultaneously with the respective hapten and the anti-IC Ab.

Folic acid and testosterone experiments were performed in the Herr Lab (UC Berkeley). All reagents for these experiments are identical to those in section 4.2.1. THC testing was performed at VTT Biotechnology (Espoo, FI). All buffers and reagents for these experiments were produced in house at VTT.

5.2.2 Chip and Immunofilter Fabrication

Standard glass microfluidic t-chips were purchased from Caliper Life Sciences. Chip preparation, including cleaning and silane treatment, was similar to that covered in section 4.2.2. Filters were fabricated from in-situ photo-patterned PA gels with a range of total gel concentrations used to produce smaller molecular weight cutoff sizes (12%T, 13.5%T, or 15%T) accommodating exclusion of 100 kDa proteins formed by the binding of anti-hapten and anti-immunocomplex antibodies (Figure 5-1). The molecular weight cut-off filter consisted of a 13.5%T (total acrylamide %T, with 3.3% cross-linker) PA region. Fabrication conditions for devices made at UC Berkeley are described in section 4.2.3. As shown in Figure 5-2, devices fabricated at VTT were made using the fluorescent light source from an Olympus IX-81 epifluorescent microscope (Melville, NY) and photolithographic patterning using a film transparency mask with a 500 µm by 4 mm opening (Fineline Imaging, Colorado Springs, CO). The open region of the mask was aligned with the desired region of the separation channel containing 13.5%T precursor and then exposed to UV power at ~ 2.04 mW/cm² for 5 min 15 s. After photopatterning of the filter, the unpolymerized 13.5%T precursor in the loading channels was exchanged with 3%T PA precursor solution through a vacuum purge. The entire unmasked 3%T loading gel adjacent to the filter was then exposed with the long-wave UV illuminator for ~5 min with an intensity of 2.7 mW/cm² (Figure 5-3).

Figure 5-1. Immunosubtraction chip fabrication at VTT. 13.5%T/3.3%C immunofilters were fabricated with a molecular weight cutoff near 100 kDa to accommodate the Fab fragment antibody cutoff.
5.2.3 Imaging and Apparatus

For experiments performed at UC Berkeley, the imaging and apparatus details are described in detail in section 4.2.3. For VTT experiments, all imaging was performed on an Olympus IX-81 epifluorescent microscope. Data were captured using a CCD camera with Cell-P image capture software. Images were collected with a 10× objective (NA 0.3), using filter cubes optimized for the sample dyes (AlexaFluor488 and AlexaFluor568). GFP detection and a 0.63× demagnifier (Diagnostic Instruments Inc., Sterling Heights, MI). An image exposure time of 250 ms was used with 2× pixel binning applied in the y-direction (transverse to separation). Image analysis was performed using Image J software as described in section 4.2.3.

The chip was seated in a custom Delrin manifold to provide large sample reservoirs for each well. 5 µL of sample was loaded into the sample well with all other wells initially filled with 60µL 1× tris/glycine native buffer. The Agilent 2100 Bioanalyzer was used as a high-voltage power supply. This required installing a custom electrode adapter to replace the microfluidic chip port (Agilent Technologies, Santa Clara CA). The electrode adapter was built in-house (UC Berkeley, Herr Lab). Caliper HTS software was used to override the native Bioanalyzer software, providing either voltage or current control of up to 16 output electrodes. The Bioanalyzer power supply was able to output between 0 and 1700 V as opposed to 0 to 2900 V for the custom Caliper HVPS. Additionally, no real-time recording of output voltage or current data was available; however proper output values were verified by external voltmeter measurements.

Figure 5-2. Long-wave UV illuminator. Low-power flood illumination light source for 3%T loading gel fabrication at VTT.
5.2.4 Immunosubtraction Protocol

The high-voltage power supply limit for the Bioanalyzer was 1700 V. Protocols were adjusted appropriately, reducing voltage output for experiments conducted at VTT. All other aspects of the immunosubtraction protocol were run for the standard t-chip as outlined in section 4.2.4.

![IX-81 Microscope (data acquisition & fabrication)](image)

**Figure 5-3.** Data acquisition and fabrication setup at VTT. Imaging was performed with an IX-81 microscope using Cell-P image capture software. The Agilent Bioanalyzer 2100 with custom electrode adapters was used as a high-voltage power supply. Filters were fabricated using the microscope UV illumination source (100 W UV mercury arc lamp).

5.3 Results and Discussion

5.3.1 Folic Acid

Folic acid (vitamin B₉) is a 441 Da vitamin essential to the development of red blood cells and other biological processes ranging from DNA synthesis to aiding in rapid cell division and
growth. Folate is the naturally occurring form of folic acid in the body. Folate deficiencies from dietary or other causes can lead to severe health problems including neural tube defects in developing embryos typically leading to death. A rapid and cheap biosensor for detection of folate levels during pregnancy would be of great benefit to prenatal care in the developing world.

Collaborators at VTT Finland developed folic acid conjugates to larger carrier proteins to facilitate detection of folic acid in mobility-shift assays and demonstrate a proof-of-concept for antibody-based detection of proteins modified by small molecules. These modifications can be naturally occurring (e.g., post-translational modifications) or unnatural (e.g., medical drug adducts including immunosuppressants, antibiotics, etc.) Fab fragment antibodies were developed with binding affinity to the epitopes present on the small folic acid molecule while conjugated to either alkaline phosphatase (90 kDa) or human serum albumin (66 kDa). Binding of the fragment antibodies with FA-AP or FA-HA antibodies in the presence or absence of native folate in biological fluids would allow straightforward detection via a competitive PAGE assay.

Experiments were run to validate detection and binding of FA-AP and FA-HA with FA Fab fragment antibodies under the on-chip immunosubtraction electrophoresis conditions. Assays were run with a 12%T/3.3%C immunofilter chip to assess binding and determine whether the Fab antibody-conjugate complexes were able to pass through the 12%T filter (Figure 5-4). For the analysis of antibody Fab fragments (50 kDa) with antigens that are larger than the Fab fragment, the goal is to exclude the complex of the joined antibody-antigen while the smaller free Fab fragments and proteins are both able to pass through the filter. This allows specific detection and identification of the labeled sample via binding and mobility of the target analytes. Figure 5-4 shows folic acid-alkaline phosphatase and folic acid-human serum albumin both labeled with Alexa Fluor 488 were separated on-chip, resolving into 2 bands at a detection point 1.5 mm down the separation channel.

Incubation of anti-folic acid Fab incubated for 1 hour with the folic acid conjugate samples prior to immunosubtraction resulted in significant reduction of both the FA-AP and FA-HAS peaks. This demonstrates that that the Fab fragment is specific to both conjugates and binds to form complexes. Given that Fab fragments are smaller than the FA-HSA, there should not be any nonspecific blockage due to addition the free Fab fragment. The specific reduction of the AP conjugate is greater than that of the HSA conjugate (70% subtraction FA-HSA, 87% subtraction FA-AP).
Figure 5-4. A) Folic acid-alkaline phosphatase and folic acid-human serum albumin resolve in < 100 s. B) Immunosubtraction performed by incubating sample with unlabeled anti-FA Fab to form large immune-complexes that were excluded from electrophoresis by the on-chip filter while free dye passes through the filter uninhibited. C) Complex exclusion for various loading currents (at 125 seconds, 3 µA separation current, 250 ms exposure).
The image montage shows the migration of the two bands for both proteins. The protein bands disperse somewhat as they migrate, leading to the wide peaks in the electropherogram. It is clear from the immunosubtraction case that there is less protein present as in the electropherograms; however this excluded protein does not appear to be contained at the interface to the 12%T filter. The excluded fluorescence at the filter does not appear to change significantly between the control and subtraction case, although there is fouling of the filter interface more than that previously observed. This appears to indicate that the antigen-antibody complexes were not actually loading onto the chip and included in the separation since they were not excluded at the filter and not demonstrated by any new bands migrating after the initial unbound proteins. In order to investigate the loading of complex, the sample was loaded from a different well to allow loading in the opposite direction. This did not result in any additional loading of the complex on the chip. Additional attempts to load the complex were made by increasing the loading current from 2 µA to 3 µA and 4 µA. This did result in the increase in visible complex excluded at the filter interface as shown in Figure 5-4C.

The results demonstrate that anti-folic acid Fab fragments bind with both the FA-HSA and FA-AP conjugates. There is slightly stronger binding affinity with the AP conjugate, possibly due to differences in protein conformation and binding site location. The 12%T filter was also shown to have a sufficient pore size to exclude both Fab-FA-HSA complex (116 kDa) and Fab-FA-AP (144 kDa). However, to make measurements of excluded complex at the filter required increasing the load current to 4 µA to decrease the amount of nonspecific protein blockage at the filter interface caused by proteins below the cutoff limit.

5.3.2 Testosterone

Testosterone is a naturally occurring steroid hormone that plays a role in male reproductive system development. Testosterone also promotes growth of bone and muscle mass in both sexes and has commonly been used by athletes to improve performance as well as men suffering from andropause. In 1990, testosterone and other anabolic steroids became a controlled substance in the U.S. due to the dangerous physical and psychological effects of long-term use. Testosterone detection by athletic anti-doping agencies is typically performed in laboratories via liquid chromatography mass spectrometry with results available in days to weeks. Thus, development of a rapid testosterone diagnostic would benefit point-of-care anti-doping testing as well as testing for andropause—the condition resulting from decreased testosterone levels with aging men.

Collaborators at VTT Biotechnology developed a novel low molecular weight compound detection system using recombinant Fab fragment antibodies. As shown in Figure 5-5, Fab antibodies were developed to undergo a structural conformational change after detection of a hapten at the binding site. A second antibody fragment was then developed with high affinity to the conformational change in the hapten-antibody complex but with low affinity to the non-complexed primary antibody in the native state. This system is amenable to mobility-shift assays using only labeled antibodies as reagents because the free Fab vs. hapten-Fab-Fab complex differ in molecular weight by 50 kDa—approximately the size of one Fab fragment. This is a significant improvement over a system using free Fab vs. hapten-Fab for size separation, which
would differ only by the size of the hapten (< 800 Da), probably rendering any mobility differences undetectable.

Figure 5-5. VTT anti-hapten and anti-hapten complex antibody fragments. A. Structural changes occur to the Fab fragment after binding a hapten such as testosterone (GRASP image). B. A detection system is developed by use of anti-immunocomplex Fab that has high affinity for binding the hapten-Fab complex but low affinity to the native anti-hapten Fab. (Images printed with permission from Prof. Juha Rouvinen, University of Eastern Finland).

5.3.2.1 Binding Study Complex Preparation:

Due to insolubility in water, the testosterone was reconstituted into a solution with 40% ethanol and 1× tris/glycine buffer to a final concentration of 0.625 mg/ml (2.22 mM). The sample was vortexed for ~30 min to dissolve and filtered with 0.2 μm syringe filter. Testosterone was to be
incubated with anti-testosterone (TES220) at a ratio of at least 50 to 1 while anti-IC would be incubated in excess with the anti-testosterone. The final concentration was 220 µM testosterone, which was incubated for 30 min with anti-testosterone at 4 µM. This sample was then incubated for 1 hour with B1 anti-IC at 8.8 µM. Due to available starting concentrations, final concentrations were slightly adjusted in the sample to the following: 220 µM testosterone, 4 µM anti-TES (TES220), and 5.72 µM B1 anti-IC.

5.3.2.2 B1 Characterization

As shown in Figure 5-6, the B1 clone of the anti-IC Fab was detectable at high concentrations. In the B1 anti-IC sample a small unknown peak migrated prior to the large Fab peak. The small peak was probably a smaller cleaved antibody fragment such as the heavy or light chain. The larger peak had difficulty passing through the 15%T immunofilter, causing dispersion and a nongaussian profile (mobility = 0.589 m²/A·s). Two possible reasons come to mind: 1) the 50 kDa Fab fragment may be very close in size to the cut off limit of the 15%T filter and 2) a high concentration of protein contributing to filter blockage was used for the assays due to difficulty detecting 5× dilutions of the samples on-chip in preliminary experiments.

![Figure 5-6. Electropherogram of separation of B1 anti-IC Fab. Free antibody heavy and light chains were found present in the sample in addition to Fab fragments (3 mm detection, 3 µA separation, anti-IC Fab 8.8 µM, AF488 labeled).](image)

5.3.2.2.1 Control: Testosterone + Anti-testosterone (TES220) Characterization

The incubated testosterone-TES220 complex was separated on-chip to assess native mobility (Figure 5-7). The labeling efficiency of the TES 220 produced a sufficient signal at a
concentration of 4 µM. For this sample labeled with the AF568 dye, the free dye peak was significantly smaller than the Fab fragment peak. In addition there were no other large species detected, such as single chain fragments detected in the B1 anti-IC sample. The migration time of the anti-TES220 Fab fragment was somewhat less than that of the B1 anti-IC Fab for 3 µA separations detected at 3 mm. Both Fab fragments were 50 kDa (mobility = 0.378 m²/A·s); however any mobility difference probably resulted from fouling at the filter interface since testing of anti-TES220 was done after significant filter usage. The anti-TES220 Fab fragment also migrated through the filter region with less dispersion and a Gaussian profile not reflected with the B1 anti-IC Fab fragment.

Figure 5-7. Electropherogram of separation of anti-TES220 Fab. A single peak migrates, indicative of high purity of Fab fragments in the sample (3 mm detection, 4 µA separation, anti-TES220 Fab 4 µM, Testosterone 220 µM, AF568 labeled).

5.3.2.2.2 Immunosubtraction: Testosterone + Anti-testosterone (TES220) + B1 Anti-IC Characterization

At VTT, experiments were performed to validate immunosubtraction in the sandwich assay format. B1 anti-IC Fab was added to anti-TES220 and testosterone, significantly reducing the amount of Anti-TES220 able to pass through the filter (Figure 5-7). Unique signals were observed between the background binding case for nonspecific binding between both Fab fragments and the immunosubtraction case where both Fab fragments were present with the target analyte (testosterone). Separation conditions were also optimized to produce electropherograms that were better resolved with less baseline offset after separation. Trypsin inhibitor was added as an internal standard to allow normalization of any loading variations between runs.

Figure 5-8 shows that separations resulted in extraction of the TES220 (4.5 µM) anti-testosterone fab antibody (labeled with AF568). B1 (4.5 µM) anti-IC fab was labeled with AF488 and not
imaged for these assays. Testosterone (17.4uM) was unlabeled. The control peak area for the TES220 Fab fragment was largest with a reduction in peak area to 29% for the background binding case. The immunosubtraction case had a wider peak and larger peak area (50%) than the background binding case which was unexpected and prevented accurate quantitation of testosterone for the B1 anti-IC Fab sample labeled with AF488.

![Figure 5-8](image)

**Figure 5-8.** Electropherogram of separation of anti-TES220 Fab. Control signal is larger than the immunosubtraction (IS) signal. The background binding signal when both Fab fragments are present without testosterone also results in noticeable decrease in signal (3 mm detection, 4 µA separation, anti-TES220 Fab 4 µM, testosterone 220 µM, AF568 labeled).

The peak area was normalized by dividing against the trypsin inhibitor standard for all cases. Background binding had a lower signal than the immunosubtraction case at the same concentration. When B1 anti-IC concentration (9 µm) was further increased to twice the concentration of anti-TES220 (4.5 µm), the IS peak (32%) area was reduced further to a level similar to the background level (29%), as shown in Figure 5-9.

An unlabeled B1 anti-IC sample was used to determine the effect of fluorescence labeling on inhibition of anti-IC binding to anti-TES220-testosterone complex. The anti-TES220-AF568 Fab and testosterone sample were the same as used in previous experiments. A background binding case was run with both Fab fragments present as well as an immunosubtraction case with the addition of testosterone. As shown in Figure 5-10, anti-IC and anti-TES220 Fab binding in the presence of testosterone resulted in significant reduction in the Fab peak area with minimal effect on the internal standard peak. The decrease in signal between background Fab binding (no testosterone present) and the immunosubtraction case (+ testosterone present) was 35%, indicating improved performance of the unlabeled B1 anti-IC Fab over the fluorescently labeled samples where background binding was indistinguishable from immunosubtraction.
Figure 5-9. Peak area measurements for testosterone immunosubtraction using fluorescently labeled B1 anti-IC Fab. The background binding peak area is 29% of the control, while immunosubtraction using equal concentrations of B1 anti-IC and anti-TES220 results in 50% of control peak area. Increasing B1 anti-IC concentration to twice that of anti-TES220 reduces subtracted peak area to 32% of control.

Figure 5-10. Improved immunosubtraction performance of unlabeled B1 anti-IC Fab. Trypsin inhibitor (TI) internal standard was reduced 17% while the anti-TES220 was reduced 52% between the background binding and immunosubtraction case.
5.3.2.3 B12 Characterization

Assays were run both at VTT and UC Berkeley to characterize the anti-hapten and anti-immunocomplex Fab detection system using the microfluidic immunosubtraction format. The B12 phage display clone of the anti-immunocomplex (anti-IC) was separated on-chip, revealing only separation of free dye into 4 peaks at 10 mm. These peaks were confirmed to be free dye due to their extremely fast migration as compared to the B1 clone anti-IC, which was characterized later. No Fab fragments or other large migrating species were detectable in the B12 sample (Figure 5-11). Production of the B12 line was determined to suffer from extremely poor labeling efficiency or depletion of Fab at the expected concentration due to production steps making, this line unusable for on-chip immunosubtraction.

![Fluorescence vs Time Graph](image)

**Figure 5-11.** B12 clone production was ineffective for on-chip immunosubtraction as labeled Fab fragments were not detectable (10 mm detection, 3 µA separation, anti-IC Fab 17.4 µM, AF488 labeled).

5.3.3 THC

Tetrahydrocannabinol (THC) is a psychoactive chemical in the cannabis plant. THC has also been reported to have mild analgesic effects useful for treatment of glaucoma, cancer, and other diseases. THC use has been linked to long-term and short-term memory loss among other adverse effects, and it is classified as a controlled substance in the U.S. The ability to detect THC use in a portable point-of-care biosensor would allow law enforcement to enforce public safety better, particularly among those driving under the influence of drugs of abuse.
5.3.3.1 Background Binding FRET Experiments

Experiments were run at VTT to validate Fab fragment sandwich type assay for THC detection. For the low molecular weight target antibody based assay, fab fragments bind to the target in a sandwich type assay with first the target Fab fragment binding the analyte (i.e., anti-THC abbreviated T3) followed by the anti-immune complex Fab fragment (anti-IC abbreviated T3-104) binding to the target-antibody complex. When T3-104 binds to T3 without the presence of THC, this nontarget binding leads to a background signal. This background signal was characterized via fluorescence resonance energy transfer (FRET) assays.

A FRET assay was run in which T3 was labeled with europium (325 nM excitation, 615 nM emission) while T3-104 was labeled with Alexa Fluor 647 (650 nM excitation/668 nM emission), and THC was unlabeled. Plate reader detection was performed to measure 665 nM fluorescence thus only complexes formed between T3 and T3-104 were measured (Figure 5-12). A negative control was run with 0 ng/well of THC as well as a positive case with 20 ng/well of THC. For both the negative and positive controls, various concentrations of T3 and T3-104 Fab fragments were tested (0 to 2 µg/well) in order to determine ratios for optimal extraction.

Results showed that there was significant background binding that occurred for the negative control that increased with increasing levels of T3 and T3-104. Subtracting the background from the THC positive cases seems to indicate an optimal binding (based on S/N) occurred with 2 µg/well T3-104 and 1µg/well T3, a ratio of 2:1 with T3-104 at the highest concentration—which was expected since T3-104 was the label being detected (Figure 5-13). When the optimum binding ratio was determined by dividing the ratio between the THC and background signal, the optimum S/N was actually reached when T3-104 was at 1 µg/well with T3 at 0.25 µg/well, giving a ratio of 4:1. Also the signal was no longer highest with the largest concentration of the labeled T3-104. The optimum concentrations determined by the net increase (plot 3, ratio 2:1 T3-104 to T3) should be used since they are more consistent with how S/N is measured and the method was used by VTT for development of optimal binding Fab fragments.
Figure 5-12. FRET plate reader assays for background binding (A) and the positive control (B) both demonstrate increased binding with increasing T3 anti-THC Fab and increasing T3-104 anti-IC Fab concentrations.
Figure 5-13. Normalized FRET signal for THC detection. A) Subtracting background binding signal from positive control results in 2:1 optimal ratio of T3-104 anti-IC to T3 anti-THC. B) Dividing positive control signal from background binding results in 4:1 optimal ratio.
5.3.3.2 On-Chip THC Assays

Assays were run to test the binding of THC with the anti-THC (T3) and anti-IC (T3-104) using the microfluidic immunosubtraction assay. A 13.5%T/3.3%C immunofilter was used for all assays (5.25 min exposure, 2.04 mW/cm²). The T3-104 sample (3 µM, AF488 labeled) was separated to assess the purity of the sample as shown in Figure 5-14. The sample separated into 4 bands after migrating across the immunofilter, probably due to degradation or cleavage into small fragments (i.e., scFv) in addition to the Fab fragments.

![Image](image.png)

**Figure 5-14.** Separation of the T3-104 sample resulted in 4 sample peaks detected in addition to the free dye peak. These peaks resulted from the presence of smaller fragments (e.g., scFv) in addition to the Fab fragments.

Specificity of the T3-104 anti-IC and T3 anti-THC samples was also tested in the immunosubtraction assay to determine suitability for on-chip electrophoretic assays. Figure 5-15 shows electropherograms comparing the control case (T3-104, 3 µM, AF488) to the background binding case (T3-104 + T3 1.84 µM, AF568) and the immunosubtraction case (T3-104 + T3 + THC 310 µM). Several bands were observed in the electropherograms—of which the two highest mobility non-dye peaks varied with the addition of THC, indicating the presence of the
binding paratopes on those fragments. Imaging only the T3-104 anti-IC, results showed that the background binding case had slightly lower peak area than the control case. The immunosubtraction case however had significantly lower peak area than the control or background binding cases, showing the efficacy of the detection system under saturated THC conditions.

**Figure 5-15.** On-chip THC immunosubtraction. Immunosubtraction (THC + T3 anti-TES + T3-104 anti-IC) results in significant reduction of peak area compared to the background binding (T3 + T3-104) case which results in a slight reduction in peak area compared to the control (T3-104) assay (320 V/cm separation, 2 mm detection).

### 5.3.4 Conclusion

Characterization of VTT affinity binders was performed using the rapid homogeneous immunosubtraction sensor technology both at UC Berkeley and VTT. A fully functional chip fabrication and data acquisition system was established at VTT capable of producing discrete polyacrylamide immunosubtraction gels on-site with the same techniques implemented in the Herr Lab. Analysis of several recombinant Fab antibody variants specific for the detection of a low molecular weight compound was performed to assess usability for on-chip electrophoretic immunoassays. Characterization results of the VTT Fab binding system are summarized in Table 5-2.
Table 5-2: VTT sample validation for on-chip immunosubtraction.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sample Purity</th>
<th>Binding Specificity</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA-AP</td>
<td>High (1 band)</td>
<td>High</td>
</tr>
<tr>
<td>FA-HA</td>
<td>High (1 band)</td>
<td>High</td>
</tr>
<tr>
<td>T3-104</td>
<td>Low (4 bands)</td>
<td>Medium</td>
</tr>
<tr>
<td>IC B1-A488</td>
<td>High (1 band)</td>
<td>Medium</td>
</tr>
<tr>
<td>IC B12-A488</td>
<td>Low (no bands observed)</td>
<td>Low (no binding)</td>
</tr>
</tbody>
</table>

The folic acid conjugates (folic acid-alkaline phosphatase and folic acid-human serum albumin) both demonstrated effective binding to the anti-folic acid Fab fragment antibodies. In less than 2 minutes, the on-chip assay performed efficient immunosubtraction detection on a multiplexed sample (70% subtraction FA-HSA, 87% subtraction FA-AP). These results indicate potential for use of the folic acid conjugate Fab fragment technology in a competitive immunosubtraction assay for folic acid detection in prenatal care.

Results showed successful on-chip immunosubtraction using the Fab fragment sandwich binding system (anti-IC Fab + anti-TES + testosterone). The B1 anti-IC clone was observable at much higher levels than the B12 anti-IC clone, allowing detection with immunosubtraction chip. The B12 clone anti-IC fragments did not present a sufficient fluorescent signal due either to poor fluorescent labeling or poor purification of the sample. The B1 anti-IC Fab sample also contained several smaller fragments from abhorrent cleavage of antibodies into additional light or heavy chains (25 kDa). Using non-fluorescently labeled B1 Fab fragments improved performance over those that were fluorescently labeled since significant detection of testosterone (Fab-Fab-testosterone binding) over background(Fab-Fab binding) was achieved.

Finally, detection of THC was also demonstrated on-chip using the sandwich Fab detectors with a data acquisition system setup at VTT. Results of both FRET and on-chip assays showed that the T3-104 anti-IC variant resulted in specific binding with T3 anti-TES in the presence of THC at a level significant over background binding of the two Fab fragments without THC. The optimal ratio of anti-IC to anti-TES was 2:1 to maximize binding. The successful proof-of-concept indicates the use of the T3/T3-104 binding pair is suitable for further characterization and potential biosensors for detecting toxins in biological samples.
6 Integrated On-Chip Sample Preparation with On-Chip PAGE Detection

Chapter Abstract: This chapter describes the design and experimental results for in-line polyacrylamide membranes used for upstream sample preparation prior to downstream on-chip PAGE or immunosubtraction analysis. Inclusion of sample preparation functionality via small pore size polyacrylamide membranes is key to automated operation of proteomic devices in point-of-care and clinical laboratory settings. A new native protein analysis format that automates and integrates all assay steps required for fluorescence-based isoform analysis was developed. The assay combines salient features of on-chip PAGE (polyacrylamide gel electrophoresis) with well-controlled protein labeling within an on-chip ‘nanoreactor’ defined by a polyacrylamide size-exclusion membrane. The method uses efficient, addressable electrophoretic transport to seamlessly integrate: native protein labeling, background signal reduction, protein enrichment, and native PAGE protein analysis. Sample preparation features integrated on-chip included sample enrichment, fluorescence labeling, buffer exchange, and mixing with capture antibody. Results demonstrated seamless integration with downstream electrophoresis for native protein ladder mass isoform detection in less than 2 minutes—a first step towards studying important disease-relevant co- and post-translational protein modifications (i.e., glycosylation). Additionally, sample preparation was integrated with downstream immunosubtraction detection of both ladder proteins and raw cerebrospinal fluid improving assay sensitivity linearly by $12 \times$ with 2 minutes of sample enrichment.

6.1 Introduction

While integration of multiple functions is a hallmark of microfluidic tools, efforts to fully incorporate sample preparation would benefit from a surge in technology development. Specifically, innovation is needed for tools that allow dynamically-adjustable protein enrichment, fractionation of complex samples based on physicochemical properties, and efficient mixing of reagents with enriched sample fractions. As is often the case with laboratory medicine assays, bottlenecks stem from both the sample preparation and assay readout steps needed to complete benchtop immunoassays including PAGE and immunosubtraction analyses. Several specific steps complicate automated and rapid completion of an assay at the macroscale; specifically: sample enrichment to extend the lower limits of detection, sample staining to enable fluorescence readout, and sample mixing with capture antibody reagents to subtract protein targets from subsequent electrophoretic analysis. A handful of notable efforts in on-chip labeling have been reported; nevertheless, simplified on-chip labeling techniques promise to expand the protein analysis application space.

As detailed in chapter 4, immunosubtraction is a widely used laboratory medicine assay often employed to quantify diagnostic proteins by antibody-based bead capture and ‘subtraction’ of target in subsequent native slab-gel polyacrylamide gel electrophoresis\(^{27, 28, 35}\) (PAGE). Target proteins are identified via comparison of PAGE electropherograms—with and without target extraction.\(^{36, 37}\) Importantly, the immunosubtraction assay described here did not employ chemical immobilization of subtraction antibody in the microchannel network. Immunosubtraction offers benefits over slab gel protein electrophoresis, ELISA, and immunostaining techniques as the assay reports target identity through both mobility (charge-to-
mass ratio) and immunoaffinity, making the assay highly specific. However, for implementation as a rapid and automated proteomics tool in the clinical laboratory or point-of-care setting, integrated raw sample preparation must be implemented to reduce sample-to-result time sufficiently to influence medical outcome.

Current advances in microfluidic integration of sample preparation with electrophoretic assays for biomarker detection surmount the bottlenecks associated with bench-top slab-gel and capillary techniques. In particular, use of microfabricated molecular weight cut-off filters in electrophoresis microchannels allows exclusion and confinement of protein targets, as is relevant to subsequent sample manipulation needed for efficient, low sample-loss preparation strategies. Recent development of discrete non-uniform in-situ fabricated polyacrylamide (PA) gels for electrophoretic immunoassays also provides a design framework for integration of assay stages in one monolithic, voltage-programmable microdevice useful for laboratory medicine and clinical chemistry. Advantages over macroscale electrophoretic assays include increased automation, more rapid analysis times, lower sample volume, reduced cost and increased sensitivity. In chapter 6, the development of a 1st generation integrated sample preparation membrane for on-chip electrophoresis is detailed followed by a 2nd generation technology integrating preparatory functions with on-chip immunosubtraction PAGE.

The first generation electrophoresis device integrates sample fluorescence labeling and enrichment to assess prostate specific antigen (PSA) isoforms. Previous efforts have utilized size-exclusion membranes fabricated using in-situ photopolymerization for enrichment and mixing of a priori labeled proteins and immunoreagents. This membrane technology was enhanced to preferentially enrich PSA with a fluorogenic dye labeling reagent in a nanoreactor region that filters out undesired background dye molecules. Measuring levels of PSA isoform ratios has been used as a putative biomarker for the onset of prostate cancer. Prostate cancer is the most common type of cancer the second leading cause of death for men in the United States. Though $320M annually is spent on testing, differential diagnosis of benign versus cancer indicating PSA isoforms is sorely needed as various isoforms (BPSA, inPSA, pPSA) test positive in free PSA tests while only pPSA is specific for cancer. Current gold standard measurements for serum free PSA levels have unacceptably high false positive (61-78%) and false negative (15-27%) rates. Altered glycan structures on PSA from tumor cells support the hypothesis that glycosylation of serum PSA could differentiate cancer from benign conditions. Thus, rapid on-chip sample processing for detection of PSA mass isoforms and glycoforms would have significant health impact.

The second generation immunosubtraction device integrated with sample fluorescence labeling, enrichment, and antibody binding for downstream immunosubtraction detection of traumatic brain injury biomarkers from raw cerebrospinal fluid. Analytical technology for longitudinal measurements of putative protein biomarkers from proximal fluids are needed to facilitate monitoring and validation of promising, yet unverified, markers of dysfunction and injury. Increased levels of the protein S100B in CSF have been linked to cellular-level brain injury in multiple sclerosis, meningitis, subarachnoid hemorrhage and cerebral infarction. S100B is in a family of calcium-modulated proteins involved with the regulation of cellular functions and associated with development, function, and disease of the nervous system. Due to low concentrations and a short half-life, S100B protein is difficult to detect and monitor in the
systemic circulation. While not routinely collected as a diagnostic fluid, CSF is collected in severe cerebral trauma cases (traumatic brain injury, TBI) when patient cognitive and motor response are impaired (indicated by a Glasgow Coma Score 8 or lower). In severe trauma cases, CSF collection is performed using external ventricular drainage (ventriculostomy) as a means to reduce intracranial pressure (acute TBI), continuously monitor intracranial pressure, and insert antibiotics directly into the CSF; all of which can be critical to preventing brain damage or death. CSF is also collected to divert blood-contaminated CSF following hemorrhage or neurosurgery. The current standard for assessment of S100B levels in collected CSF relies on labor intensive and slow diffusion based techniques including immunoradiometric assays and sandwich enzyme immunoassays. Fine time-point monitoring via on-chip immunosubtraction would provide rich and currently missing information for biomarker validation, as well as the potential to monitor therapeutic efficacy (i.e., ventriculostomy) during treatment in cases of severe TBI.

In this chapter we present 1) integration of the flexible on-chip PAGE and immunosubtraction formats with three key on-chip preparatory steps, 2) characterization of PSA mass isoforms indicative of prostate cancer, and 3) identification of CSF trauma biomarkers via quantitative measurements of target mobility and binding specificity. The integrated on-chip sample preparation and immunosubtraction assays conducted using the new device are also characterized to establish optimal assay conditions. The study suggests the usefulness of the integrated sample preparation and immunosubtraction tool to automate other complex laboratory medicine techniques.

6.2 Materials and Methods

6.2.1 Reagents and Proteins

Solutions of 30% (29:1) acrylamide/bis-acrylamide, 3-(trimethoxysilyl)propyl methacrylate (98%), and glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO). The water soluble photoinitiator 2,2’-azobis[2-methyl-N-(2-hydroxyethyl)propionamide] (VA-086) was purchased from Wako Chemicals (Richmond, VA). Premixed 10× Tris-glycine native electrophoresis buffer (25 mM Tris, pH 8.3, 192 mM glycine) was purchased from Bio-Rad (Hercules, CA). Recombinant S100 beta protein (S100B), mouse monoclonal antibody to S100B (S100B antibody) and mouse monoclonal antibody to C-reactive protein (CRP antibody) were purchased from Abcam (Cambridge, MA). CRP purified from human serum was purchased from CalBiochem/EMD (Gibbstown, NJ). Ovalbumin (OVA, AlexaFluor 488 conjugated), Carbonic Anhydrase (CA), Chicken Serum Albumin (CSA), Trypsin Inhibitor (TI) and Immunoglobulin G (IgG) were purchased from Invitrogen (Carlsbad, CA). Human cerebrospinal fluid from pooled samples of healthy individuals was purchased from Biological Specialty Corp. (Colmar, PA). PSA isoforms were obtained from collaborators at Stanford University School of Medicine (Palo Alto, CA). Unlabeled proteins were fluorescently labeled in-house using Alexa Fluor 488 protein labeling kits (Life Technologies, Carlsbad, CA). Purification of labeled proteins was performed with P-6 and P-30 Bio-Gel columns (Bio-Rad, Hercules, CA). Post-labeling, proteins were diluted with 1× Tris/Glycine native buffer to attain desired concentrations. On-chip labeling was conducted using reagents from the Quant-iT Protein Assay Kit from Invitrogen Molecular Probes (Eugene, OR). The Quant-iT dye reagent was mixed with
1× Tris/Glycine buffer at a ratio of 1:4. Proteins were stored at 4°C in the dark until use. For off-chip preparation, protein-antibody complexes were formed by incubating the target protein with the relevant antibody for at least 1 hr at room temperature.

6.2.2 Chip Fabrication and Surface Preparation

Commercially available chip formats were selected to foster translation of the homogenous immunosubtraction assay to laboratory medicine and clinical labs. Two formats of soda-lime glass microfluidic chips were purchased from Caliper Life Sciences (Hopkinton, MA). A t-junction geometry was used for the sample preparation integrated with on-chip electrophoresis. Two intersecting channels terminated in fluid wells denoted here as sample (S), sample waste (SW), buffer (B), and buffer waste (BW). A separation channel was 2.5 mm in length, ~80 μm wide, and 20 μm deep. For the integrated sample preparation concatenated with the homogeneous immunosubtraction a more complex channel network was employed (see Figure S-1). This integrated chip consisted of the four wells used for immunosubtraction plus three additional reagent wells used for dye, antibody, and wash buffer all connected to a separation channel that was 2.05 mm in length, ~80 μm wide, and 15 μm deep.

Channel walls were prepared for in-situ gel polymerization by washing with 1M NaOH for 5 min to remove debris from the channels followed by a wash with deionized water to remove the NaOH. This was followed by channel wall functionalization to enable covalent linkage to PA with a 2:3:5 ratio solution of 3-(trimethoxysilyl)propyl methacrylate, glacial acetic acid, and deionized water loaded via capillary action. After 30 min the surface preparation solution was vacuum purged and channels were flushed twice with 30% glacial acetic acid followed by two washes with deionized water.

6.2.3 Sample Preparation Membrane Fabrication and Functions

A sample preparation membrane was fabricated in-situ with a photo-polymerization process similar to the immunosubtraction filter. A 40%T 6%C acrylamide/bisacrylamide solution with 0.2% VA-086 photoinitiator was sonicated and degassed for 5 min then introduced into the microfluidic channels. To photo-polymerize the sample preparation membrane, a sheet of UV light from the high intensity laser was used to: 1) localize the feature in the microchannel network and 2) yield sharp, well-defined boundaries. The sample preparation membrane was photo-polymerized with a 2 kW 355 nm Nd:YAG laser (Teem Photonics, Lafayette, CO) directed through cylindrical focusing optics and a 100 μm slit in a chrome on glass mask (Photo Sciences, Torrance CA) for 75 s. This resulted in a ~100 μm wide PA sample preparation membrane with a pore size sufficient to prevent passage of S100 (11 kDa) and all larger proteins while allowing free dye to pass through. The 3%T loading gel and 12%T immunosubtraction filter were fabricated adjacent to the 40%T sample preparation membrane in the manner described previously. The sample preparation membrane was used for enrichment of proteins and mixing of samples with required reagents for on-chip labeling and binding at discrete intervals. Samples and reagents were both loaded to the membrane and eluted by applying 0.1 μA (1100 V/cm) across the appropriate current path followed by subsequent immunosubtraction or electrophoresis.
Figure 6-1. UV laser fabrication system. A) A 355 nm ND:YAG laser (2 kW) was shaped with cylindrical focusing optics to precisely pattern PA gels within microfluidic chips. B) 100W UV Lamp.

6.2.4 Immunofilter Fabrication and Function Contiguous with Sieving Gel

A step decrease in PA gel pore-size at the start of an electrophoretic separation channel defines a molecular weight cut-off filter and electrophoretic sieving gel. During electrophoretic analysis of a protein sample, the cut-off filter acts as an immunofilter, as bound capture antibody and protein target are excluded from entering the separation gel. Immunofilters were fabricated from in-situ photo-patterned PA gel precursor solutions made by diluting the total volume of the 30%T (w/v) acrylamide/bisacrylamide solution to the desired ratio and adding 0.2% (w/v) VA-086 photoinitiator. All PA gel precursor solutions were sonicated and degassed 5-10 min prior to loading into channels for photo-polymerization. The molecular weight cut-off filter consisted of a 12%T (total acrylamide %T, with 3.3% w/w cross-linker, %C) PA region. 12%T filters were empirically determined to have a pore-size cutoff near 150 kDa, making this filter composition relevant to subtraction of S100B-antibody complexes. Adjacent to the filter a 3%T loading gel was polymerized creating a step discontinuity in the gel from large to small pore-size between the loading gel and filter region, allowing sample stacking at the interface for improved resolution and unbiased protein loading. Filters were fabricated via photolithography using a film transparency mask with a 500 µm × 4 mm opening (Fineline Imaging, Colorado Springs, CO). The open region of the mask was aligned with the desired region of the separation channel containing 12%T precursor solution then exposed to UV power (~5.8 mW/cm²) for 4 to 5 min. UV illumination was accomplished via a 100W mercury arc lamp and a 4× UV objective on an Olympus IX-50 microscope (Melville, NY) in conjunction with neutral density filters to achieve the desired power. After photo-patternning of the filter, the unpolymerized 12%T precursor solution in the loading channels was exchanged with 3%T PA precursor solution through a vacuum purge. Using a UV lamp in order to expose the entire surface area of the microfluidic channel geometry, the unmasked chip was then subjected to flood illumination for 10 minutes at a distance of 18 cm from the 100W UV lamp (UVP B100-AP, Upland, CA) at ~10 mW/cm².

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6.2.5 **Homogeneous Electrophoretic Immunosubtraction**

To initiate electrophoretic immunosubtraction, sample was loaded onto the chip by applying a 1.5 µA current (~300 V/cm) between S and SW, while B and BW were maintained at 0 µA current. For sample injection to the PA filter, 3 µA (~300 V/cm) was applied between B and BW, while S and SW were maintained at 0 µA (floating potential). For baseline cases in which no antibody was present, sample proteins were mixed just prior to performing separations. Conditions for the immunosubtraction cases integrated with on-chip sample preparation were similar as detailed in Figure 6-2. After each assay, protein complexes excluded at the filter were removed by reversing the electrical potential to apply 3 µA (~300 V/cm) from BW to SW, while B and S were maintained at 0 µA (floating potential). Channels were electrophoretically flushed with 1× Tris/Glycine buffer prior to each new assay. To generate electropherograms, the separation channel was imaged at 1.5 mm downstream of the separation junction. The channel was also imaged at the filter interface (250 µm downstream of the separation junction) to yield measurements of total excluded complex fluorescence. Total assay time including sample loading, separation, and removal of sample from the filter was ~5 min per run. Increasing concentrations of IgG (6 nM to 5 µM) were incubated for 1 hr with S100B (234 nM) prior to running on-chip immunosubtraction.

6.2.6 **Apparatus and Imaging**

Glass chips were seated in a custom Delrin manifold to provide sample reservoirs for each well. 5 µL of sample was loaded into the sample well while all other wells were filled with 60 µL 1× Tris/Glycine native buffer. Electrophoretic loading of sample was performed with a custom-built, programmable high-voltage power supply. Platinum wire was used as electrodes. Imaging was performed with inverted epi-fluorescence microscopes (IX-70, Olympus, Melville, NY and Diaphot 200, Nikon Instruments, Melville, NY). Images were collected with a 10× objective (NA 0.3), using a filter cube optimized for GFP detection and a 0.63× demagnifier (Diagnostic Instruments Inc., Sterling Heights, MI). A 1392 × 1040 Peltier-cooled interline CCD camera (CoolSNAP HQ2, Roper Scientific, Trenton NJ) was used to record images of the protein migration. An image exposure time of 250 ms was used with 2× pixel binning applied in the y-direction (transverse to separation). Image analysis was performed using Image J software (National Institutes of Health, http://rsbweb.nih.gov/ij/). All images were background subtracted to account for variability in background signal. Electropherograms were generated by measuring fluorescence intensity in a region of interest (ROI) occupying the width of the channel at a single axial position (ROI ~ 160 µm × 80 µm) ~1.5 mm downstream from the immunosubtraction filter interface. Variations in signal intensity due to CCD noise or external light fluctuation were normalized with the recorded signal to either a free-dye internal standard or total fluorescence of the injected sample in the separation channel.
6.3 Results and Discussion

6.3.1 Integrated fluorescence-labeling and polyacrylamide gel electrophoresis for analysis of protein isoforms

As part of the needed on-chip preparatory tool repertoire, protein labeling with fluorescent dye is essential to realize the “sample-to-answer” paradigm of many lab-on-a-chip systems. The technology introduced herein maximizes protein labeling, while minimizing background signal from unconjugated dye molecules. Small pore-size polyacrylamide membranes yield an on-line ‘nanoreactor’ defined by the volume of fluid in front of the membrane forming a streamlined method from sample preparation. The size-exclusion properties of the membrane allow elimination of background fluorescence from small dye molecules and provide a confined geometry for well-controlled protein-dye interactions. Subsequent electrophoretic protein analysis occurs rapidly in a contiguous polyacrylamide gel.

Figure 6-2 shows the small pore-size polyacrylamide gel membranes (15-22%T, 3.6-6%C) fabricated on a standard t-junction chip. The assay was run by performing the following steps: 1) enrich protein samples at the membrane interface using purely electrophoretic flow, 2) co-localize non-covalent dye with the enriched protein sample in an aqueous environment, and 3) subsequently elute and analyze labeled proteins using on-chip polyacrylamide gel electrophoresis (PAGE; 6%T, 3.6%C). Figure 6-3 provides a schematic overview of the technique. Key features of the assay design include: i) filter-based reduction of background fluorescence arising from unconjugated dye molecules, ii) dynamic protein enrichment, iii) well-controlled and adjustable protein-dye ratios (using timed electrophoretic loading of each species), and iv) use of a confined geometry at the membrane interface to actively co-locate protein and dye during labeling incubation.

A fluorogenic dye (Quant-iT, Invitrogen) that fluoresces after interacting with protein molecules was used as the labeling reagent (excitation/emission maxima: 470/570 nm). The non-covalent labeling reaction occurs within milliseconds and avoids contribution of interfering signals from free amino acids, nucleic acids, solvents, and common contaminating salts. Figure 6-4 shows non-fluorescent dye loading against and interacting with protein enriched at the membrane over 10 minutes. As the dye interacts with the protein the fluorescent signal increases substantially. Experiments performed showed that a minimum level of detectable fluorescence becomes identifiable after 50 seconds of labeling the enriched proteins at the membrane. This time is significantly reduced from the recommended protocol incubation step of 15 minutes for achieving maximum signal when labeling by off-chip incubation—again illustrating the improvement of electrokinetically driven on-chip mixing over benchtop diffusion based mixing and reaction.
Figure 6-2. In-Situ photopolymerization of sample preparation membrane. A 100 µm polyacrylamide membrane with precise features for size-exclusion is patterned with a UV laser in the microfluidic channel.

Figure 6-3. Size exclusion membrane allows on-chip protein labeling and subsequent native PAGE analysis. An electric current is used to drive proteins from the sample well to enrich at the membrane. Non-fluorescing dye is driven to label proteins at membrane and, upon interaction, the protein-fluorophore complex fluoresces. After labeling native PAGE separation commences.
Figure 6-4. Image sequence shows on-chip labeling of unlabeled native proteins. (Initial concentration: 126 µM BSA and 7 µM Urease).

Figure 6-5 shows that efficient separation of a native protein occurred within 48 seconds. Proteins were electrophoretically loaded against the membrane to enrich the concentration followed by Quant-iT loading. The labeled proteins were then separated via electrophoresis down a separation channel with single point fluorescence imaging. The first peak was indicative of unbound Quant-It as evidenced by the Quant-iT only negative control. A significant increase in fluorescence occurred after the Quant-iT label bound to the urease trimer or hexamer isoform. The urease trimer peak shows a S/N ratio of 145 while the hexamer peak has a S/N of 35. Also noticeable was the lack of background artifacts or signal drop often observed with other on-chip labeling and separation techniques. Additional experiments were run to show multiplexed labeling. Figure 6-6 shows results for near-baseline native PAGE analysis of a complex protein sample. After on-chip labeling, native PAGE separations were complete in less than 2 minutes generating distinguishable fluorescence peaks from various species and isoforms in the native molecular weight ladder.

Figure 6-5: Electropherogram for native Urease separation after labeling and enrichment at the sample preparation membrane.
Figure 6-6. Proteins are separated post on-chip labeling. 1) unbound dye (Quant-It)  2) Chicken Albumin (45 kDa) 2) BSA (66kDa) and 3) mass isoforms of Urease (272 kDa, 545 kDa)

Finally experiments were run to validate the integrated enrichment and PAGE technique for detection of isoforms from clinical samples. World health organization standard purified PSA samples were labeled off-chip with Quant-iT prior to on-chip enrichment and separation (Figure 6-7). Four PSA isoforms were rapidly identified with a 15 s separation, a result showing promise for development of a rapid on-chip diagnostic to stratify benign PSA isoforms from prostate cancer biomarkers.

Figure 6-7. WHO standard purified free PSA (1.5 ng/µl) labeled with Quant-iT (off-chip) rapidly reveals at least 4 isoforms in under 15 seconds.
6.3.2 Integrated Sample Preparation with On-Chip Immunosubtraction

On-chip immunosubtraction assays consisted of two electrophoretic separations: 1) a baseline sample separation in which no capture antibody was present and 2) a subsequent separation after capture antibody was incubated with the sample. Contiguous to the separation channel was a small pore-size membrane that allowed integration of several sample preparation functions needed to implement immunosubtraction in an automated manner as shown in Figure 6-8. After sample preparation, both sample aliquots were serially injected into the separation channel which contained a PA sieving gel. A sharp step discontinuity from large-to-small pore-size gel was located at the start of the PA sieving gel, somewhat similar to a stacking gel in a slab gel system. This immunosubtraction filter reversibly excluded antigen-antibody complex. Thus, when both protein target and capture antibody were present, the resulting large immune-complex was excluded from entering the PAGE separation region. Consequently, the target analyte peak was not present in the PAGE assay. When no capture antibody was present, the sample contained all protein peaks. Comparison of a baseline assay (Ab not present) and an immunosubtraction assay (Ab present) resulted in an apparent “subtraction” of the target peak. All assays were conducted under native (non-reducing, non-denaturing) conditions, to preserve the affinity of target protein to antibody.

Figure 6-8. Sample preparation integrated with downstream PAGE for immunosubtraction electrophoresis in a homogeneous format. (A) The discrete pore-size discontinuity acts as an immunosubtraction filter when complexing antibody is present, (+ Ab) thus excluding target sample peaks from the resultant PAGE electrophoretogram. Comparison to a baseline electrophoretogram (No Ab) reveals analyte mobility and interaction. (B) Photo-patterning of
contiguous PA gel features with different pore-sizes allows definition of preparative and analytical functions in one monolithic device. (C) The sample preparation membrane is used in conjunction with electrophoretic analyte transport to enable sample enrichment, mixing of sample with antibodies, and fluorescence labeling of the sample.

6.3.2.1 Integration of Preparatory Functions

The integration of multiple sample preparation steps needed to provide a complete and rapid assay that minimized laborious and manual preparatory functions was facilitated by the use of a microfluidic format with in-situ fabricated PA gels. Several discrete PA regions of varying pore sizes formed distinct reaction chambers within the microfluidic channels that provided capability to perform various functions prior to the immunosubtraction assay including: 1) in-line electrophoretic enrichment of low concentration analytes, 2) on-chip fluorescent labeling of samples, and 3) rapid on-chip binding and incubation of antibody and target proteins.

First, enrichment was achieved by electrophoretically loading sample towards a 100 µm wide PA sample preparation membrane (40%T/6%C, Figure 6-8) in the 7 well chip (Figure 6-9). The small pore size of the 40%T sample preparation membrane allowed the passage of small free dye molecules (640 Da) while preventing passage of all sample proteins, of which S100B (11 kDa) was the smallest. A sample containing model proteins (TI, OVA, and CRP) was enriched at the sample preparation membrane prior to electrophoresis resulting in a total protein enrichment factor of 12\(\times\) over the baseline in 2 minutes (Figure 6-10A). No fluorescent signal was detectable without enrichment prior to electrophoresis, thus 10 s enrichment was used as a baseline value to assess the increase in sensitivity due to enrichment. The protein enrichment, or signal enhancement, factor increased linearly with loading time for the model system explored (\(y = 0.099x + 0.346, R^2 = 0.992\)), with the rate of enrichment of each individual protein dependent upon the electrophoretic mobility as described in previous studies\(^9\),\(^10\) (Figure 6-11). As the system sensitivity, or lower limit of detection, is proportional to the enrichment factor, the LLOD would also be expected to increase directly with enrichment time until reaching the point where concentration polarization limits effectiveness of continued electrophoretic enrichment.\(^9\) The ability to confine sample at a location—without immobilization—in a homogeneous assay format allowed further manipulation to concentrate the sample downstream while obviating the need for slow and labor intensive bench-top enrichment techniques including vacuum centrifuge and evaporative concentrators.

Next, on-chip fluorescence labeling of samples was achieved via electrophoretic transport and mixing of a fluorogenic dye (Quant-iT) with target proteins at the sample preparation membrane. Quant-iT, a non-covalent binding dye, has minimal fluorescence in free solution however undergoes significant fluorescence enhancement when bound to proteins. Therefore the Quant-iT dye is useful for immunosubtraction assays since all sample proteins are labeled before comparison of a baseline and antibody subtraction PAGE run. Quant-iT dye (1:4, reagent to 1×Tris/Glycine) was initially injected into the loading channel adjacent the sample preparation membrane (see Figure 6-9). An unlabeled sample of model proteins (S100B 1.21 µM, CA 1.36 µM, CSA 888 nM) was simultaneously injected into the same channel co-locating with the free dye and initiating the labeling interaction. The dye and protein were then electrophoretically transported to the sample preparation membrane confining them into a small reaction region
facilitating more efficient binding. Figure 6-10B shows a linear increase in protein signal with labeling time following injection of the fluorescently labeled proteins into the separation channel for subsequent PAGE \((y = 0.284x - 0.168, R^2 = 0.989)\). Labeling with Quant-iT for 10 s resulted in approximately 4× increase in detectable signal for each sample protein (S100B 4.1×, CA 4.3×, CSA 4.1×). With inclusion of the necessary reagent loading and transport steps, on-chip fluorescent labeling was achieved in 70 s compared to the off-chip labeled assays which required anywhere from 1 hr to overnight to incubate and prepare samples for covalent labeling with AlexaFluor 488 dye.

**Figure 6-9.** Schematic of integrated sample preparation and immunosubtraction achieved by electro-kinetic transport on a microfluidic chip. Protein enrichment was achieved by loading sample across the 40%T PA sample preparation membrane (S→SW). Sample fluorescence labeling was achieved by co-loading protein and the fluorescent label (Quant-iT) into a channel where binding occurred as both reagents were concentrated at the membrane (S→SW, L→SW). Ab binding and incubation were performed by loading the antibody to mix with a previously enriched and off-chip labeled sample adjacent to the membrane (Ab→SW). For all cases, subsequent separation across the immunofilter was implemented by eluting the concentrated sample off of the membrane (B1→BW) then applying an electric field to bypass the membrane (B2→BW) and inject proteins into the 12%T separation gel (immunofilter). \(E = 1100\) V/cm for all sample preparation steps. \(E = \sim200\) V/cm for elution and bypass separation.
Figure 6-10. Sample preparation including sample enrichment, on-line fluorescent labeling, and antibody mixing are integrated using a combination of electrophoretic transport and a small pore-size PA gel sample preparation membrane. (A) Sample enrichment at the sample preparation membrane significantly increases enrichment factor of 3 pre-labeled model proteins (TI 200 nM, OVA 97 nM, CRP 393 nM). Protein enrichment increases linearly with increasing electrophoretic loading to the PA membrane resulting in enrichment factors of $18\times$, $11\times$, and $7\times$ respectively for TI, CRP, and OVA in under 2 min. (B) Non-covalent fluorescent labeling and enrichment of sample is achieved by co-loading a non-covalent fluorogenic dye (Quant-iT) with model proteins (S100B 1.21 µM, CA 1.36 µM, CSA 888 nM). The signal of each protein increases $4\times$ with 10 s dye/enrichment time due to labeling. (C) On-chip mixing and incubation of pre-labeled sample with antibodies (TI 200 nM, OVA 97 nM, CRP 393 nM). Mixing with antibody against CRP (4.4 µM) is performed just prior to downstream baseline (no Ab, 0 s) and immunosubtraction (+Ab, 30 s and 120 s) PAGE resulting in increased specific CRP target extraction with Ab load/enrichment time (78% extraction at 120 s). $E = 1100$ V/cm across the sample preparation membrane for all sample preparation steps. $E = 200$ V/cm separation with imaging at 1.5 mm. In each panel, electropherograms are slightly offset (y-axis) to aid interpretation.

On-chip antibody binding and incubation were also incorporated at the sample preparation membrane with the goal of integrating all required preparatory functions into the immunosubtraction assay. CRP antibody (4.4 µM) was electrophoretically introduced to the sample preparation membrane where previously enriched model proteins were present (TI 200 nM, OVA 97 nM, CRP 393 nM; proteins labeled off chip). Continuous introduction of antibody increased antibody-antigen binding and removal of the target analyte during subsequent immunosubtraction PAGE analysis (Figure 6-10C). Results showed the rapid immunosubtraction of 69% of the CRP peak with only 30 s of antibody binding time. Further increase in antibody binding time resulted in more modest improvement, achieving maximum target extraction of 78% CRP with an antibody binding time of 120 s. Since co-location of antigen and antibody was electrophoretically driven in this homogeneous system, binding occurred faster than in diffusion based systems with no separate incubation step required. Antibody immobilized on surface substrates or Sepharose beads typically requires at least 30 minutes of incubation with the antigen while standard off-chip free solution incubation requires at least an hour to achieve maximum binding—thus the on-chip sample preparation format
achieved antibody binding and incubation an order of magnitude faster. Programmable on-chip binding also afforded the flexibility to rapidly establish ideal antibody-antigen ratios to maximize target extraction while providing multiplexing capabilities by introducing distinct antibodies specific for individual targets simultaneously or within consecutive assay runs.

**Figure 6-11.** Signal enhancement with enrichment of model proteins at the sample preparation membrane (Trypsin Inhibitor 200 nM, Ovalbumin 97 nM, and CRP 393 nM). Each protein enriched and enhanced detection signal linearly as a function of enrichment time as follows: (TI) $y = 0.152x - 0.259$, $R^2 = 0.996$; (OVA) $y = 0.053x + 0.818$, $R^2 = 0.986$; (CRP) $y = 0.091x + 0.480$, $R^2 = 0.984$. The linearity of total protein enrichment was $y = 0.099x + 0.346$, $R^2 = 0.992$. Enrichment at the sample preparation membrane for 2 min increased the signal 18×, 11×, and 7× respectively for TI, CRP, and OVA with a 12× signal increase for total protein in the sample.

To validate the capability to perform sample preparation on proteins in their native biological matrix, enrichment was performed on CSF and nasal discharge (Figure 6-12). Results demonstrated that pre-labeled CSF and nasal discharge samples had increased signal with increased electrophoretic concentration at the membrane from 10 s to 30 s prior to PAGE separation. For additional labeling experiments, the CE-503 fluorogenic dye was chosen for labeling proximal fluid samples because the labeling chemistry does not alter the native charge (or electrophoretic mobility) of proteins. Like the Quant-iT dye, CE-503 is also negatively charged in 1× tris/glycine buffer as are the proteins of interest thus facilitating electrophoretic co-migration for mixing. The fluorogenic enhancement of proteins happens almost immediately upon electrophoretic co-location of the two species at an on-chip junction resulting in significant fluorescence enhancement as shown in Figure 6-13.
**Figure 6-12.** Enrichment of proteins in biological matrices. Proteins are enriched at the sample preparation membrane (40%T 6%C) directly from (A) cerebrospinal fluid and (B) nasal discharge showing increased signal with enrichment time (3 µA separation, 1.5 mm detection, AlexaFluor 488 labeled off-chip).
Figure 6-13. Inverted fluorescence image of fluorogenic dye labeling on-chip. CE-503 fluorogenic dye is electrophoretically driven to a junction with an unlabeled protein ladder (TTR, HSA). A labeling reaction occurs at the intersection of the flows resulting in significant fluorescence increase as the small dye molecules diffuse into the protein stream (0.2 µA loading).

Labeling of a CSF sample with CE-503 resulted in detectable signal with 10 s of labeling that increased with labeling time (Figure 6-14) demonstrating the ability to effectively label proteins within a raw human CSF matrix on-chip. Unlike Quant-iT, the CE-503 dye was found to react with 1× tris/glycine buffer increasing fluorescence within 5 mins; so the reagent was diluted 5× in water, under which conditions the dye still maintained a negative charge for electrophoretic loading. The labeling dynamics were also investigated to optimize dye to protein concentration. Ideally the dye reagents would be present at an excess concentration in the nanoreactor region adjacent the membrane to ensure that the reaction was occurring at the maximum rate and not transport limited. Results showed that the fluorogenic labeling was indeed occurring in the reaction limited regime because increasing the amount of Quant-iT dye reagent while maintaining the protein concentration constant had minimal effect on the fluorescent signal (Figure 6-15). However, increasing the amount of protein at the sample preparation membrane while maintaining a constant dye concentration resulted in an increase in fluorescent signal.
Figure 6-14. On-chip labeling of protein ladder with CE-503. A ladder representative of CSF (TTR 83.3 µg/ml and HSA 208 µg/ml) was co-loaded with CE 503 (5× dilution in water) for simultaneous loading and enrichment. The signal was visible after 15 s and increased over time up with up to 2 mins of enrichment (3µA separation, 40%T 6%C membrane; 3%T/13.5%T immunofilter).

Figure 6-15. Quant-iT Fluorogenic Labeling Optimization. Labeling with Quant-iT occurs in the reaction limited regime as evidenced by the increase in signal when (A) only protein concentration is increased and dye remains constant at the membrane versus (B) a minimal signal increase when only dye reagent is increased and protein concentration is held constant. Labeling with Quant-It dye (1:20, reagent to 1× tris/glycine). 3µA separation, 40%T 6%C membrane.
6.4 Conclusion

A 1\textsuperscript{st} generation technology was demonstrated for protein analysis that automates and integrates native protein labeling, background signal reduction, protein enrichment, reagent mixing, and native PAGE protein analysis. Labeling conditions for native PAGE assays were optimized for analysis of PSA isoforms. PSA isoform analysis resulted in the rapid detection (120 s) of four peaks indicative of mass isoforms (or abhorrent glycosylation) and useful for prostate cancer diagnostics—a first step towards studying important disease-relevant co- and post-translational protein modifications (i.e., glycosylation).

The 2\textsuperscript{nd} generation on-chip integrated sample preparation and homogeneous immunosubtraction technology introduced here yielded detection of target analytes via rapid PAGE to provide data on mobility and specificity of target molecules. Introduction of sample preparation functions on-chip including protein enrichment, labeling, and antibody-binding were shown to significantly reduce labor intensive effort and time required for the total assay including preparatory functions from 4-12 hours to 5-10 min;\textsuperscript{28-30, 36} while also improving system sensitivity 12\times. Performing immunosubtraction on-chip in an automated and programmable manner on a uniform platform opens up the application space to detecting numerous biomarkers for various proximal fluid diagnostics, as well as for serum and urine analyses.
Early Traumatic Brain Injury Screening Diagnostics: CSF Rhinorrhea Biomarker Validation via On-Chip Immunosubtraction

Chapter Abstract: To expedite and automate microfluidic separation techniques for clinical diagnostics, on-chip immunosubtraction is introduced for detecting cerebrospinal fluid (CSF) rhinorrhea. In particular, cerebrospinal fluid (CSF) rhinorrhea, a potentially dangerous condition identified by leakage of CSF into the nasal cavity due to traumatic brain injury, neurosurgery, spontaneous leakage or other causes; stands to benefit from a rapid and non-invasive diagnostic. Recent studies show potential for differentiation between CSF and nasal leakage through analysis of proximal fluid specific biomarkers including transthyretin (TTR) in CSF. On-chip immunosubtraction is achieved by photo-fabricating precise features within an in-situ sieving matrix contained within the channel network. By miniaturizing the immunosubtraction assay onto a microfluidic format, the assay is performed in less than 5 min with integrated sample preparation steps including on-chip labeling, enrichment, and binding for on-the-fly multi-target identification. This saves significant time when compared to sample preparation bottlenecks for current gold standard techniques including slab gel or capillary electrophoresis with biomarker extraction via immobilized beads. Results demonstrate rapid subtraction of ~95% endogenous TTR from CSF while confirming no TTR detection within nasal secretion indicating applicability of this technique for rapid CSF rhinorrhea detection.

7.1 Introduction

This chapter introduces an on-chip sample preparation and immunosubtraction diagnostic for emergency room screening of cerebrospinal fluid (CSF) rhinorrhea. CSF rhinorrhea is a dangerous condition occurring when CSF from the cranial cavity leaks into the nasal cavity owing to head trauma, neurosurgery, invasive tumors, congenital defects, or non-traumatic causes including spontaneous leakage or repetitive strenuous exercise that elevates intracranial pressure. The result is a low-viscosity, clear unilateral or bilateral nasal secretion resembling mucus that may increase output as the patient leans forward and is often accompanied by headaches. Regardless of etiology, this disruption of the brain—sinus barrier can potentially lead to bacterial meningitis (10% risk annually, 40% long-term) or viral infection, intracranial hypotension, pneumocephalus, anosmia, frontal lobe abscess or even stroke. Early detection and treatment are critical to patient prognosis and outcome given the potentially fatal risks and complications associated with CSF rhinorrhea, thus screening techniques stand to benefit from rapid on-chip laboratory diagnostics.

Presently, state-of-the-art CSF rhinorrhea detection includes a combination of CT scan, MRI, endoscopic examination, or ELISA. Optimized algorithms for diagnosing CSF leakage rely on a combination of detection methods based on the etiology, however most algorithms recommend performing non-invasive biomarker screening assays first to indicate the presence of CSF leakage prior to more thorough otorhinolaryngologic examination to locate and visualize possible defects for surgical treatment. Despite the optimal diagnosis protocol, the lack of available rapid biomarker screening methods can lead practitioners to opt directly for more costly and
invasive diagnosis (imaging, endoscopy) or unnecessary reparative procedures for rhinorrhea pathologies falsely presumed to be caused by CSF leakage. In particular, frequently used techniques such as high resolution CT scans and endoscopy with intrathecal application of fluorescein dye for leakage localization can have serious side effects. Endoscopic nasal procedures create potential for further tissue damage while addition of fluorescein to the intracranial cavity is not manufacturer nor FDA approved and at sufficient doses has been correlated to grand mal seizures and spinal headaches. Therefore, these procedures should be limited to implementation only after results of a positive screening assay. Additionally, given that 1.4 million Americans suffer traumatic brain injuries annually, there are many emergency situations in which a rapid and portable biomarker assay would be the only available diagnostic option; such as for emergency first responders, patients at rural clinics, and for use at forward operating base medical facilities for U.S. military personnel—for whom traumatic brain injuries have increased more than 200% in the last ten years. Consequently, due to the morbidity risk of invasive techniques and the need for increased automation and portability of emergency diagnostics, CSF rhinorrhea detection would benefit from an automated, rapid and non-invasive protein biomarker based nasal discharge screening assay for CSF-specific proteins.

This chapter summarizes the development of a fully-automated sample preparation and homogeneous immunosubtraction device on a microfluidic format capable of detecting a panel of diagnostic protein biomarkers to quantify and differentiate endogenous CSF proteins from mucus proteins in raw nasal secretions. Currently, there have been no published reports on a microfluidic assay to diagnose CSF rhinorrhea. Recent reports show the crucial need for increased automation and speed needed for proteomic tools and the benefits of realizing these with homogeneous immunoassays that integrate sample preparation and biomarker measurements. This assay, the first work from the Herr Lab on raw nasal secretion analysis, builds upon previous published work from the author and others on the analysis of CSF collected from trauma surgery patients or lumbar puncture—a difficult means of sample collection not amenable to point-of-care assays. Homogeneous immunosubtraction is a powerful laboratory medicine assay reporting both protein mobility and binding specificity of target analytes within a complex biological matrix—typically implemented via antibody-based bead capture used to ‘subtract’ a target analyte from subsequent native polyacrylamide gel electrophoresis (PAGE). Identification of target analytes is performed by comparing PAGE electropherograms run with and without target subtraction. The on-chip CSF rhinorrhea diagnostic technology automates several sample preparation techniques including sample dilution and mixing, analyte enrichment, buffer exchange, matrix protein depletion, antibody-antigen binding, and fluorescence labeling necessary to detect putative biomarkers via immunosubtraction electrophoresis. Advances with in-situ photo-polymerization are essential to integrating sample preparation with analytical functions harnessing small transport length scales within integrated architectures. Precise functionalized gel regions are fabricated contiguously within the glass microchannel geometry with UV photolithography to form discrete reaction zones with unique physical properties (e.g. polymer cross-linking ratio to control pore size) or chemical properties (e.g. biotin incorporation for protein fixation). Within the customizable and distinct gel reaction zones, the multiple sample preparation steps required for raw biological sample processing are performed prior to analyzing the biomarkers of interest. Previous reports have shown various aspects of raw sample preparation including electrokinetic mixing, labeling, or immuno-depletion via on-chip or hybrid means; however none have
Herein, an automated microfluidic tool for proteomic analysis and diagnostics of raw nasal secretions from CSF rhinorrhea patient cohorts is detailed. Multiplexed detection is achieved for a panel of putative differentiating CSF biomarkers indicative of head trauma in raw nasal discharge (prolactin inducible protein—PIP, transthyretin—TTR, and beta2-(tau)-transferrin—β2TF). Recent proteomic studies using two-dimensional electrophoresis with isoelectric focusing and western blot approaches have shown potential for differentiating CSF and nasal leakage through analysis of more than five CSF, nasal mucus, and plasma specific markers including TTR, PIP, and β2TF. Additional studies have focused on exclusively detecting β2TF or β-trace protein as a means to assess aqueous rhinorrhea, however reported false positives and false negatives as well as slow assay times (> 1 hr) with these techniques point to the utility of introducing rapidly assessable biomarker panel diagnostics.

In chapter 7, technology is presented that surmounts major diagnostic gaps in emergency medicine CSF rhinorrhea screening techniques by quantifying multiple putative biomarkers in raw nasal mucus, CSF, and combinations of both biological specimens. Details of advances in several areas are shown including: 1) engineering design to extend complete on-chip sample preparation capabilities, including confounding matrix protein depletion, for difficult to detect co-migrating putative CSF biomarkers (i.e., β2-(tau)-Transferrin) and 2) demonstration of biomarker panel analysis and validation for diagnosis of clinical samples from a healthy and injured patient cohort examined at the University of California San Francisco otolaryngology clinic. Results show that translating this technology from laboratory to clinical practice holds considerable promise for both point-of-care diagnostics and laboratory medicine by implementing on-chip CSF rhinorrhea testing that addresses the limitations of current techniques often used for screening including: limited availability and high cost (CT Scan, MRI), invasive protocols with potential to cause further damage (endoscopic methods and intrathecal dyes) or long assay times with large sample volume requirements and intensive manual labor steps (ELISA).

7.2 Materials and Methods

7.2.1 Reagents and Proteins

Solutions of 30% (29:1) acrylamide/bis-acrylamide, 3-(trimethoxysilyl)propyl methacrylate (98%), and glacial acetic acid were purchased from Sigma-Aldrich (St. Louis, MO). The water soluble photoinitiator 2,2’-azobis[2-methyl-N-(2-hydroxyethyl)propionamide] (VA-086) was purchased from Wako Chemicals (Richmond, VA). Premixed 10 × tris/glycine native electrophoresis buffer (25 mM Tris, pH 8.3, 192 mM glycine) was purchased from Bio-Rad (Hercules, CA). Ladder proteins including TTR, S100, and HSA along with polyclonal antibodies to TTR and HSA were purchased from Invitrogen (Carlsbad, CA). Monoclonal antibodies for beta-2-transferrin, prostaglandin-D synthase, and secondary HRP conjugated anti-rabbit and anti-mouse antibodies were purchased from Abcam (Cambridge, MA). CSF obtained via lumbar puncture was purchased from Biological Specialty Corporation (Colmar, PA). Human cerebrospinal fluid from pooled samples of healthy individuals was purchased from Biological Specialty Corporation (Colmar, PA).

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Biological Specialty Corp. (Colmar, PA). Human nasal mucus was collected directly from healthy human subjects. Unlabeled proteins were fluorescently labeled in-house using Alexa Fluor 488 protein labeling kits (Life Technologies, Carlsbad, CA). Purification of labeled proteins was performed with P-6 and P-30 Bio-Gel columns (Bio-Rad, Hercules, CA). Post-labeling, proteins were diluted with 1× tris/glycine native buffer to attain desired concentrations. On-chip labeling was conducted using reagents from the CE 503 dye kit from Active Motif (Eugene, OR). The CE 503 dye reagent was mixed with 1× tris/glycine buffer at a ratio of 1:4. Proteins were stored at 4°C in the dark until use. For off-chip sample preparation, protein-antibody complexes were formed by incubating the target protein with the relevant antibody for at least 1hr at room temperature. SDS-PAGE and western blot assays of biological samples were performed using 4-20% Tris-HCL precast gels (Biorad, Hercules CA) with the XCell Surelock Mini-cell gel electrophoresis system (Invitrogen, Carlsbad CA) for separation and blotting to PVDF membranes. Novex HRP Chromogenic substrate was purchased from Invitrogen (Carlsbad, CA).

7.2.2 Chip Fabrication and Surface Preparation

Commercially available chip formats were selected to foster translation of the homogenous immunosubtraction assay to laboratory medicine and clinical labs. Two formats of soda-lime glass microfluidic chips were purchased from Caliper Life Sciences (Hopkinton, MA). A t-junction geometry was used for the homogeneous immunosubtraction assay characterization. Two intersecting channels terminated in fluid wells denoted here as sample (S), sample waste (SW), buffer (B), and buffer waste (BW). A separation channel was 2.5 mm in length, ~80 µm wide, and 20µm deep. For the integrated sample preparation concatenated with the homogeneous immunosubtraction a more complex channel network was employed (see Figure S-1). This integrated chip consisted of the four wells used for immunosubtraction plus three additional reagent wells used for dye, antibody, and wash buffer all connected to a separation channel that was 2.05 mm in length, ~80 µm wide, and 15µm deep.

Channel walls were prepared for in-situ gel polymerization by washing with 1M NaOH for 5 min to remove debris from the channels followed by a wash with deionized water to remove the NaOH. This was followed by channel wall functionalization to enable covalent linkage to PA with a 2:3:5 ratio solution of 3-(trimethoxysilyl)propyl methacrylate, glacial acetic acid, and deionized water loaded via capillary action. After 30 min the surface preparation solution was vacuum purged and channels were flushed twice with 30% glacial acetic acid followed by two washes with deionized water.

Immunofilter Fabrication and Function Contiguous with Electrophoretic Sieving Gel

A step decrease in PA gel pore-size at the start of an electrophoretic separation channel defines a molecular weight cut-off filter and electrophoretic sieving gel. During electrophoretic analysis of a protein sample, the cut-off filter acts as an immunofilter, as bound capture antibody and protein target are excluded from entering the separation gel. Immunofilters were fabricated from in-situ photo-patterned PA gel precursor solutions made by diluting the total volume of the 30%T (w/v) acrylamide/bisacrylamide solution to the desired ratio and adding 0.2% (w/v) VA-086 photoinitiator. All PA gel precursor solutions were sonicated and degassed 5 to10 min prior to loading into channels for photo-polymerization. The molecular weight cut-off filter consisted
of a 12%T (total acrylamide %T, with 3.3% w/w cross-linker, %C) PA region. 12%T filters were empirically determined to have a pore-size cutoff near 150 kDa, making this filter composition relevant to subtraction of S100B-antibody complexes. Adjacent to the filter a 3%T loading gel was polymerized creating a step discontinuity in the gel from large to small pore-size between the loading gel and filter region, allowing sample stacking at the interface for improved resolution and unbiased protein loading. Filters were fabricated via photolithography using a film transparency mask with a 500 µm × 4 mm opening (Fineline Imaging, Colorado Springs, CO). The open region of the mask was aligned with the desired region of the separation channel containing 12%T precursor solution then exposed to UV power (∼5.8 mW/cm²) for 4 to 5 min. UV illumination was accomplished via a 100W mercury arc lamp and a 4× UV objective on an Olympus IX-50 microscope (Melville, NY) in conjunction with neutral density filters to achieve the desired power. After photo-patterning of the filter, the unpolymerized 12%T precursor solution in the loading channels was exchanged with 3%T PA precursor solution through a vacuum purge. Using a UV lamp in order to expose the entire surface area of the microfluidic channel geometry, the unmasked chip was then subjected to flood illumination for 10 minutes at a distance of 18 cm from the 100W UV lamp (UVP B100-AP, Upland, CA) at ∼10 mW/cm².

Homogeneous Electrophoretic Immunosubtraction
To initiate electrophoretic immunosubtraction, sample was loaded onto the chip by applying a 1.5 µA current (∼300 V/cm) between S and SW, while B and BW were maintained at 0 µA current. For sample injection to the PA filter, 3 µA (∼300 V/cm) was applied between B and BW, while S and SW were maintained at 0 µA (floating potential). For baseline cases in which no antibody was present, sample proteins were mixed just prior to performing separations. For the immunosubtraction cases with off-chip sample preparation, target protein was incubated for one hour with the relevant antibody prior to loading. The immunosubtraction cases conducted with on-chip sample preparation are detailed in the Results and Discussion section. A minimum of three replicate runs for each sample were performed. After each assay, protein complexes excluded at the filter were removed by reversing the electrical potential to apply 3 µA (∼300 V/cm) from BW to SW, while B and S were maintained at 0 µA (floating potential). Channels were electrophoretically flushed with 1× Tris/Glycine buffer prior to each new assay. To generate electropherograms, the separation channel was imaged at 1.5 mm downstream of the separation junction with the exception of the high resolution measurements which were detected at 12mm. The channel was also imaged at the filter interface (250 µm downstream of the separation junction) to yield measurements of total excluded complex fluorescence. Total assay time including sample loading, separation, and removal of sample from the filter was ∼5 min per run.

Sample Preparation Membrane Fabrication and Functions
A sample preparation membrane was fabricated in-situ with a photo-polymerization process similar to the immunosubtraction filter. A 40%T 6%C acrylamide/bisacrylamide solution with 0.2% VA-086 photoinitiator was sonicated and degassed for 5 min then introduced into the microfluidic channels. To photo-polymerize the sample preparation membrane, a sheet of UV light from the high intensity laser was used to: 1) localize the feature in the microchannel network and 2) yield sharp, well-defined boundaries. The sample preparation membrane was photo-polymerized with a 2 kW 355 nm Nd:YAG laser (Teem Photonics, Lafayette, CO) directed through cylindrical focusing optics and a 100 µm slit in a chrome on glass mask (Photo
Sciences, Torrance CA) for 75 s. This resulted in a ~100 µm wide PA sample preparation membrane with a pore size sufficient to prevent passage of S100 (11 kDa) and all larger proteins while allowing free dye to pass through. The 3%T loading gel and 12%T immunosubtraction filter were fabricated adjacent to the 40%T sample preparation membrane in the manner described previously. The sample preparation membrane was used for enrichment of proteins and mixing of samples with required reagents for on-chip labeling and binding at discrete intervals. Samples and reagents were both loaded to the membrane and eluted by applying 0.1 µA (1100 V/cm) across the appropriate current path followed by subsequent immunosubtraction as described previously.

7.2.3 Apparatus and Imaging

Glass chips were seated in a custom Delrin manifold to provide sample reservoirs for each well. 5 µL of sample was loaded into the sample well while all other wells were filled with 60µL 1× tris/glycine native buffer. Electrophoretic loading of sample was performed with a custom-built, programmable high-voltage power supply. Platinum wire was used as electrodes. Imaging was performed with inverted epi-fluorescence microscopes (IX-70, Olympus, Melville, NY and Diaphot 200, Nikon Instruments, Melville, NY). Images were collected with a 10× objective (NA 0.3), using a filter cube optimized for GFP detection and a 0.63× demagnifier (Diagnostic Instruments Inc., Sterling Heights, MI). A 1392 × 1040 Peltier-cooled interline CCD camera (CoolSNAP HQ2, Roper Scientific, Trenton NJ) was used to record images of the protein migration. An image exposure time of 250 ms was used with 2× pixel binning applied in the y-direction (transverse to separation). Image analysis was performed using Image J software (National Institutes of Health, http://rsbweb.nih.gov/ij/). All images were background subtracted to account for variability in background signal. Electropherograms were generated by measuring fluorescence intensity in a region of interest (ROI) occupying the width of the channel at a single axial position (ROI ~ 160 µm × 80 µm) ~1.5 mm downstream from the immunosubtraction filter interface. Variations in signal intensity due to CCD noise or external light fluctuation were normalized with the recorded signal to either a free-dye internal standard or total.

7.3 Results and Discussion

7.3.1 Sample Preparation Protocol

The operation of the CSF rhinorrhea immunosubtraction device consisted of performing a series of on-chip sample preparation steps prior to downstream immunosubtraction PAGE for quantification of the target analyte (Figure 7-1). All sample loading and manipulation on-chip is achieved electrophoretically via a programmable, high voltage power supply to apply an electric field through the desired channel paths. Rapid loading and sample dilution through a large pore size polyacrylamide gel (3%T, 3.3%C) is used to introduce the sample proteins to the sample preparation membrane while suppressing electroosmotic flow. A minimum of 5 µL of raw CSF or nasal discharge was loaded into the sample wells shown in step 1. Samples were electrophoretically diluted on-chip to the desired ratio by precise control of the loading currents, a technique that has been previously shown to provide accurate dilution while saving significant time over manual diffusion based dilution via pipetting. Samples were loaded 1 min to a sample waste well to ensure reaching equilibrium during dilution. After sample dilution the
current was applied from the sample wells across the 40%T 6%C sample preparation membrane where large target proteins enrich and buffer exchange occurs between the sample and 1× tris/glycine load buffer as the target proteins are too large to pass through the membrane but smaller ions pass freely (Figure 7-1, step 2 and 3). Simultaneously, during this time other sample preparation reagents were electrophoresed to the reaction area adjacent to the membrane including the fluorescent label and immunosubtraction target antibody contained in distinct reagent wells. Fluorescence labeling was performed using CE 503 (dye reagent diluted 5× in diH2O), a fluorogenic reagent that undergoes a 50-fold increase in quantum yield upon binding proteins via a non-covalent reaction. CE 503 was chosen because the reaction chemistry maintains the positive charge of amine groups after conjugation and does not alter native protein charge—thus signal masking and band broadening from differential labeling are reduced while native electrophoretic mobility of proteins are unaffected.

After sample/reagent reaction was completed at the sample preparation membrane, the sample was electrophoresed across a customizable matrix depletion gel (3%T, 3.3%C, + streptavidin-biotin-Ab linkage) used to remove confounding proteins present in high abundance that may interfere with the detection signal (Figure 7-1, step 4). Anti-HSA (human serum albumin) antibodies were immobilized in the depletion region because HSA composes ~67% of all CSF protein and migrates with similar mobilities to potential biomarkers of interest present at lower quantities. (Figure 1, step 4). As the sample band traverses the depletion gel a portion of the HSA is removed after binding to the gel immobilized antibody. All other proteins without affinity to anti-HSA pass through to the downstream immunosubtraction region (Figure 7-1, step 5). Upon reaching the immunosubtraction PAGE region proteins electrophoresed across a discrete step decrease from a large pore size loading gel (3%T, 3.3%C) to a small pore size separation gel (12%T 3.3%C). This interface acted as a size-based immunofilter excluding large proteins and capture antibodies over 150 kDa. Thus any target analyte binding to the capture antibody formed a large complex excluded from subsequent PAGE analysis. Comparison of control (no antibody) and immunosubtraction (+ antibody) runs allowed identification and quantitation of biomarker content within complex biological matrices via downstream single point fluorescence detection and comparison of electropherogram peak area profiles.
Figure 7-1. Raw sample preparation integrated with immunosubtraction PAGE. 1) Electrophoretic sample loading and dilution are performed through a 3%T loading gel at a channel junction. 2) Small pore size sample preparation membrane (100 µm; 40%T, 6%C) allows protein enrichment as small ions pass through but large proteins are blocked. 3) Reaction zone adjacent the membrane is used for efficient electrophoretic mixing of fluorogenic dye (CE 503) and immunosubtraction target antibodies. 4) Streptavidin-acrylamide gel (3%T) with immobilized anti-HSA antibody depletes albumin from migrating sample band. 5) Detection of target analyte performed via comparison of control (no Ab) and immunosubtraction (+ Ab) PAGE where the target analyte is excluded at the discrete border between the 3%T loading gel and 12%T immunosubtraction gel.
Figure 7-2. Homogeneous assay enables immunosubtraction of putative protein biomarkers from complex biological fluids and as multi-analyte panels. A) Conductivity of a dilute rhinorrhea ladder containing nasal discharge and CSF decreases linearly as a function of nasal discharge concentration. Mobility decreases as a function of nasal discharge concentration non-linearly as there is a plateau region in the middle where mobility is constant as a function of sample concentration due to buffer exchange (nasal discharge samples consist of 10× dilution of nasal mucus in 1× tris/glycine, CSF samples are undiluted). B) Labeling with simultaneous enrichment at the membrane for HSA with CE 503 dye increases signal sensitivity linearly by 15× in 2 min. Labeling and enrichment of TTR increases at a lower rate non-linearly by a factor of 2× in 2 min. C) Albumin depletion via immunosubtraction decreases albumin concentration 50% while allowing TTR concentration to increase by 50% due to less filter blockage. Albumin depletion via the 3%T immobilized depletion gel showed preferential removal of albumin (20%) compared to non-specific removal of TTR (10%). D) Immunosubtraction of TTR from a model system (S100 and TTR in 1× tris/glycine) results in 100% peak subtraction while subtraction of 95% endogenous TTR from human CSF is achieved in 2 min.

7.3.2 Integrated Sample Preparation Results
The integrated sample preparation techniques allowed for rapid and automated sample processing of CSF or nasal discharge with seamless on-chip downstream detection. Each sample preparation step was assessed to verify proper function when integrated into the device starting with the electrokinetic dilution and buffer exchange (Figure 7-2A). Raw CSF samples and 10× diluted nasal discharge samples (diluted 10× in 1× tris/glycine to prevent detector saturation) were electrophoretically diluted at various sample ratios in order to develop a calibration curve.
Flux density, and thus applied current are directly proportional to the sample analyte concentration during electrophoresis in a polyacrylamide gel where all transport is assumed to result from only applied electric fields and all particles are assumed to be charged,

\[ \vec{J} = -D_i \mu_{ep} c \vec{E} = \sigma \vec{E}, \]

and

\[ i = \vec{J} \cdot \vec{E} \]

where \( J \) is flux or current density, \( D_i \) is diffusivity, \( \mu_{ep} \) is electrophoretic mobility, \( c \) is concentration, \( \sigma \) is conductivity and \( i \) is current. Thus as we see the sample conductivity should be directly proportional to the concentration as shown in blue for off-chip conductivity measurements performed on various nasal discharge dilutions into CSF. Conductivity decreases linearly (\( R^2 = 0.981 \)) from 13 mS/cm to 2 mS/cm when diluting the more highly concentrated CSF sample with the 10× nasal discharge sample. On-chip measurements of inverse mobility, which should increase linearly as conductivity increases linearly, reached a plateau increasing non-linearly in a manner consistent with a third order polynomial (\( R^2 = 0.798 \)). This indicated an effect of the buffer ion exchange that occurred on-chip as the finite sample volume injected to the PAGE assay diffuses into 1× tris/glycine buffer in the reaction zone and at the discrete immunosubtraction interface.

Labeling and enrichment were performed by co-loading a raw CSF sample and CE503 dye across the sample preparation membrane at constant current (3 µA) for increasing durations ranging from 15 s to 2 min (Figure 7-2B). Results indicated successful on-chip electrophoretic labeling of endogenous proteins in a raw CSF sample matrix for the first time. Greater than 10-fold enrichment for HSA and 4-fold enrichment for TTR over the baseline signal (15 s signal) was detected in 2 min. HSA enriched linearly (\( R^2 = 0.996 \)) while TTR concentration increased logarithmically (\( R^2 = 0.979 \)) over time. This result was expected as previous work with electrophoretic enrichment of model proteins in buffer at porous membranes has shown that larger proteins often enrich at a higher rate as they are less likely to be forced through the membrane pores after long loading durations34.

Two methods of albumin depletion were performed on chip to assess which would be most effective in reducing the albumin signal present in downstream immunosubtraction (Figure 7-2C). The first method was by loading anti-HSA (0.2 mg/ml) incubated with 80% CSF off-chip then running immunosubtraction PAGE on-chip and comparing to a control sample (80% CSF, no Ab). This resulted in the ability to extract approximately 50% of the albumin peak area while the TTR peak area increased 50% likely due to reduced interaction with non-specific blockage of HSA-Ab complex present at the filter interface. The second method tested was using the anti-HSA immobilized via a streptavidin-biotin linkage into a 3%T loading gel for 1.5 mm between the sample preparation membrane and the immunosubtraction filter. This method resulted in the removal of 20% of the HSA protein while the TTR protein decreased by 10% likely due to non-specific interactions with the blotting region. The immunosubtraction method appeared more effective in albumin removal due to the higher density of Ab that could be localized at the filter interface improving binding kinetics compared to the incorporation of anti-HSA into the acrylamide gel limited by the density of streptavidin biding sites and the porosity of the 3%T gel.
After all integrated upstream sample preparation functions were verified, immunosubtraction of endogenous CSF proteins were verified (Figure 7-2D). Results indicated that in a 1× tris/glycine buffer model system 100% of the TTR peak (0.01 mg/ml) was subtracted by addition of polyclonal anti-TTR Ab (0.0375 mg/ml). Subtraction of 95% endogenous TTR from a 90% CSF sample (diluted 1/10 with Ab) was achieved by addition of the same polyclonal anti-TTR Ab (0.17 mg/ml). In both the model system and CSF, detection was achieved in less than 2 min demonstrating the ability for rapid and effective protein target identification via both binding affinity and native mobility quantitation.

7.3.3 On-chip immunosubtraction PAGE vs. slab gel PAGE

Automated sample preparation with immunosubtraction detection is performed under non-denaturing non-reduced conditions to allow verification of target analyte mobility and antibody-antigen binding specificity during native PAGE while also facilitating sample preparation steps by preventing detergent interference with fluorogenic reactions during on-chip labeling. The resulting detection is comparable to that performed by diffusion based immunosubtraction using Sepharose beads and native slab gel electrophoresis or SDS-PAGE with subsequent western blotting, however can be performed with automated sample preparation in under 5 minutes as opposed to 4 to 10 hr.81 Slab gel SDS-PAGE was performed on undiluted CSF and nasal discharge samples using a 4-12%T precast 6cm polyacrylamide gel followed by Coomassie blue staining or western blotting to a PVDF membrane for three putative biomarkers (beta-2-transferrin, prostaglandin-D synthase, and transthyretin with HRP conjugated secondary antibody). The SDS-PAGE and western blot process required approximately 12 hr and resulted in a complex peak pattern (Figure 7-3). By visual inspection 3 to 4 bands can be clearly seen in the CSF lane with the largest peak presumably albumin (66 kDa). The nasal discharge sample resulted in a minimum of 7 bands that were poorly resolved after SDS-PAGE. Subsequent western blotting for the three putative unique CSF biomarkers80 showed detection (brown signal) in the CSF lane however also showed some signal cross reactivity with bands in the nasal discharge lanes possibly due to bleed over from adjacent lanes. As expected, interpretation to distinguish between CSF and nasal discharge via slab gel PAGE and western blot was slow and non-quantitative. A profile plot of the SDS-PAGE pixel intensity allowed assessment of signal-to-noise ratio of the slab gel detection technique vs. the on-chip detection technique with single point detection at 12 mm. Slab gel SDS-PAGE resulted in maximum peak S/N = 128 for undiluted CSF and S/N = 187 for undiluted nasal discharge after 5 hrs separation and Coomassie staining. Significant increase in signal was achieved with rapid (2min) on-chip separations across the discrete immunofilter resulting in S/N = 11667 or undiluted CSF and S/N = 2381 for 10× diluted nasal discharge. The signal increase was ~ 100× for the rapid on-chip assay with fluorescence detection performed in 1/30th total assay time. Peak resolution was also analogous between the native on-chip page (CSF 6 peaks, nasal discharge 7 peaks) and slab gel SDS-PAGE.
Figure 7-3. On-chip immunosubtraction PAGE facilitates assay interpretation over slab gel SDS-PAGE and western blotting. A) Slab gel SDS-PAGE performed in 4-12% T gel with subsequent Coomassie Blue staining requires ~5hrs. Slab gel SDS-PAGE followed by western blotting with HRP conjugated secondary antibody requires ~12hrs. The protein band profile for CSF differs significantly from nasal discharge in intensity and protein content with 3 putative biomarkers identifiable qualitatively via western blot (brown signal). B) Comparison of slab gel signal intensity profile with the on chip electropherograms for undiluted CSF and nasal discharge (on-chip nasal discharge diluted 10×). On-chip total assay requires 5-10 min compared to 5 hrs off-chip yet results in ~100 fold increase in S/N ratio.

7.3.4 CSF Rhinorrhea Biomarker Quantitation

Automated on-chip immunosubtraction was used to validate endogenous CSF and nasal discharge proteins for efficacy in CSF rhinorrhea diagnostic panels. Two target proteins were assessed for efficacy in a biomarker panel, prolactin inducible protein (PIP) and transthyretin (TTR). PIP (15 kDa) is used in regulation of H₂O transport in serous cells of the salivary and submucosal glands and has been shown to be a specific indicator for nasal mucus while TTR (55 kDa) is a serum and CSF carrier of thyroxine and retinol reported as a putative biomarker for CSF via 2-D electrophoresis studies. Assays were run to develop a calibration curve for both PIP and TTR signal present in dilutions of healthy human CSF samples with healthy patient nasal discharge samples (10× diluted). Results showed the ability to identify the high mobility PIP peak only in samples that contained nasal discharge while the TTR peak was significantly larger in the 100% undiluted CSF sample (Figure 7-4A). Analysis of the peak areas demonstrated that TTR concentrations increased 14-fold between the nasal discharge sample and the CSF sample whereas the PIP decreased from baseline to zero over the same dilutions (Figure 7-4B). These results supported the assertion that that TTR and PIP can comprise an effective protein biomarker panel for quantitation of nasal mucus or CSF content in unknown nasal discharge samples from rhinorrhea patients. Additionally, these preliminary calibration curves were rapidly generated (15 min) via integration of upstream on-chip sample dilution.
Figure 7-4. Sample matrix protein characterization of putative CSF rhinorrhea biomarkers (PIP and TTR). A) Levels of key putative biomarkers (PIP and TTR) vary with sample dilution creating distinct biomarker profiles observed after rapid PAGE (75 s per run) of raw human CSF and nasal discharge (10× dilution). B) Mixing of CSF into nasal discharge (10× dilution) from 0 to 100% results in the decrease of PIP content and increase of TTR content. PIP is uniquely found in nasal discharge while TTR is enriched in CSF compared to nasal discharge (10-fold enrichment). The entire dose-response study is completed in less than 15 min using on-chip sample dilution with downstream immunosubtraction electrophoresis.

7.4 Conclusion

A microfluidic device with integrated sample preparation was introduced for the detection of cerebrospinal fluid rhinorrhea. While CSF rhinorrhea is a serious condition, current state-of-the-art diagnostics are expensive or slow (MRI, CT scan or ELISA). A microfluidic immunoassay was demonstrated to accept and processes raw biological fluids with integrated sample preparation steps (enrichment, labeling, dilution, buffer exchange, matrix depletion, and Ab binding) prior to characterizing biomarker content via immunosubtraction PAGE. Miniaturizing the assay onto a microfluidic chip with integrated sample preparation offered advantages for detecting CSF rhinorrhea including increased automation, portability, multiplexed detection of biomarker panels (PIP, TTR, HSA) and reduced assay time from 5 to 12 hours for slab gel SDS-PAGE with western blot to 5 min on-chip analysis.

Results demonstrated the ability to rapidly identify and differentiate CSF from nasal mucus based on PIP and TTR quantitation as differentiating biomarkers, the efficacy of which was validated via on-chip calibration curve generation in less than 15 min. TTR increase (7% to 100%) and PIP decrease (100% to 0%) were shown to correlate to increased presence of CSF in nasal discharge. Results point to the efficacy of the on-chip technique for CSF rhinorrhea confirmatory diagnosis and monitoring in clinical labs and for emergency medicine to promote more widespread implementation of efficacious diagnostic protocols while reducing cost and assay time to result.
Chapter abstract: This chapter summarizes the development of microfluidic diagnostics tools to replace recombinant immunoblot assays (RIBA) for identifying the presence of viral antibodies from biological samples. Two novel microfluidic separation techniques were used: pore-limit electrophoresis (PLE)—a gradient gel sizing technique commonly used in slab-gel analysis, and pore-limit isotachophoresis (PLITP)—a novel dispersionless concentration and separation technique introduced for the first time on or off-chip in this dissertation work. PLE and PLITP were developed via customized and functionalized polyacrylamide gels fabricated in-situ to facilitate electrophoretic protein transport, separation, and detection within minutes as opposed to hours required for current diffusion based assays like ELISA and RIBA strip tests. Additionally, the microfluidic technology allows the assay to be performed with minimal sample volume (5 µL) on a small footprint format that is amenable to portable, low-cost, and disposable use at the point-of-care. The pore-limit sieving technology uses novel chemical and physical based techniques to separate antibodies useful for the detection of hepatitis C directly from raw serum or other biological fluids by immobilizing the antibodies into the sieving matrix. Results show that the pore-limit separation techniques allowed rapid detection of clinical levels of antibodies for 4 recombinant antigens (NS3, c100, core protein, and peptide) in less than 30 min detection using raw serum samples.

8.1 Introduction

Infectious diseases infect millions of people annually in the developed and developing world—often times with dire consequences. Of the top ten diseases resulting in mortality annually worldwide, three of these diseases are caused by viral pathogens resulting in numerous deaths annually (HIV/AIDS 2.8 million, measles 0.6 million, and hepatitis B 0.1 million). Viruses are typically diagnosed by assessing the concentrations of viral pathogens (antigens) or antibodies produced in response to the pathogens present in the blood. Viruses are often more difficult to detect than bacterial or parasitic pathogens due to their varying and potentially low concentrations between individual patients as well as their small size. Thus, the primary methods for diagnosing viral pathogens include immunoassays such as lateral flow assays, ELISA, and RIBA amongst other techniques. Many diseases require complex immunoassays looking for multiple biomarkers (biomarker panels) at various concentrations in systemic or proximal fluids. The aforementioned diagnostic methods are all diffusion based and thus are time consuming, manual-labor intensive, and potentially expensive to run while often requiring special expertise, training, or laboratory infrastructure for running assays effectively. Among these viral diagnostics, screening test assays for hepatitis C virus are performed using recombinant immuno-blot assay (RIBA) strip tests that require multiple technician-assisted wash and incubation steps over 7 hours to run up to 5 assays in parallel. The resulting complex assay readout requires visual inspection to interpret slight color changes, leaving potential for misdiagnosis. Viral immunoassay diagnostics such as these and others for infectious diseases can greatly benefit from advances in technology to increase automation, reduce cost, decrease assay time, and increase portability and ease of use at the point-of-care. These improvements will all serve to increase the efficacy of diagnosing and treating infectious diseases through increased
adoption of accurate diagnostics in developing and developed countries thus potentially saving millions of lives.

Pore-limit electrophoresis assays have previously been demonstrated on-chip for detection of antibodies in buffer systems as well as zymography studies. The technique consists of electrophoretic loading of a sample plug along a microchannel with a gradient polyacrylamide gel from large to small pore sizes. As various protein species within the injected sample reach their size exclusion limit they are immobilized by physical exclusion by pores smaller than a particular protein species—thus separating according to molecular weight along the gradient gel. The technique is amenable to use in a single microchannel format and provides a detectable signal that can be interrogated even after the separation current is removed. In this chapter we extend the on-chip PLE technique for detection of proteins from biological matrices with large quantities of interfering proteins (e.g., HSA, IgG, etc.). Challenges were addressed to achieve clinical limits of detection as well as prevent undesired pore blockage due to high protein concentration.

Pore-limit isotachophoresis was developed and implemented as a means to minimize sample injection dispersion in single channel assays while simultaneously providing sample enrichment during protein sizing assays. ITP is a moving-boundary concentration technique in which an ionic target analyte concentrates in a zone formed between a higher-mobility leading electrolyte buffer and a lower-mobility trailing electrolyte buffer. As fast moving anions in the leading electrolyte move towards the anode, a charge void is created at the boundary with the slow trailing electrolyte which is filled with analytes having mobilities between the two—typically the sample analytes desired to be enriched. All analytes are concentrated in the zone as a function of their mobility, moving with the constant boundary speed. ITP is an electrokinetic concentration method; however several efforts have been made previously to integrate it with separation techniques including transient ITP prior to electrophoresis. This technique is common in both the macroscale stacking gel (Laemmli system) and on-chip where it has been used to achieve million-fold sample stacking with analyte separation. However, this approach produces a loss of analyte band stacking as the switch from ITP to electrophoresis occurs causing the previously tightly-packed analyte zones to disperse. In this chapter a technique is introduced for the first time to size proteins using only low-dispersion isotachophoretic transport and sample stacking to pattern proteins at their pore-limit cutoff in a gradient polyacrylamide gel. This method, pore-limit isotachophoresis, is used for sample preparation and sizing of raw serum proteins in a single channel immunoassay for hepatitis C.

This chapter details the development of electrokinetically driven sizing immunoassays in pore-limit gradient gels. These assays enable integrated sample preparation and detection for raw serum biomarker analysis in simple single channel formats with key features including: 1) customized non-linear gradient gels to facilitate targeted protein sizing, 2) ordered electrokinetic sample injection and Ab binding, and 3) low concentration sample enrichment. The on-chip pore-limit sizing techniques driven with electrokinetic sample manipulation discussed herein have many advantages over macroscale diffusion-based binding immunoassay techniques such as RIBA strips or ELISA assay. Assay times (30 min vs. 3 h for ELISA or 7 h for RIBA), sample consumption (5 µL vs. 20 µL RIBA), power consumption, and cost per assay are all reduced on-chip compared to the macroscale. The on-chip methods also reduce manual labor.
intensive steps and decrease the overall footprint of the device, making the tools useful in traditional laboratory medicine facilities and at the point-of-care for portable or low resource setting diagnostics. To demonstrate proof-of-concept, the on-chip PLE and PLITP immunoassays are used for confirmatory diagnostics of hepatitis C via quantitative viral antibody measurement of 4 target recombinant antigens (NS3, c100p, core protein, core peptide).

8.2 Materials and Methods

8.2.1 Reagents and Proteins

Solutions of 30% (29:1) acrylamide/bis-acrylamide, 3-(trimethoxysilyl)propyl methacrylate (98%), glacial acetic acid, NaCl, HEPES, and human serum samples were purchased from Sigma-Aldrich (St. Louis, MO). Recombinant S100 beta protein (S100B) and mouse monoclonal antibody to S100 (S100 antibody) for ladder assays were purchased from Abcam (Cambridge, MA) while trypsin inhibitor (TI) was purchased from Invitrogen (Carlsbad, CA). The water soluble photoinitiator 2,2’-azobis[2-methyl-N-(2-hydroxyethyl)propionamide] (VA-086) was purchased from Wako Chemicals (Richmond, VA). Premixed 10× tris/glycine native electrophoresis buffer (25 mM tris, pH 8.3, 192 mM glycine) was purchased from Bio-Rad (Hercules, CA). Recombinant proteins NS3, c100p, core protein, and core peptide along with monoclonal antibodies anti-NS3, anti-c100p, and anti-core were provided directly from collaborators at Novartis Diagnostics (Emeryville, CA). Unlabeled ladder proteins were fluorescently labeled in-house using Alexa Fluor 488 protein labeling kits (Life Technologies, Carlsbad, CA). Purification of labeled proteins was performed with P-6 and P-30 Bio-Gel columns (Bio-Rad, Hercules, CA). Post-labeling, proteins were diluted with 1× tris/glycine native buffer to attain desired concentrations.

8.2.2 Chip Fabrication and Surface Preparation

Commercially available chip formats and custom designed chips were used for assays. All chip formats were fabricated with soda-lime glass at Caliper Life Sciences (Hopkinton, MA). All chips consisted of straight channels connecting 2 wells. For standard chips, dimensions were 10.4 mm length, 100 µm width, and 15 µm depth. Custom chips were 4 mm long, 35 µm deep, and 120 µm wide. Channel walls were prepared for in-situ gel polymerization by washing with 1 M NaOH for 5 min to remove debris from the channels followed by a wash with deionized water to remove the NaOH. This was followed by channel wall functionalization to enable covalent linkage to PA with a 2:3:5 ratio solution of 3-(trimethoxysilyl)propyl methacrylate, glacial acetic acid, and deionized water loaded via capillary action. After 30 min the surface preparation solution was vacuum purged and channels were flushed twice with 30% glacial acetic acid followed by two washes with deionized water.

8.2.3 Gradient Gel Fabrication and Function

A gradient increase in total polyacrylamide %T was used to create a gradual decrease in PA pore size from large pores at the start of the separation channel to small pores at the end. During electrokinetic separations migrating protein species (including target antibodies) will reach their molecular weight cut-off, causing immobilization at various distances along the channel based
upon PA pore-size. Once the target antibodies are entrapped within the channels, they can then be probed with labeled affinity reagents (e.g., recombinant antigen proteins or secondary labeling antibodies, etc.) that are used to detect the presence of the viral antibody in the patient as well as quantify the concentration. The schematic in Figure 8-1 shows the detection methodology for quantifying specific HCV diagnostic antibodies present in serum. After serum is electrokinetically loaded onto the device where any antibodies are immobilized, spatial or multispectral measurements can be used to detect and confirm various biomarkers.

Figure 8-1. Schematic for rapid on-chip electrokinetically driven binding RIBA test. The microfluidic device functions by physically entrapping endogenous antibodies from patient serum within a microfluidic channel filled with a gradient pore-size PA sieving matrix. Chip geometry allows either spatial or spectral multiplexing.

Both linear and nonlinear gradient gels were photopatterned in-situ for various applications (figure 8-2). Linear PA gels were fabricated by loading a high precursor concentration (i.e., 20, 30, or 40%T) into the exit channel and a low precursor concentration (i.e. 3%T) into an inlet well and then allowing the solutions to diffuse to equilibrium (3 h minimum). Plastic pipette tips were used as fluid reservoirs to hold 20 µL of gel solution in required wells. Nonlinear gels were made in a similar fashion; however the loading well and channel were first filled with the low concentration precursor solution prior to addition of the high concentration precursor in the outlet well at which point diffusion was allowed to occur for a time shorter than necessary to reach concentration equilibrium (20 min to 1 h). To stop pressure driven flow due to inequality in precursor solution between the loading well and exit well, the loading well was typically partially polymerized (4 min, ~5 mW/cm²) with a UV lamp (UVP B100-AP, Upland, CA) using a transparency photomask (Fineline Imaging, Colorado Springs, CO). After diffusion for the required time to reach the desired gel gradient properties, the entire chip was exposed to UV lamp flood illumination (6 min, ~ 5 mW/cm²). All precursor gel solutions 30%T or less were mixed by diluting the total volume of a 30%T (w/v) acrylamide/bisacrylamide solution to the desired ratio and adding 0.2% (w/v) VA-086 photoinitiator. 40 %T solutions were mixed from 100% acrylamide solution diluted with water and mixed with lyophilized bisacrylamide to the desired concentration. Precursor solutions were sonicated and degassed 5 to 10 min prior to loading into channels for photo-polymerization.
Figure 8-2. Linear and non-linear gradient pore-size gel composition and assay operation. A) Linear gradient gels were fabricated by diffusing a high concentration precursor (40%T) at the channel outlet with a low concentration precursor solution at the inlet (3%T) until the solutions reached equilibrium, followed by photopolymerization. B) Nonlinear gradient gels were fabricated in the same way; however the high concentration precursor solution was added last and not allowed to diffuse a sufficient time to reach equilibrium. All assays were run by loading samples at the cathode (-) and electrokinetically driving them towards the anode (+), where various species immobilize upon reaching their molecular weight pore-limit within the gradient gel.

8.2.4 Pore-Limit Electrophoresis and Pore-Limit Isotachophoresis Operation

Two different protocols (2-step, 4-step) were used for pore-limit electrophoresis. To initiate pore-limit electrophoresis, the positive serum samples (pre-incubated with HCV antibody) or negative serum samples (no antibody) were loaded into the sample well (~ 5 µL). The outlet well was filled with 1× tris/glycine buffer. An electric field (~300 V/cm) was applied between the sample-inlet well (S) and the sample-waste exit well (SW). Serum proteins were loaded for between 5 and 7 minutes. The sample was then removed via pipette from (S) and replaced with 1× tris/glycine buffer which was loaded for 5 min to (SW) to stack the finite volume of loaded serum proteins in the channel at their pore-limits. For the third step, fluorescently labeled recombinant HCV antigens were loaded from (S) to (SW) by applying an electric field for 3 to 5
min. As the antigens reached any HCV antibodies, they bound, showing a signal at the antibody pore-limit in the gradient gel. For the last assay step, 1× tris/glycine buffer was again loaded from (S) to (SW) to remove any fluorescent signal from unbound antigen from the well. Since the smaller antigens had a smaller pore-limit, the majority of the unbound signal was concentrated into a sample band very near the channel exit and distinct from the Ab-Ag complex band (Figure 8-3). A simplified 2-step assay was also implemented for pore-limit electrophoresis. The first step required pre-incubation of positive or negative HCV serum with the labeled HCV antigens for a minimum of 5 min. The serum/antigen sample was then loaded from (S) to (SW) electrophoretically (300 V/cm) for up to 30 min. The sample was then removed and 1× tris/glycine buffer was loaded from (S) to (SW) to remove any free antigen and concentrate the Ab-Ag complex at the gel pore-limit. Data collection was performed at the end of the assay by imaging fluorescence over the entire length of the channel and generating a fluorescence profile plot.

Figure 8-3. Pore-limit electrophoresis. A) Negative control: fluorescently labeled ladder proteins separate into bands in a gradient gel. B) Positive control: addition of unlabeled antibody for the target protein (NS3) results in the formation of a large NS3-Ab complex band indicating the presence of Ab in the sample.

The pore-limit isotachophoresis assay consisted of a simple single step sample loading/separation assay. The positive or negative serum was pre-incubated with the HCV antigens and mixed into a buffer containing the trailing electrolyte (HEPES 5-20 mM). The chip was previously stored with the leading electrolyte (NaCl 10 mM) in both the sample and waste wells allowing it to diffuse into the entire channel (typically overnight after initial chip fabrication). To start the assay the leading electrolyte was removed from (S) and replaced with the sample/TE solution. An electric field was applied across (S) to (SW) of ~2000 V/cm. The current drop was observed and eventually after 10 to 15 min depletion of ions from the separation channel initiated the start of ITP and a stacked band of all serum proteins and dye reagents with mobilities between the TE and LE migrated along the channel at constant speed (Figure 8-4). During the migration process proteins that reached their molecular weight cutoff limit were immobilized in the gradient gel and removed from the ITP stack. The electric field was maintained for ~30 min which was the time required for the ITP stack to migrate across the entire channel. Data collection was performed at the end of the assay by imaging fluorescence over the entire length of the channel and generating a fluorescence profile plot.
8.3 Results and Discussion

8.3.1 Pore-limit Electrophoresis

Pore-limit electrophoresis experiments were performed to detect HCV antibodies directly from serum. Analysis of serum proteins provided many challenges including preventing non-specific blockage of the gradient gel by matrix proteins present in high concentrations. In particular, the large concentration of albumin proteins were shown to block smaller proteins from passing through the channel to their molecular weight cutoff as they stacked behind the high concentration of albumin proteins immobilized at the albumin pore-limit. Additionally albumin proteins presented other challenges including the ability to cause co-ion charge exclusion from concentration polarization of the region adjacent to their pore-limit, thus contributing another challenge to accurate protein sizing via PLE. To mitigate the issues of non-specific pore blockage, serum assays were performed with a 20× serum dilution (5% serum final concentration) with 1× tris/glycine buffer. This dilution factor was similar to that of the standard benchtop RIBA assay. Additionally, non-linear gradient gels were implemented to reduce the degree of preferential albumin loading compared to target antibody loading. Loading of proteins is proportional to their electrophoretic mobility. In larger pore size (lower %T) gels the difference between mobilities of large and small molecules is minimized. This was exploited in non-linear gels with large pore size regions over the majority of the channel so that the ratio of albumin (66 kDa) loading to antibody (150 kDa) loading was decreased.

Initial assays with the NS3 Ab (700nM) in a 3-40%T linear gradient gel are shown in Figure 8-5. Results indicated the ability to accurately detect binding in a model buffer system, however the signal was significantly reduced in 10% serum either as a result of reduced Ab-Ag binding or
reduced loading of Ab into the channel. Assays were also run to detect a clinically relevant concentration of NS3 Ab in serum (Figure 8-6). Results demonstrated that NS3-Ab complex was detectable at 35 pM (5 ng/ml), a detection limit 5× below that of the benchtop RIBA assay (Figure 8-7). The separation of a negative control revealed that there was significant cross-reactivity and binding of NS3 to non-target IgG which makes up upwards of 20% of serum protein. Additionally the free NS3 antigen separated into 2 bands. This was either caused by mass isoforms of complexed antigen or from NS3 bands passing through a co-ion exclusion zone caused by previous immobilization of unlabeled native serum albumin at the pore-limit.

![Figure 8-5. Detection of NS3 antibody in PLE gradient gel. The negative control in buffer shows a single band of NS3 antigen. The addition of unlabeled NS3 Ab to buffer results in NS3-Ab complex formation. Complex formation signal in 10% serum is significantly inhibited compared to buffer due to the presence of other non-specific matrix proteins (700 nM Ab, 0.1 μA separation).](image)

![Figure 8-6. Fluorescence profile of NS3 detection. Lower limit of detection for NS3 Ab (35 pM) is demonstrated in 5% serum. A baseline non-specific signal is present during PLE from interaction of NS3 antigen with non-specific serum IgG.](image)
Figure 8-7. Dose-response for NS3 Ab detection in 5% serum. 35 pM NS3 antibody is detectable with S/N > 3. The sensitivity of the on-chip method for NS3 detection is 5× greater than that of the benchtop RIBA assay.

Assays were also run to detect other RIBA antibodies including core and c100p. Core Ab was detectable at (28 nM) with both the core protein antigen and the core peptide antigen (Figure 8-8). The signal increased proportionally with total separation time as this allowed for increased mass of Ab-Ag complex to electrophorese to the pore-limit. Assays were also runged to detect c100p (2.9 nM) showing the capability for multiplexed detection of all antibodies present on the RIBA benchtop assay with a rapid on-chip PLE immunoassay.

Figure 8-8. Effect of separation time on PLE assays of core Ab detection. Increasing separation time for the final step 1× tris/glycine load resulted in increased signal as additional complex at the start of the channel is stacked at the pore-limit.
8.3.2 Pore-Limit Isotachophoresis

PLITP was demonstrated in a simple and rapid one-step assay to load, enrich, and detect HCV antibodies present in raw serum. A positive control was made by spiking HCV antibody (17 nM) into raw human serum. This positive sample was then diluted to 5% serum using 1× tris/glycine buffer for analysis with the PLITP chip. Results show that the PLITP detection results in a significant increase in peak capacity and decrease in band dispersion over the 2-step PLE assay (Figure 8-9). The sample is both loaded into the channel and traverses the channel as a result of ITP thus all proteins of a given mobility reach their pore-limit at the same time and are patterned into concentrated bands with bandwidths similar to their ITP stacked bandwidth. This differs significantly from PLE where the initial sample load is performed over several minutes causing some proteins to load for longer durations than others of the same species within a sample. This results in significant dispersion in PLE since the gradient gel molecular weight pore-limit is not absolute but a function of loading field strength and duration. Other sources of dispersion eliminated with PLITP include non-specific pore blockage and associated concentration polarization resulting from high concentrations of albumin protein present in serum typically present at more than 1000× the analyte of interest. The large albumin concentration can physically block pores and cause co-ion exclusion of the fluorescent detection antigen increasing dispersion of the detection bands. However, all of these sources of dispersion are eliminated with PLITP resulting in a high fidelity assay with increased sensitivity and peak capacity with decreased assay time (20 min) and complexity (1-step assay). Dose-response studies with PLITP also demonstrated the ability to detect clinically relevant HCV antibody concentrations (25 ng/ml) within 5% serum (Figure 8-10).

![Figure 8-9. Pore-limit isotachophoresis assay results compared to pore-limit electrophoresis. PLITP single-step assay shows significantly decreased dispersion and increased sensitivity compared to NS3 detection with PLE.](image-url)
Figure 8-10. NS3 calibration curve with PLITP one step assay. Clinical levels of HCV NS3 antibody (25 ng/ml) were detected with the 1-step PLITP assay in 20 min.

8.4 Summary

The on-chip electrokinetic assays presented in this chapter were used to identify the presence of various HCV antibodies in the human serum with a rapid and portable assay. Both PLE and newly introduced PLITP achieved clinical levels of detection (25 ng/ml, NS3) and multiplexed detection (core, NS3, c100p) in a fraction of the time (30 min) necessary for the current diffusion based confirmatory RIBA technology (7 hours). The presented technology has many advantages and potential uses including low cost and low power point-of-care testing of viral and bacterial infection from blood and proximal fluids (e.g. urine, saliva, sweat, cerebrospinal fluid, etc.). PLITP in particular was found to significantly decrease band dispersion for electrokinetically driven protein sizing given that proteins are exclusively loaded and migrated across a sizing membrane by non-disperse stacked isotachophoretic transport. The proof-of-concept with the PLITP and PLE on-chip serum assays points to the ability for their use in developing future multiplexed immunoassays for other infectious diseases of interest including Herpes Simplex Virus (HSV) and Human Immunodeficiency Virus (HIV) for use in traditional clinical laboratories or remote developing communities where traditional ELISA and RIBA assays are not available.
Table 8-1. Summary of on-chip HCV Assay Performance Metrics

<table>
<thead>
<tr>
<th>Assay Performance</th>
<th>HCV Antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NS3</td>
</tr>
<tr>
<td>Ab Capture in PLE Gel</td>
<td>✓</td>
</tr>
<tr>
<td>PLE assay Ab/Ag binding (in 5% serum)</td>
<td>✓</td>
</tr>
<tr>
<td>Clinically relevant sensitivity demonstrated</td>
<td>✓</td>
</tr>
<tr>
<td>RIBA LLOD demonstrated</td>
<td>35 pM</td>
</tr>
<tr>
<td>Multiplexed on-single chip</td>
<td>✓</td>
</tr>
<tr>
<td>Sample Volume &lt; 20 μL (RIBA)</td>
<td></td>
</tr>
<tr>
<td>Assay Speed &lt; 7 hr (RIBA)</td>
<td></td>
</tr>
</tbody>
</table>
9 Conclusion

In this dissertation, various high-throughput protein analysis platforms with integrated sample preparation were developed to address the identified technology gaps in high-throughput biomarker validation, clinical diagnostics, and point-of-care diagnostics. The goals of the technology were to automate and integrate protein sample preparation with electrokinetic separations, implement biomarker detection assays capable of processing raw biological fluids, and perform high-throughput protein assays useful for targeted disease diagnosis. A hallmark of microfluidic technology, which is a common underpinning through all of the assays developed within this dissertation, is the integration of multiple functions. In addition to integration and novel assay development, translation of technology from the laboratory to the clinic was a key feature of the dissertation work, thus all assays were developed with clinical applications and samples in mind along with their associated challenges. Application of novel functionalized, polyacrylamide gel in-situ fabrication techniques allowed for the successful development of a range of rapid, low-cost, and portable microfluidic technologies as summarized herein.

In chapters 2 and 3, the characteristics and detailed fabrication protocol for the polyacrylamide gel sieving matrix and sample preparation features were presented. The polymer synthesis parameters that affect the pore size of a bis/acrylamide size-exclusion membrane were modeled and simulated. The most important factor in determining pore size theoretically was found to be the monomer to cross-linker ratio. The membrane pore size in the biologically relevant protein range varied exponentially with cross-linker ratio suggesting the potential difficulty in fabricating pore sizes to filter similarly sized proteins and pointing to the need to experimentally characterize pore size cut-off limits for accurate assay development.

In chapter 4, a high throughput assay was developed useful for protein biomarker detection. The on-chip immunosubtraction protocol demonstrates accurate detection of target analyte via rapid PAGE to provide data on mobility and specificity of target molecules. LLLOD for determining specificity and mobility of proteins was 1.1 nM, with specificity-only based detection of excluded proteins at the filter interface reaching a limit of 193 pM. Optimization of antibody-antigen ratio and applied separation current resulted in the ability to immunosubtract up to 95% of target analyte while extraction of non-target proteins did not reach below 35% for single analyte detection in a model system. The on-chip immunosubtraction device was used for detection of S100B (65 nM) spiked into a pooled sample of human cerebrospinal fluid in under 2 minutes using 5 µl of sample, suggesting usefulness for assessing putative protein markers of traumatic brain injury. The immunosubtraction protocol was performed on-chip in an automated and programmable manner vital to facilitating integration of further sample processing steps with the detection assay.

In chapter 5, the versatility of the immunosubtraction assay was translated for point-of-care detection of low molecular weight compounds. Detection of folic acid, testosterone and THC were made in assays requiring less than 5 min per run on a portable on-chip format. The viability of novel folic acid small compound-large compound conjugates as well as Fab fragment immunocomplex sandwich binding antibodies provided by collaborators at VTT was also proven. These results point to the usefulness of low molecular weight compound detection via
electrophoretic separations previously not possible in the length scales of a microfluidic format. Thus the proof-of-concept technique has further implications for development of portable biosensors for prenatal healthcare (folic acid), anti-doping (testosterone), and drugs-of-abuse (THC).

In chapter 6, a 2nd generation immunosubtraction detection assay was integrated on-chip with automated sample preparation including native protein labeling, background signal reduction, protein enrichment, reagent mixing, and native PAGE protein analysis. The new integrated sample preparation and detection device was used for rapid PSA isoform detection (120 s) of four peaks indicative of mass isoforms (or abhorrent glycosylation) and useful for prostate cancer diagnostics. Model proteins systems as well as proximal fluids (CSF and nasal mucus) were also characterized showing the ability to enrich proteins linearly with loading time with an 18× increase achieved in just 2 min. The total integrated sample preparation and biomarker detection assay was run on-chip in 4-12 min, which was significantly faster than the benchtop technique which would require 5-10 h.

In chapter 7, immunosubtraction technology was further translated to the clinical laboratory setting for cerebrospinal fluid proteomics and diagnosis of cerebrospinal fluid rhinorrhea. CSF rhinorrhea, a serious condition typically requiring expensive or slow diagnostic techniques (MRI, CT scan, ELISA, endoscopy), was assessed on-chip via rapid protein biomarker based detection. The microfluidic immunosubtraction assay developed accepted raw biological fluids (CSF, mucus) quickly differentiated between CSF and nasal mucus based on detection of two putative biomarkers; TTR and PIP respectively. TTR increase (7% to 100%) and PIP decrease (100% to 0%) were shown to correlate to increased presence of CSF in nasal discharge. Results indicate the efficacy of the on-chip technique for CSF rhinorrhea confirmatory diagnosis and monitoring in clinical labs.

Finally, in chapter 8, novel detection assays were introduced that were useful for the detection of hepatitis C virus, requiring mitigation of interference from the numerous human serum matrix proteins. The on-chip electrokinetic assays (pore-limit electrophoresis and pore-limit isotachophoresis) presented in this chapter successfully identified the presence of various HCV antibodies in the human serum at clinical levels of detection (25 ng/ml, NS3), and multiplexing (core, NS3, c100p) was performed in a fraction of the time (30 min) necessary for the current diffusion based confirmatory RIBA technology (7 hours). PLITP required only manipulation of buffer conditions to implement sample preparation including enrichment and species-ordered loading, and the technique was found to also significantly decrease band dispersion for electrokinetically driven protein sizing. The proof-of-concept assay results encourage further development of point-of-care infectious disease diagnostics for additional diseases including herpes simplex virus and HIV.
10 References

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11 Appendices

A. Custom Chip Schematic

Four custom chip designs were fabricated at Caliper Life Sciences for the PLE assay using a standard well plate format (MCF 14 shown below). The first two designs (Y1 and Y4) use short straight channels to reduce channel resistances. The Y1 layout has 50 µm by 3 mm channels while the Y4 design has µm by 3 mm channels. The 3rd design allows a multiplexed assay in 2 long separation channels or in the 6 shorter branching channels (Y2). All channels in the Y2 layout are 50 µm wide. The Y2 layout is useful for running a single assay for spatially multiplexed detection of several HCV antibodies simultaneously because of the several wells for isolating samples from assay reagents. The 4th design allows for simultaneous acquisition of several replicate data points during the same assay from the parallel channels (Y3). All channels in the Y3 layout are 50 µm wide. All chips were etched 40 µm deep to increase sensitivity over the standard chip formats (typically 20 µm).
B. Pore-Limit Serum Assay Tips

Pore-limit electrophoresis assays using serum suffer from blockage of the channel during loading due to the high concentration of serum matrix proteins. As proteins in high abundance such as human serum albumin (~67% of serum protein) begin to reach their pore limit in the gradient gel, they immobilize causing a co-ion exclusion zone. This phenomenon, known as ‘concentration polarization’, was observed in several early PLE assays as shown in the image below. There is a noticeable dark area adjacent to the channel exit representing the ion exclusion zone where the fluorescently labeled NS3 proteins cannot load. As noted with increased load time from 0 to 30 min the ion exclusion zone continues to grow preventing the small NS3 antigens (30 kDa) from reaching their pore limit at the end of the channel. It was also noted in additional experiments with higher serum concentrations (50% and 100%) that the accumulation of the concentrated albumin proteins were capable of degrading the gel and causing bubbles likely due to the high accumulation of charge at the albumin pore-limit.

![Image showing concentration polarization](image)

The difficulties in loading of small antigens to their pore-limit for accurate sizing in the presence of confounding matrix proteins led to the development of the ordered loading provided by the pore-limit isotachophoresis approach (described in chapter 8). Additional methods for implementing the pore-limit isotachophoresis assays avoiding the confounding signal of the free antigen are shown in the schematics on the following page.
Selection of buffer systems can be optimized to establish ITP concentration zones that exclude free Ag and HSA from the channel (option 1) or prevent concentration of free antigen (option 2).

**Option #1**

Load sample with LE, Ag > LE > Ab/Ag > TE

Sample (Ag, HSA, Ag/Ab, etc.) + LE

Only Ab/Ag complex and slower proteins load via ITP

Ab/Ag complex at pore limit

**Option #2**

Load sample with TE, Ag > LE > Ab/Ag > TE

Sample (Ag, HSA, Ag/Ab, etc.) + TE

Only Ab/Ag complex concentrates via ITP

Faster proteins migrate quickly to well dispersing

Ab/Ag complex at pore limit
C. Chip Fabrication and Tips

During fabrication of PA gels using the standard protocol as described in chapter 3, it was found that occasionally gels began to polymerize quickly in ambient light due to the photoinitiator. This was observed particularly when working with high %T gels such as those used for the immunofilter (12%T) or sample preparation membrane (40%T). The best solution to prevent this undesired polymerization during the degassing and other steps was to use opaque 1.7ml tubes or keep clear tubes in opaque boxes during fabrication.

During fabrication, it was often desirable to begin the silanization process for multiple chips simultaneously in order to minimize time spent on the long silane incubation step. However, it was noted that if chips that were previously silanized were stored too long than the functionalization of the channel walls degraded resulting in bubbles or movement of the gel within channels upon use. The best solution for long-term silanized chip storage was found to be use of a desiccator under vacuum to prevent oxygen contact with the silanized chips. Chips were also stored within an opaque box in the desiccator to prevent exposure to ambient light which also appeared to contribute to degradation of the silane channel coating.