

**DEVELOPMENT OF MICROSCALE SEPARATION AND BLOTTING METHODS
FOR ANALYSIS OF SMALL-VOLUME BIOLOGICAL MIXTURES**

by

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ABSTRACT

DEVELOPMENT OF MICROSCALE SEPARATION AND BLOTTING METHODS FOR ANALYSIS OF SMALL-VOLUME BIOLOGICAL MIXTURES

by

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A microscale Western blotting system based on separating sodium-dodecyl sulfate protein complexes by capillary gel electrophoresis followed by deposition onto a blotting membrane for immunoassay is described. In the system, the separation capillary is grounded through a sheath capillary to a mobile X-Y translation stage, which moves a blotting membrane past the capillary outlet for protein deposition. The blotting membrane is moistened with a methanol and buffer mixture to facilitate protein adsorption. In the first instrument discrete protein zones could be detected, but bands were broadened by about 1.7-fold by transfer to membrane. A complete Western blot for lysozyme was completed in about one hour with 50 pg mass detection limit from low microgram per milliliter samples. To increase throughput, a multiplexed CE-Western apparatus was constructed. To improve stability and allow for speedier separation, formulations for dextran sieving media were tested. Reduced band broadening and separation of 100 kDa proteins in 20 min was achieved. Up to four capillaries were operated in parallel, separating and transferring cell lysate proteins to a single blot for immunoassay for total analysis in 1 hour. Each capillary performed multiple injections before capillary regeneration,

further increasing throughput. Sub-microgram/mL levels of actin were easily detected in cell lysate samples. These results demonstrate substantial reduction in time requirements and improvement in mass sensitivity compared to conventional Western blots. Dot blotting using capillaries for deposition is also reported. By delivering nanoliter-microliter volumes to a narrow spot, sensitivity is enhanced. For a dot 1000x larger in volume, a 7x larger diameter dot was observed, representing orders of magnitude in concentrating effects for larger volume accumulations. Western blotting using capillary electrophoresis and capillary dot blotting show promise to analyze low volume samples with reduced reagents and time. These methods retain the information content of a typical immunoblot and extend analytical capabilities for both Western and dot blotting.

CHAPTER 1

INTRODUCTION

Separations methods coupled with affinity interactions are important techniques for life science research and biotechnology. Western blotting, affinity chromatography, Southern blotting, and gel-mobility shift assays are examples of widely used techniques that combine separation methodology with selective binding to improve target identification and selectivity. Of the techniques that combine separations and affinity interactions, Western blotting is among the most widely used. The method is routinely implemented for basic research and as a confirmatory test for clinical assays and regulatory tests.

Capillary gel electrophoresis (CGE) is a powerful separations technique which allows for rapid separations with small sample requirements, typically nanoliters or less. When capillary gel electrophoresis is employed to separate proteins, time of analysis decreases relative to slab gel electrophoresis. The use of CGE and membrane capture of separated proteins provides a microscale Western blotting method that combines the utility and flexibility of traditional Western blotting with increased throughput and low sample volumes of CGE. V

In scenarios where a separation is not required, the advantages of microscale accumulations of complex mixtures for immunoanalysis are also demonstrated. It is hoped that the microscale Western blotting and dot blotting presented will be used in other laboratories to reduce the time and costs associated with traditional Western blotting or protein arrays.

Western Blotting Background

Western blotting is a pervasive biochemical technique used to identify specific proteins from a complex mixture. The use of the term “Western blotting” to describe this protein separation and identification method is a play on words related to an analogous technique, Southern blotting. Southern blotting, the separation and probe of DNA sequences, was named for its inventor Edwin Southern in 1975. When RNA separation and identification was published in 1977 it was named Northern blotting. To complete the cardinal directions, an affinity probe of protein post-translational modifications is termed Eastern blotting.

Western blotting is highly adaptable to a wide range of biochemical problems and protocols, so what follows are some typical, but not exclusive, descriptions. The first step is sample preparation. Cellular or tissue samples are homogenized and lysed with ice-cold lysis buffer. This buffer contains harsh detergents to lyse cell walls, as well as protease inhibitors to inhibit protein degradation. Lysis buffer choice is informed by the particular experimental aims. For instance, Radio Immuno Precipitation Assay (RIPA) buffer is appropriate for whole cell extraction, because it contains ionic detergents that more readily solubilize membrane-bound and nuclear proteins. In other experiments, buffers designed to liberate proteins of a particular sub-cellular fraction are helpful. Proteins released from lysed cells or tissue samples are quantified by a colorimetric (Bradford or bicinchonic acid) assay.

Typically, desired total protein concentration for analysis is 1-5 mg/mL, of which target protein would be a small percentage. The protein sample, in buffer with an excess of sodium dodecyl sulfate (SDS) and beta-mercapto ethanol (BME), is then heated to denature the protein,

reduce disulfide bonds, and surround the extended protein structure with dominating negative charge from the sulfate ions. It has been shown the ratio of SDS-to-protein for most globular proteins is constant at 1.4 mg SDS/1 mg protein.¹ The accepted model is a flexible structure where sulfate ions are dispersed all along the polypeptide chain. The hydrophobic alkyl chains of SDS molecules align to hydrophobic regions of the protein and then charge repulsion from the sulfate groups favors an extended, unfolded structure.² Because of this uniform charge extending the length of the polypeptide chain the charge of the protein is overwhelmingly due to the SDS. The variable from one SDS-protein complex to another no longer relates to particular amino acid sequences, but rather the length of the polypeptide chain, i.e. the size of the protein.

With the application of an electric field, SDS-protein complexes migrate through a cross-linked gel in a size-dependent manner, in a technique known as SDS-polyacrylamide gel electrophoresis (SDS-PAGE).³ The logarithm of the protein molecular weight is proportional to the SDS-protein complex mobility. As the name suggests, the cross-linked separation matrix (“PAGE gel” or “slab gel”) typically includes a linear acrylamide monomer as well as varied amounts of a crosslinker, bis acrylamide. While polyacrylamide is the most prevalent matrix, other gels have been reported and are of historical interest, in particular agarose gel.⁴

Following separation, proteins are transferred from the slab gel to a blotting membrane to preserve the separation and allow for immunological characterization. Transfer of proteins to the membranes in their relative quantities can be problematic. Additionally transferring a wide molecular weight range with one set of conditions is difficult.^{5,6} Transfer can be achieved via diffusion (also termed gravity), vacuum or application of an electric field parallel to the direction of separation. Since it was first introduced by Towbin *et al.* in 1979, electro-blotting has proven popular.⁵ Variations of this method, such as wet transfer (membrane submersion in a buffer

tank) and semi-dry transfer (membrane pressed between wetted filter papers) have found use for extended and fast transfer, respectively. The substrate that binds eluting proteins, the blotting membrane, is typically either nitrocellulose or polyvinylidene fluoride (PVDF). Compared to nitrocellulose, PVDF is preferred for most SDS-PAGE applications because it is mechanically strong, binds proteins even in the presence of SDS, and has good protein binding capacity, 150-300 $\mu\text{g}/\text{cm}^2$.

After preparation, separation and transfer, the membrane blot is exposed to antibodies in an immunoassay to probe for the presence of a specific protein target. Primary and secondary antibody wash concentrations are prepared and optimized by varying parameters with controls spotted on a membrane (dot blot). The membrane is subjected to blocking, primary antibody and secondary antibody washes, with rinse steps between stages. The blocking protein solution binds to available sites on the membrane surface to reduce non-specific binding of the antibodies. The primary antibody binds protein target. A secondary antibody binds the primary antibody, and serves to label the target with a tag for detection. This tag may be a radiolabel, a fluorophore, or an enzyme that is used to generate a colorimetric, fluorescent or chemiluminescent response. Fluorescence and chemiluminescence are favored for the lowest limits of detection.

Among the many choices for immunoassays is whether to use monoclonal or polyclonal antibodies. Monoclonal antibodies recognize one epitope from one antigen, and are produced in immortalized cell lines for extremely high batch-to-batch consistency. Polyclonal antibodies are a heterogeneous mixture of antibodies that may each respond with varying affinity to different epitopes on an antigen (or may cause some undesirable cross-reactivity). While monoclonal antibodies are highly specific and reproducible they are also expensive. Polyclonal antibodies are often of greater utility for denatured proteins as the chance that one (or many) antibodies will

still recognize a target is increased. Another advantage of polyclonal antibodies is that when multiple antibodies bind a single protein this results in signal amplification for increased sensitivity.

The region of the target protein that is recognized by the antibody is called an epitope. Epitopes may be either conformational or linear epitopes, which are comprised of discontinuous or continuous amino acid sequences respectively. Conformational epitopes rely on tertiary interactions that create a three-dimensional structure specifically recognized by the antibody. Antibodies can also recognize linear epitopes that are based on the primary structure, i.e. fewer than ten amino acid residues or monosaccharides. Linear epitopes are more easily characterized as these can be probed in a variety of denaturing conditions and by isolating the minimum peptide sequence needed for antibody recognition.⁷ SDS is necessary for separation in PAGE gel and migration of proteins from PAGE gel to PVDF membranes, but the surfactant is then diluted by incubation in blocking buffer and repeated rinse steps. In the end antibodies bind a linear epitope on the protein target in the absence of SDS, which might otherwise hinder recognition.

Western blotting is a workhorse method in basic biochemical research to confirm the identity of a protein in a complex mixture, and gather quantitative information. Measurement of changes in relative protein amounts, after treatment of one or more groups, is a common analysis. Western blotting is also used to confirm the operation of a transfected gene, with immunoassay for the downstream protein product in a host animal. Additionally, Western blotting has some important clinical uses as a definitive test for diagnoses of some prion diseases, as well as HIV and Lyme disease.⁸ Western blotting is not well suited to large-scale surveys of many experimental conditions or screens of protein activity with a library of

substrates. The lack of automation (manual manipulation of gel and membrane, incubation rinses), time constraints (several hours), and significant sample requirements (several μL) prohibit those types of experiments. The large volume requirements also limit application of Western blotting to study single cell or micro-organ environments. Better analytical tools to study small volumes may be achieved by scaling down the instrumentation to match the samples of biochemical interest. One such microscale technology is capillary electrophoresis, where the dimensions of the capillary require less sample and make possible higher mass sensitivity. Capillary electrophoresis is also fast and may be multiplexed, so that throughput can be increased. With increased sensitivity and speed Western blotting may become even more prevalent, and be used in wider avenues of research.

Principles of Capillary Electrophoresis and Capillary Gel Electrophoresis

Capillary electrophoresis has been in research use as a high speed, high resolution separation method for approximately 30 years. Separation of proteins with the use of an electric field has been of interest for over a century. At the turn of the 20th century, Hardy established that proteins displayed distinctive electrophoretic mobilities. In 1909 Michaelis was able to manipulate proteins in an electric field based on their isoelectric points and coined the term “electrophoresis”. The father of electrophoresis, Arne Tiselius, was the first to separate proteins (several classes of globulins from albumin and antibodies) in blood.⁹ He also advanced several facets of electrophoresis with the introduction of cooling to reduce distortion created by convection currents, an optical detection method based on refraction, and use of opposing flow at the separation outlet to increase separation time and improve resolution. His student, Hjertén, was the first to separate proteins in a narrow glass tube into discrete zones for absorbance detection, which he termed “free zone electrophoresis”.¹⁰ The potential for extremely efficient

capillary electrophoresis separations was demonstrated by Jorgenson and Lukacs in the early 1980s.¹¹ Over time, an array of electrophoresis-based separation techniques have been developed, all with the parent term “capillary electrophoresis.” These include capillary zone or free solution electrophoresis (CZE), capillary electro-chromatography (CEC), micellar electrokinetic capillary chromatography (MEKC), capillary isoelectric focusing (C-IEF), and capillary gel electrophoresis (CGE). The separation in all of these modes is confined in a capillary that has an inner diameter (i.d.) ranging from 5 to 200 μm and a length from 5 to 100 cm.

Free solution capillary zone electrophoresis is the simplest format of electrophoretic separation in capillaries. In CZE bands of analytes migrate in the background electrolyte (BGE) solution when an electric field is applied across the capillary. The speed and direction of analyte migration are governed by two phenomena: electrophoresis and electroosmotic flow (EOF). Electrophoresis is the migration of charged particles experiencing an electric field. Its speed is measured by electrophoretic mobility (μ). Electrophoretic mobility is an intrinsic property of the analyte and is described by:

$$\mu = \frac{q}{6\pi\eta r} \quad (1.1)$$

where q is the charge of the particle, η is the viscosity of the electrophoresis buffer, and r is the Stoke’s radius of the particle. The charge-to-size ratio of the analyte determines its electrophoretic mobility. The second contribution to analyte velocity is electroosmotic flow, which directly results from ionized silanol groups on the inner capillary wall. The charged silanol groups cause a net positive charge for the bulk electrophoresis buffer and under an electric field the bulk solution migrates toward the cathode. The electroosmotic mobility (μ_{eo}) is defined as:

$$\mu_{eo} = \frac{\varepsilon \zeta}{\eta} \quad (1.2)$$

where ε is the permittivity, ζ is the potential in the Helmholtz plane, and η is the viscosity of the solution. EOF is directly dependent on the ζ potential, which in turn is determined by the ionic properties at the capillary/buffer interface. This holds in any capillary with charged walls, even if the material is not fused silica (and the resultant silanol surface moieties). The overall migration velocity (v) of the analyte is then proportional to the electric field strength (E) and given by:

$$v = (\mu_{eo} + \mu)E \quad (1.3)$$

Since the thickness of the diffusion layer at the capillary/buffer interface is negligible compared to the capillary i.d., there is very little distortion in the flow of analyte across a cross-section of the capillary. EOF is said to have a flat-fronted or plug-shaped velocity profile, compared to the parabolic profile of a pressure-driven laminar flow. This plug-shaped profile results in much less band broadening in CE compared to techniques such as HPLC. In the ideal situation, when factors such as Joule heating, injected sample plug width and adsorption are controlled or inconsequential, diffusion is the only dispersive phenomenon contributing to the peak broadening, quantified as:

$$\sigma^2 = \frac{2D_i L_d}{(\mu_{eo} + \mu)E} \quad (1.4)$$

where σ^2 is the variance of the zone. The equation reinforces some intuitive conclusions: high diffusion coefficients (large D_i), long migration length (large L_d) and slow migration velocity (a small denominator) result in wider peaks.

To measure the merits of a separation there are several key values and terms in common usage. N , the number of theoretical plates, is a historical term first developed to describe

distillation columns. A large value for N is optimal. N can be calculated with these equations:

$$N = \frac{L^2}{\sigma^2} = \frac{\mu_{ave} V}{2D_i} \quad (1.5)$$

where μ_{ave} is the average mobility of the analytes and L is the migration length. Since N is proportional to the voltage applied, the highest voltage (V) that can be applied without causing significant Joule heating or arcing is always desirable for achieving efficient electrophoretic separation. For CE, 10^5 - 10^6 of theoretical plates are possible. Other useful metrics to evaluate electrophoretic separation include resolution (R_s) and peak capacity (n_c). R_s is a numerical index assigned to neighboring peaks to assess the ability to discriminate between them:

$$R_s \equiv \frac{\Delta x}{(4\sigma)_{avg}} \quad (1.6)$$

where Δx is the distance between the peaks in time units, and $(4\sigma)_{avg}$ is the average width of the peaks in time units. Peak capacity (n_c) is the number of peaks it is theoretically possible to resolve in the course of the separation:

$$n_c = \frac{L}{4\sigma R_s} \quad (1.7)$$

where L is the time frame in which peaks could appear, in time units.

Parallel to the development of free zone electrophoresis was the development of gel electrophoresis. In the mid-20th century sucrose was used as an electrophoretic medium to separate proteins (analogous to centrifugal separations with sucrose gradients still in use today). In 1955, Smithies introduced starch as a gel electrophoresis medium.¹² In 1959, Ornstein and Davis dramatically changed the field with the introduction of controlled pore sizes in polyacrylamide gel and discontinuous buffer systems.¹³ Gel electrophoresis was finally transferred to a microscale format with the first capillary gel electrophoresis paper by Karger and

Cohen, in 1987.¹⁴ In the 1990s, buffered entangled polymers or “sieving gel media” replaced cross-linked gels in capillary gel separations.^{15, 16} This allowed for stable, reproducible operation and easier automation.

Capillary Gel Electrophoresis (CGE) is governed by all the same principles and equations as other forms of capillary electrophoresis. As the name suggests, it is distinct from CZE because instead of background electrolyte, buffered entangled polymers fill the capillary. This allows for size-based sieving just as in a polyacrylamide slab gel. Another feature of CGE is that charge on the capillary walls is often chemically modified to be extremely low so that analyte velocity, which in CZE would be controlled by both electrophoretic mobility and electroosmotic mobility (equation 1.3), is now dominated by electrophoresis. The strategies for and ramifications of this are discussed in Chapter 3.

The pore regions in a polyacrylamide or agarose slab gel can effectively prevent loss of resolution due to convective distortion of the analyte bands. Likewise, the microscale separation space in a capillary also prevents convective distortion. The capillary format offers distinct advantages over traditional gels in that its high surface area-to-volume ratio enables efficient dissipation of Joule heat. Capillaries allow an electric field of up to 10^3 volts per centimeter to be applied (10^2 V/cm for CGE), compared to 15-40 V/cm for gel electrophoresis.¹⁷ As seen in equation 1.5, separation efficiency in electrophoresis, expressed as the number of theoretical plates, scales with voltage, and so CGE in theory is able to improve both separation speed and resolution relative to slab gels.

Capillary Gel Electrophoresis of Proteins

CGE first came to prominence as a valuable tool in accelerating progress of gene sequencing in the Human Genome project and subsequent genome sequencing efforts. In DNA

sequencing, use of primers and chain terminators result in samples with DNA sequences of every length. Size-based separation can then inform on the order of bases in the sequence. Several characteristics of CGE made it attractive first for DNA and subsequently for other classes of analytes, such as proteins. Capillaries can be easily regenerated with rinsing between runs. Automation for increased throughput is possible because no manual input is required to refill capillaries or make injections. Capillaries are also flexible and easily directed to sample wells for automated injections. Lastly, the need for high throughput gene sequencers in the 1990s drove the development of capillary arrays, with up to hundreds of capillaries bundled together and operated simultaneously.^{18, 19}

Capillary gel electrophoresis, as reviewed in Chapter 3, has advanced so that there are that there are now many reported entangled polymer solutions for sieving separation of proteins. Many water soluble polymers have been assessed for use as sieving media, with linear polyacrylamide providing very good resolution but long analysis times (min-hours). Applied fields are about 200-300 V/cm for capillaries between 10 and 50 cm.^{20, 21, 22} Times of analysis and automation are strengths of the method, though operation in series (rather than parallel gel lanes) and reproducibility are challenges that need further innovation.²³ Figure 1.1 shows two CGE separations published in recent years, representing efforts towards very high resolution (~150,000 theoretical plates for 1.1-A) and sensitivity (50-300 nM LODs for ladder proteins 1.1-B). Both of these represent ladder separations of proteins between 15 and 100 kDa. A recent review²³ is a good summary of reported formulations.

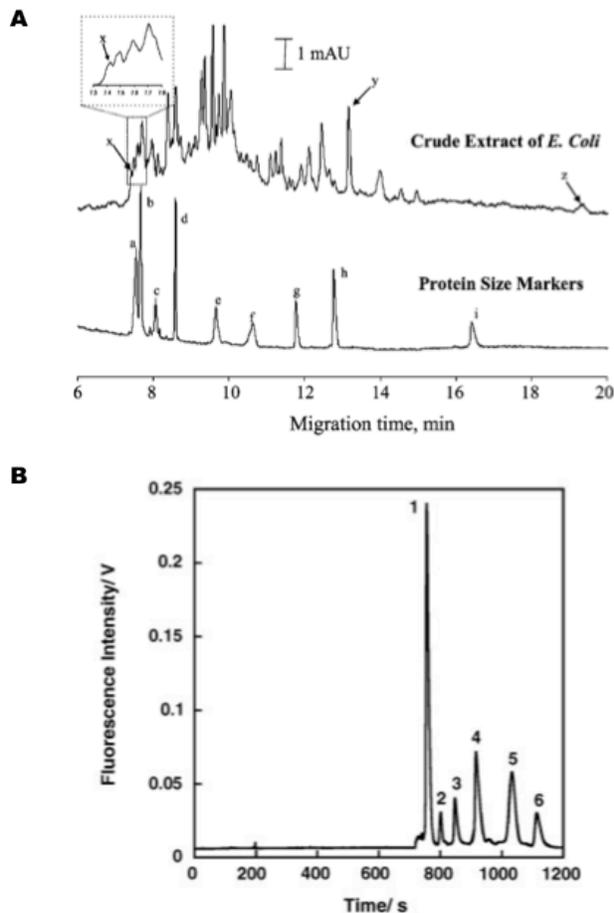


Figure 1.1. Examples of recent CGE separations in capillaries. Capillaries were 75 μm i.d., 30 cm effective length in both cases; crosslinked polyacrylamide and a static coating operated at 290 V/cm (A) and 2% polyethylene oxide 250 V/cm (B). Reproduced with permission from Lu, J. J.; Liu, S. R.; Pu, Q. S., *Journal of Proteome Research* **2005**, *4* (3), 1012-1016 and Kaneta, T.; Yamamoto, D.; Imasaka, T., *Electrophoresis* **2009**, *30* (21), 3780-3785. Copyright American Chemical Society.

Entangled polymer solutions have been discussed extensively in the literature, with respect to effect pore (or “mesh”) size and viscosity.^{24 25} Glycerol has been mentioned as an additive that can change pore sizes, though to date this has only been described in one paper that addresses DNA separations in a cellulose sieving gel.²⁶ Subsequent work does incorporate glycerol to increase retention or improve sample loading, without mention to changes in sieving gel structure.²⁷ A picture of the proposed interactions between glycerol, borate buffer ion, and cellulose polymer is shown in Figure 1.2. This paper is a key inspiration for the work presented

in Chapter 3, where a sieving formulation that is relatively high speed and low viscosity is sought and formulated using glycerol as a sieving buffer additive.

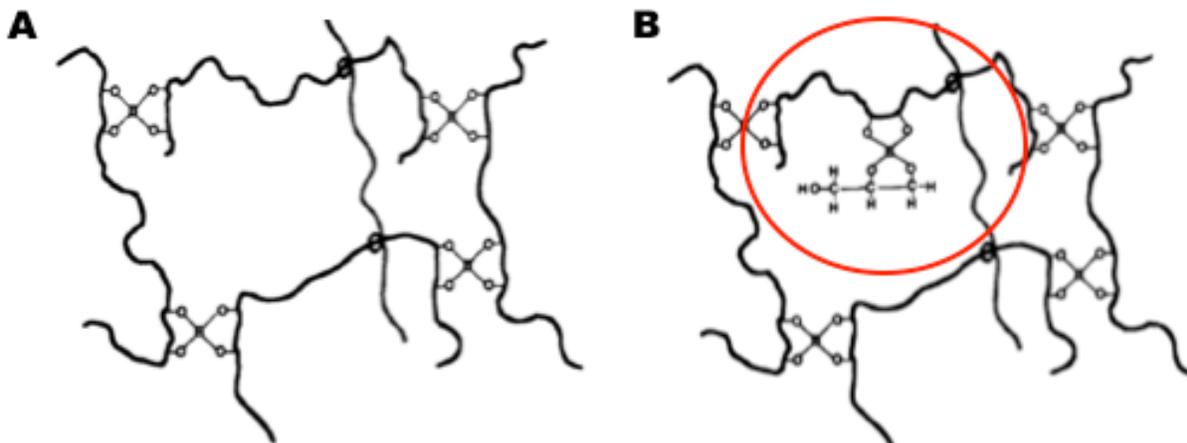


Figure 1.2. Glycerol effects on pore structures for sieving matrices in CGE. (Hydroxypropyl) methyl cellulose entangled structure without (A) and with (B) glycerol as a media additive. Figure reproduced with permission from Cheng, J.; Mitchelson, K. R., *Analytical Chemistry* **1994**, *66* (23), 4210-4214. Copyright 1994 American Chemical Society.

Capillary electrophoresis also continues to advance with the use of microfluidic devices. Microfluidics is the patterning of small features on glass, silicon or polymer substrates, often used for microscale fluid handling. This allows for a reduced footprint, integration of multiple functions (injection, mixing, separation, etc.) and fast operation in CE experiments. Of particular interest to this work is on-chip capillary gel electrophoresis. Protein sizing using microfluidic devices has been demonstrated in both one and two dimensions. High speed separations are possible, such as in a highly influential paper by Bousse *et al.* that first demonstrated the potential for high speed (~1 min) protein sizing on chip (Figure 1.3).²⁸ Since that time many gel separations on chip have been reported including two-dimensional electrophoresis, typically with isoelectric focusing in the 2nd dimension.^{29,30}

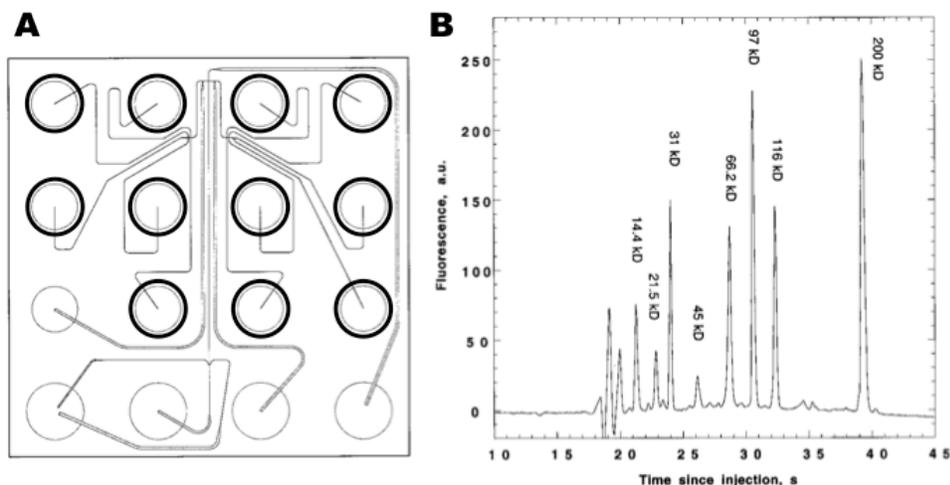


Figure 1.3. Microfluidic chip schematic for high speed CGE. (A) An electropherogram and schematic (B) of a protein sizing CGE chip. 11 sample chambers, which were injected sequentially, are highlighted in black. With a separation channel length of 1.25 cm and an applied field of 274 V/cm, separation of a protein ladder with proteins up to 200 kDa was achieved in about 40 s. Reproduced with permission from Bousse, L.; Mouradian, S.; Minalla, A.; Yee, H.; Williams, K.; Dubrow, R., *Analytical Chemistry* **2001**, 73 (6), 1207-1212. Copyright 2001, American Chemical Society.

Affinity Techniques in Capillary Electrophoresis

In addition to Western blotting, there are several schemes in which affinity interactions add resolution or chemical information to electrophoretic separation. Some affinity-CE techniques include capture agents such as antibodies and aptamers³¹, as well as study of chiral mixtures, protein conformations and protein-drug interactions.³² Antibodies are used in preparative affinity-CE to separate particular species from bulk fluids. They have also been used on-chip for competitive immunoassay, in which a labeled sample is not necessary to quantify analyte with fluorescence detection.^{33 34} Finally, CE has been used to characterize antibody performance to tailor antibodies for specific analytical challenges.³⁵

Commercial Products related to Western Blotting

Scientific advances in aspects of Western blotting will be noted in the text as applicable, but it is worth noting that due to its role as a workhorse technique in biochemical and clinical

applications many commercial products have been developed to address one or more of the stages of Western blotting: separation, transfer to membrane, and immunoassay. This is an ever-growing field, especially during the time frame of this dissertation, and so what follows is a partial list.

Agilent acquired Caliper Technologies, which developed a high-speed microfluidic gel electrophoresis protein separation platform (Agilent 2100 Bioanalyzer). The instrument automates conditioning, sample loading, separation and detection. There is no immunoaffinity component to this instrument.

Available technology for electro-transfer has not advanced beyond optimizing buffers and minimizing consumables. The most promising transfer module among many is from Thermo Scientific Pierce, which sells a semi-dry electro-blotting module that reduces transfer time after assembly to about 10 minutes. This still requires several gel/buffer equilibration steps, adding another 20-30 minutes.

For immunoassay, Millipore has commercialized a module called SNAP i.d. that decreases time and labor using principles of vacuum filtration. Blocking buffer, antibody and rinse buffers are pulled through the PVDF membrane with either house vacuum or a vacuum pump. Using vacuum instead of incubating the membrane in antibody solutions (which relies on diffusion) allows for a much more rapid assay, about 30 min.

While the scientific and commercial product advances mentioned in this review have contributed to steady improvement of Western blotting, there is still a need for faster multiplexed Westerns with higher mass sensitivity. If Western blotting were amenable to sensitive, high throughput analyses new types of scientific questions could be asked and answered. While capillary electrophoresis has found application in many bioanalytical laboratories (particularly

for genome sequencing), we surmise that capillary electrophoresis has not replaced PAGE separations because, even with dramatically improved separation performance, until now there was not a blotting component. With introduction of CE-Western blotting we see a broadening role for CE in protein biochemistry laboratories, where faster operation and smaller sample volume requirements may improve throughput and ultimately improve biological understanding.

Specific Aims Summary

In Chapter Two an instrument designed to reduce time and improve Western blotting performance is presented. This work utilizes the separations power of capillary electrophoresis combined with standard immunoassay protocols to work towards a faster Western blot without any loss in versatility. A separate transfer step to deliver proteins to the substrate is not required, which further streamlines the method.

In Chapter Three, many improvements are made to CE-Western instrument. These included implementation of a sieving media better suited to this particular analytical challenge, refined sample preparation protocols and investigation of online preconcentration. Simultaneous separations in parallel capillaries are performed, and there are demonstrations of these improvements with analysis of a complex mixture, i.e. cell lysate.

Chapter Four represents somewhat of a departure in that there is not a separation component, rather microscale dot blotting is investigated. Many protein-membrane interactions observed in CE-Western prove helpful in development of these low volume, cost-effective multiplexed protein arrays.

Chapter Five reiterates the advancements made in CE-Western blotting and microscale immunoblotting, and includes commentary on how these topics could be furthered in future work.

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CHAPTER 2

WESTERN BLOTTING USING CAPILLARY ELECTROPHORESIS

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INTRODUCTION

In a Western blot, proteins are separated by size and then immunoassayed to detect target protein. The technique is powerful because it provides selective protein detection based on size and antibody binding in a semi-quantitative assay. The method is also characterized by simplicity, reliability, and facile methods development.

Despite the great utility of Western blots, they have several well known limitations. Western blots are manually intensive and time consuming, generally requiring 4 to 24 h when taking into account gel preparation, separation, electro-blotting, and multiple incubations. Sensitivity is typically in the nanogram range making them incompatible with sample limited analysis. Analysis of large proteins is hindered by difficulty of transferring them from slab gels.¹ Finally, the method has not been miniaturized, which wastes materials and reduces sensitivity.

Recently, efforts to reduce time and reagent requirements and introduce automation for Western blotting have been reported. A semi-automatic platform has been developed that uses vacuum filtration to pull blocking, antibody and reagent solutions through the membrane to decrease time required for the immunoassay procedure.² Microfluidics technology has been used to create flow channels for applying blocking and antibody solutions over the surface of the blot,

which is advantageous in reducing reagent consumption and optimizing conditions.¹⁰ These advances improve the immunoassay, but do not address other aspects of the Western such as separation and blotting. A more comprehensive approach is integration of separation with faster blotting in a microfluidic format.^{3,4} In this method, antibodies pre-immobilized in the chip selectively capture protein targets from a high speed gel separation. In one example, 500 nM free prostate specific antigen was separated, transferred and blotted in < 5 min with a signal-to-noise ratio of 40. This promising technique drastically reduces timescales and offers potential for automation; but the requirement for microfabrication, pre-labeling analyte proteins with fluorophore, and pre-loading of antibody presents barriers for some applications. Further development is expected to lower these barriers.

Interestingly, Western blots based on capillary electrophoresis (CE) have not been reported even though a variety of affinity-CE techniques have been developed as described in Chapter One. The closest approximation of a CE-Western blot that has been reported uses capillary isoelectric focusing to separate proteins which are then immobilized by photoactivated cross-linking to the capillary surface.⁵ The captured proteins are detected in the capillary by immunoassays performed by flushing antibodies and reagents through the capillary. The method provides excellent sensitivity and separation; but, the instrument used is expensive and the method does not provide the size-based separation of a classical Western blot.

Although a CE-based Western blot has not been described, several relevant technologies have been reported. For example, methods for capturing proteins separated by CE onto a membrane have been described.⁶ Most of this work was directed towards off-column mass spectrometry detection, although one study did use antibody detection.⁷ None of this prior work on membrane capture of proteins separated by CE used size-based separations; therefore, it did

not provide the platform needed for Western blotting. Of course, size-based separation of proteins in capillary and chip-based gel electrophoresis formats has been demonstrated.^{8,9} These studies show that smaller channel dimensions reduces sample volumes and enables higher electric fields (typically up to about 300 V/cm) relative to slab gels.

In this work, we describe a capillary gel electrophoresis (CGE)-Western blot that builds on these previous developments. A gel-filled capillary is interfaced with a blotting membrane, which captures protein as it migrates from the capillary and eliminates the need for a separate electro-blotting step. The use of CE leads to better mass sensitivity as well as faster separation when compared to slab gel Western blots. CE also allows use of entangled polymer solutions, instead of cross-linked gels. Entangled polymer solutions facilitate automation and reduce time for gel preparation because capillaries can be emptied and refilled by pumping as necessary to maintain consistent separation performance. This is unlike cross-linked gels which must be formed within the capillary.¹⁰ Faster separation, the elimination of an electro-blotting step, and recent improvement in commercial immunoassay instrumentation combine for a reduction in total Western blot analysis time to under two hours. The technique is promising for improved throughput, automation, and mass sensitivity while retaining the information content and ease of use of a traditional Western blot.

EXPERIMENTAL METHODS

Materials and Reagents. Fluorescein isothiocyanate (FITC) and FITC-labeled insulin were purchased from Invitrogen (Carlsbad, CA). ECL Plus chemifluorescence kit and polyvinylidene fluoride (PVDF) membranes were purchased from GE Healthcare (Piscataway, NJ) and GE Osmonics (Minnetonka, MN) respectively. Rabbit anti-lysozyme was purchased from Millipore (Bedford, MA) and rabbit anti-carbonic anhydrase from Genway Biotech (San

Diego, CA). FITC-labeled bovine serum albumin (BSA), and the secondary antibody, horseradish peroxidase-conjugated anti-rabbit IgG produced in goat, were purchased from Sigma (St. Louis, MO). Other unlabeled proteins and all other chemicals were purchased from Sigma. Fused silica capillaries were from Polymicro (Phoenix, AZ). All solutions were made using Milli-Q (Millipore) 18 M Ω deionized water. Phosphate buffer solution (PBS) was 100 mM Na₂HPO₄ adjusted to pH 7.5 with 100 mM NaH₂PO₄. Electrophoresis buffer for free solution CE was 100 mM Tris adjusted to pH 8.8 with HCl. Sieving media was a proprietary solution of entangled polymers designed for resolution of proteins from 10 kDa to 225 kDa (part number 390953 from Beckman-Coulter, Brea, CA). This media had a kinematic viscosity of 78 cP at 40 °C as measured using a glass semi-micro viscometer (Cannon Instrument, State College, PA).

Sample Preparation. Proteins were denatured by heating at 70 °C for 5 min in denaturation buffer consisting of PBS, 3% sodium dodecyl sulfate (SDS) and 5% β -mercaptoethanol (BME). In some instances, protein samples were then dialyzed against PBS using mini-dialysis cups (Pierce Biotechnology, Rockford, IL). Protein samples for injection were diluted from these sample stocks with 25 mM Tris adjusted to pH 8.8 with HCl. In some experiments, proteins were first labeled with FITC, then denatured and dialyzed. FITC prepared at 1 mg/mL in dimethyl sulfoxide was diluted to a final concentration of 100 μ g/mL and incubated for 1 h with protein at 1-5 mg/mL in PBS for labeling.

Apparatus and Procedure for CE-Western Blot. A diagram of the apparatus used to couple the separation capillary to the blotting membrane is shown in Figure 2.1. High voltage was applied at the separation capillary inlet reservoir and grounded at the stage. The separation capillary outlet was surrounded by a gel-filled sheath capillary that made contact with a PVDF membrane secured to the X-Y translational stage. During injection and separation the sheath

capillary, fixed at an angle of approximately 45°, was positioned to make light contact with the membrane (determined by slowly lowering the capillary until it bent). The stage moved the blot past the capillary at a rate of 3 mm/min, which was the slowest setting possible for the motorized stage. A LabVIEW program controlled stage motion and application of high voltage. The maximum distance traveled by the stage, as constrained by the stepper motor track length, was 13 cm. Typically, a 0.5 x 12 cm blotting membrane was mounted on the stage, though these proportions are generous and a separation may require a shorter length. The footprint of the apparatus, including the stage, stepper motors, and capillary positioner is about 40 x 40 cm.

Polyimide-coated fused silica capillaries were used for both the separation and sheath capillaries. The separation capillary (20 cm in length, 50 μm i.d., 185 μm o.d.) was threaded through a sheath capillary (6.5 cm long, 250 μm i.d., 360 μm o.d.) and fixed in place using a PEEK tee (VICI Valco Instruments, Houston, TX). The sheath capillary extended past the separation capillary by 0.5 mm. During separations electrophoresis gel was pumped through the sheath capillary at 10 nL/min using a syringe pump (Fusion 400, Chemyx, Stafford, TX).

Separation capillaries were conditioned by sequential rinsing with 0.1 M NaOH, 0.1 M HCl, and water for 10, 5, and 2 min, respectively, followed by pumping gel through the capillary (10 min) as recommended by the manufacturer. Gel-filled capillaries were used for 3-5 injections before regeneration with further conditioning.

The PVDF membrane mounted on the stage was kept wet with a wick that overlaid the membrane prior to contact with the capillary. The wick was kept stationary so that the membrane on the stage moved out from under it just before contact with the capillary. Both the membrane and wick were wetted with 50:50 (v:v) methanol and electrophoresis buffer. After a region of membrane passed the capillary it was allowed to dry.

Detection on Membrane. Fluorescent proteins were detected on the membrane by direct imaging using a Typhoon 9410 variable mode imager (GE Healthcare) in fluorescence mode. For Western blot analyses, immunoassays were performed per instructions of the manufacturer using a SNAP i.d. unit (Millipore), which partially automates antibody incubation and wash steps. In this system, 10 mL of blocking solution, 1 mL volume of antibody solutions, and tens of mL of rinse buffer, are poured over the surface of the blot (28 cm² in area in this case) in the normal sequence. After incubation (10 min for each antibody step) solution is forced through the PVDF membrane using a vacuum pump. Design of the unit, including a cartridge which holds the blot stationary, prevents membrane drying. The primary antibody sera to each target protein was diluted to 1:1700 and the secondary antibody was diluted to 1:33000. All dilutions were made with electrophoresis buffer. 0.5% nonfat dry milk and 0.5% Tween 20 were used in the blocking and rinse steps, respectively, per manufacturer suggestions. Signal generation, using hydrogen peroxide and acridan ester substrates in a chemifluorescence kit (ECL Plus, GE Healthcare), was catalyzed by horseradish peroxidase-conjugated secondary antibody.

Capillary Gel Electrophoresis. For comparison to the X-Y translational stage apparatus, experiments were conducted using a P/ACE MDQ capillary electrophoresis unit equipped with an LIF detector (Beckman-Coulter, Fullerton, CA). The detector used 5 mW of 488 nm light from an Ar⁺ laser (Model: IMA101015B0S; Melles Griot, Carlsbad, CA) for excitation. Emission was detected after passing through a 488 nm notch filter and a 520 (10 nm band-pass filter. Capillaries had an effective length of 20 cm, 50 μm i.d., and 360 μm o.d.. Separation capillaries were treated as described above for the membrane capture experiments. Fluorescence imaging of proteins migrating from separation capillary outlet into the sheath capillary region was performed using an inverted epi-fluorescence microscope (IX71, OlympusAmerica, Inc.,

Melville, NY) and CCD camera (C9100-13, Hamamatsu Photonic Systems, Bridgewater, NJ) as described in detail elsewhere.¹¹ In these experiments the capillaries were of similar dimensions to those of the CE-Western blot, except that the sheath capillary extended 5 cm beyond the separation capillary and was grounded in a gel reservoir rather than to a moving surface.

RESULTS AND DISCUSSION

SDS-protein complex capture from CGE. Our approach to CE-based Western blot was to capture SDS-protein complexes onto a membrane as they migrate from the column using the system shown in Figure 2.1 and then detect target protein analyte using conventional immunoassay. Initial experiments were directed at identifying conditions that would allow protein capture with minimal band spreading using fluorescently-labeled proteins as a model. Figure 2.2 illustrates images from the direct detection on a PVDF membrane of captured FITC-labeled proteins that had been separated by CGE as SDS-complexes. PVDF membrane was chosen as the blotting membrane because of its reported high binding capacity and mechanical strength¹²; however it is anticipated this method could also be used with different substrates. Earlier work with nitrocellulose did allow for electrophoresis, though the membrane was brittle and difficult to manipulate (data not shown). Using PVDF membranes, proteins are captured in discrete zones, preserving the separation. Furthermore, proteins migrated according to the logarithm of the molecular weight as expected for CGE of SDS-complexes (Figure 2.2).

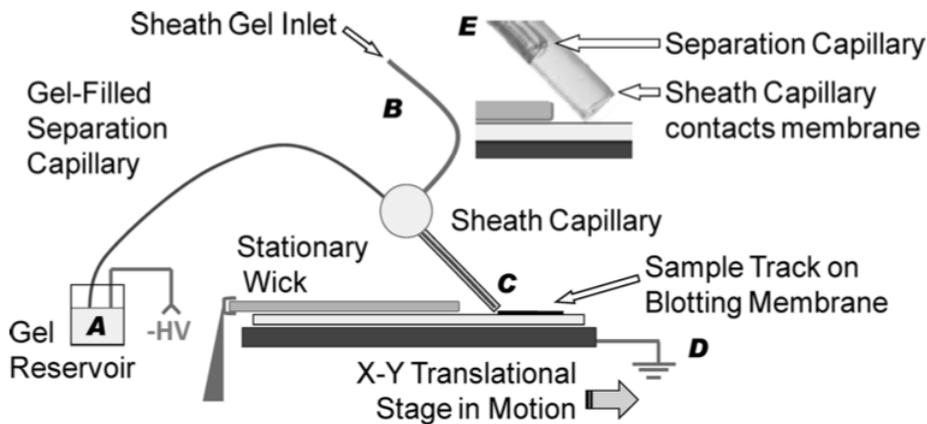


Figure 2.1. Instrument overview. Sample is injected at the inlet of the separation capillary (A). The protein mixture migrates the gel-filled capillary under an electric field that is generated by the application of negative high voltage (A) and ground (D). Proteins exit the capillary as it drags over the surface, and deposit on the blotting membrane (C). A translational stage moves the blot past the end of capillary to preserve the protein separation on the membrane. Gel is pumped through a sheath capillary (B) that surrounds the latter portion of the separation capillary and makes direct contact with the blotting membrane (E). The blotting membrane (and wick overlay) are moistened with 50:50 (v:v) methanol: electrophoresis buffer.

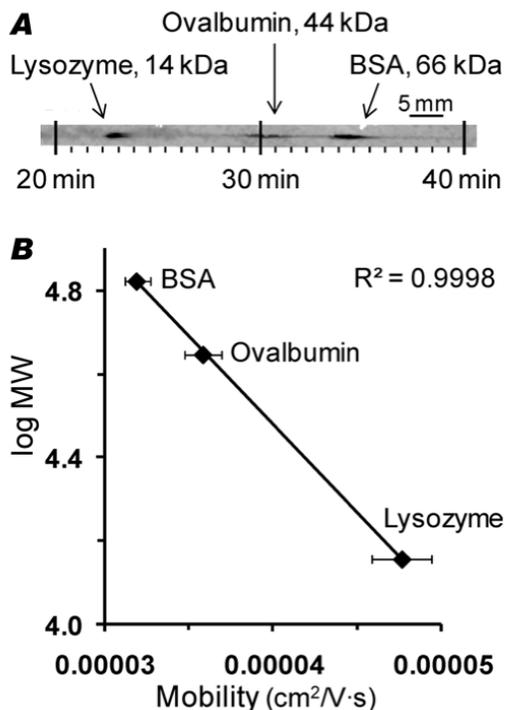


Figure 2.2. Size-dependent separation of standard FITC-labeled proteins. (A) 3 proteins, prepared in stock samples of 100-300 mg/mL. The molecular weight for unlabeled protein is noted beside each observed peak. (B) Plotting log MW as a function of mobility yields a linear plot for these FITC-labeled proteins.

Although previous work had already demonstrated capturing native proteins on surfaces after separation by free solution CE^{6,7}, we found that several modifications to conditions used in those reports were necessary to capture proteins separated as SDS complexes by CGE and achieve detection of discrete bands as shown in Figure 2.2. In particular, it was necessary to wet the capture membrane with a methanol: electrophoresis buffer mixture (a 50:50 v:v mixture was used). Without methanol present during capture either no bands or diminished signal was observed. Methanol helps to wet the PVDF membrane and dissociate SDS-protein complexes, which improves adsorption onto the hydrophobic membrane.¹³ A wick overlaid the membrane prior to capture, and helped to prevent evaporation of the methanol mixture during CGE separation.

A second modification from previous work was use of a gel-filled sheath capillary around the outside of the separation capillary (Figure 2.1). Without a sheath capillary, electrophoresis current was unstable and bubble formation was observed in the separation capillary a few minutes after voltage was applied. If gel in the sheath was static, i.e. without flow, migration times were irregular and the current was only stable for about 15 min before drift occurred. Pumping the sheath gel at 10 nL/min alleviated these problems. Higher sheath gel flow rates (50-200 nL/min) were found to smear protein on the blot. The underlying causes of these effects were not investigated, but they are consistent with electrolysis induced alterations in electrolyte at the outlet. Specifically, it has been observed in CZE separations that when the outlet reservoir has a small volume, hydroxide ions formed at the outlet. Electrolysis products migrated towards the inlet to cause changes in pH and conductivity in the capillary.¹⁴ With a thin layer of buffer on the blotting membrane the same conditions may apply. The sheath capillary will dilute ions formed and create a low field region that slows migration towards the inlet. With

a supplemental flow, ions may be prevented from entering the separation capillary at all. The sheath capillary also prevents a sharp electric field boundary along with buffer change at the outlet of the separation capillary, which may also contribute to the stability observed.

Band broadening. Although proteins are detected in discrete bands, we found that the system produced bands that were approximately 1.7 fold broader than on-column detection. To determine the source of band broadening, zones detected on membrane were compared to those detected on-column and within the sheath flow capillary. For these experiments, the membrane capture experiment was performed as before (e.g., Figure 2.2-A). A separate measurement was done to image protein leaving the separation capillary and entering the sheath gel using an imaging system under similar separation conditions (i.e., sample composition, column length, column i.d., buffer, and electric field). Separate experiments were required because of the impracticality of detecting on-capillary while also capturing zones on a membrane. For both measurements, FITC-BSA was used as a test compound. Peak variance for FITC-BSA increased from 230 s^2 on capillary to 610 s^2 in the sheath (350 mm downstream of the separation capillary) to 670 s^2 on the membrane (see Figure 2.3-A). These results indicate that the majority of band broadening in transfer from the capillary to membrane occurs within the sheath itself.

Images of the transfer from capillary to sheath (Figure 2.3-B) provide insight into some of the reasons for the observed band broadening. The parabolic flow induced in the sheath, evident from the bullet shaped zone, contributes to spreading. (Recall that flow was important to maintain stable current). The decreased electric field in the sheath capillary, due to the wider bore and lower electrical resistance compared to the separation capillary, may also contribute to the band broadening. These results suggest that fabrication of a sheath with narrower bore, through microfabrication methods, would decrease band broadening and improve resolution. The

high viscosity of the commercial separation media is also hypothesized to exacerbate band broadening in the sheath media, which could be reduced with implementation of a different, less viscous sieving media. The imaging results also show that the zone is not focused by the sheath flow, as is observed with less viscous sheath media and higher flow rates.¹⁵

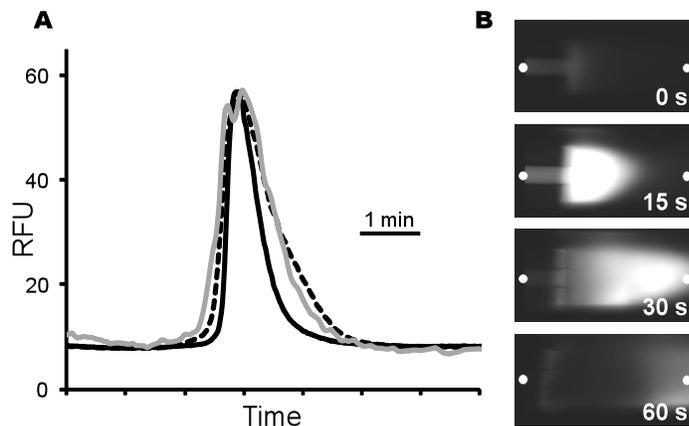


Figure 2.3. Measurements of band broadening inside the sheath capillary and on membrane. (A) Comparison of peak width for on-column detection (black line), in sheath 350 μm beyond the exit of the separation capillary (dashed line), and on membrane after traveling through 500 mm of sheath (gray line). The on-column and in sheath measurements were taken from the same separation. The membrane data was from a separate injection. All separations used 150 $\mu\text{g/mL}$ FITC-BSA as the sample separated at 300 V/cm with an effective capillary length of 20 cm. Capillaries were not thermostatted. (B) Selected images of protein exiting the separation capillary and entering sheath capillary. Time zero represents time zone first appears at exit of separation capillary and is 32 minutes after sample injection. The white dots indicate where signal was measured to construct Figure 2.3-A.

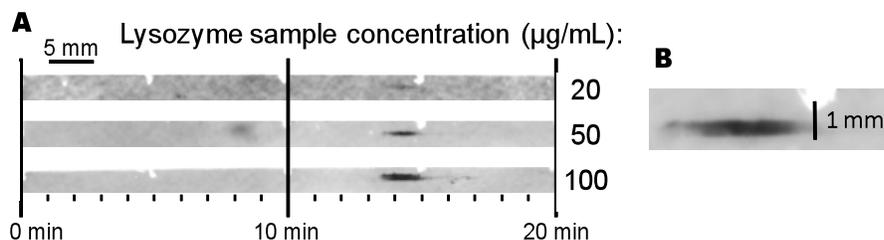


Figure 2.4. CE-based Western blot of lysozyme at 3 different concentrations. Samples were separated at 300 V/cm and the resulting membranes probed with antibody using an automated system for applying reagents. Analysis time for an individual assay was about 60 min, though the immunoassay and detection were performed in parallel. Enlargement shows that zones spread perpendicular to the deposition track. The sheath capillary was 250 µm in diameter but the zone is 450 µm wide.

When SDS-protein complexes migrate onto the membrane, most of the spreading on the blot appears to be laterally across the surface, rather than down into the membrane. Evaluation of spreading in the dimension perpendicular to the motion of the capillary indicates a typical trace width of approximately 450 µm, about 200 µm larger than the width of the sheath capillary inner diameter (Figure 2.4). Yet in the vertical dimension, protein was not detected beyond the first 150 µm thick membrane when deposition was performed onto a stack of membranes. This result is in contrast to previous work that indicated some protein penetrated a first membrane and bound the second.⁷ In that work, 20 ng of protein was deposited whereas in this work picograms of proteins were deposited, so it could be that the spread through the membrane is too low for detection via immunoassay. These results support the idea of rapid protein capture, which is interrogated further in Chapter 4.

Immunoblotting after capillary separation. Figure 2.4 illustrates a CGE-Western blot for lysozyme at different concentrations. As with traditional slab gel Western blots, this technique is semi-quantitative, allowing for differences in protein concentration to be determined by the intensity of the band detected, as shown in Figure 2.4. An advantage of the CE method

over traditional Western blots is the elimination of a separate electro-blotting step because protein is directly delivered to the membrane as it is separated. This saves time as transfer requires up to 1 h in a conventional slab gel system.

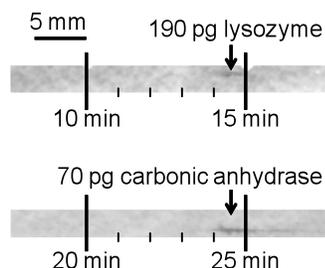


Figure 2.5. Immunoassay of unlabeled sample proteins at low levels. Estimated quantities of proteins calculated from injection length, elution time and sample concentrations are displayed for different proteins.

Limits of detection. The limit of detection was investigated by performing CGE-Western blot of carbonic anhydrase and lysozyme at concentrations that produced weak signals (Figure 2.5). In these experiments, sample concentrations of 25 and 20 $\mu\text{g/mL}$ resulted in peaks with signal-to-noise ratio (S/N) of 22 and 12 for carbonic anhydrase and lysozyme, respectively. Assuming a linear response, this S/N corresponds to a LOD of 3 $\mu\text{g/mL}$ or 10 pg injected for carbonic anhydrase and 5 $\mu\text{g/mL}$ or 50 pg injected for lysozyme. (Amounts injected were estimated using injection duration, sample concentration and sample mobility.) The manufacturer reports the LOD for the chemifluorescence assay kit to be low picograms, so the sensitivity of our scheme is on par with the capabilities of the detection method. The good mass LOD for this system is attributed to use of small bore capillaries and small capture zones. Further, recovery may be higher in CGE because proteins are migrated from a low volume sieving polymer matrix rather than electro-transferred out of a cross-linked slab gel for blotting.

The rate of stage motion past the capillary may also impact LOD and resolution. Lower speeds are preferable to capture the band in a small zone and improve sensitivity by keeping the

zone concentrated; however, if the speed is too low, zones would be captured with little distance between them, and resolution may be lost. In our experiments we used the lowest speed of the translational stage because no resolution was lost when compared to higher speeds, but the zones were more concentrated on the membrane. A stage with slower translational velocity therefore may allow better sensitivity.

Incorporation of size standards. An important component of a Western blot is providing size information on the detected proteins in addition to immunoaffinity. The size of the target protein is estimated by comparison of its mobility to the mobility of a different proteins of known size separated in an adjacent lane. One approach for calibration in the CGE system is to add fluorescently-labeled protein size standards to the sample mixture and analyze the membrane by both direct fluorescence and immunoblotting. Results from such an experiment are illustrated in Figure 2.6. In the initial fluorescence scan, the added calibrants, FITC- insulin and FITC-BSA, are detected to provide reference positions on the membrane. The membrane is then probed with antibodies specific for the analyte protein, carbonic anhydrase. The signal for this protein is detected as a band between the reference bands, as anticipated because of its intermediate molecular weight. During the second analysis of the membrane, the standard proteins are barely detectable. This may be because the chemifluorescent signal from the secondary antibody conjugate is more intense than the fluorescent ladder proteins. The signal of the ladder proteins may also be reduced by photobleaching or rinsing prior to the second detection step.

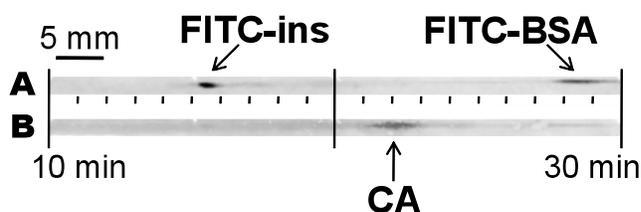


Figure 2.6. Immunoassay of unlabeled peak with labeled size standards. (A) Initial fluorescence scan displays FITC-labeled size standards at approximately 16 and 28 minutes for insulin (FITC-ins) and bovine serum albumin (FITC-BSA). (B) Upon immunoassaying this PVDF membrane with anti-carbonic anhydrase IgG, the intermediate MW protein, carbonic anhydrase (CA), is detected at 22 minutes.

Potential for improvements. These experiments prove the feasibility of performing CE-based Western blots. The prototype system allows separation and blotting to occur at the same time thus eliminating the time and inefficiencies of electro-transfer. Although the described approach shows promise for reducing sample requirements, automating, and reducing time of Western blots, improvements in time of analysis, throughput, robustness and sensitivity are possible. Among the improvements we envision for faster analyses are active cooling of the separation capillary, use of a smaller i.d., or separation with a different sieving matrix. All of these measures would be directed towards application of higher electric fields, which in turn would return faster, more efficient separations. Addition of cooling complicates the implementation of this technique, but it may serve to push the boundaries in separation efficiency and speed. As indicated by microfluidic gel sieving, extremely fast protein size separations are possible in miniaturized formats, suggesting the possibility of reducing the separation and blotting time to less than 1 min.^{4,8} In addition to faster separation, throughput would also be improved by use of capillaries in parallel. Parallelization would also allow ladder proteins to be run in a separate capillary for sizing if desired. Further throughput improvements are possible by automating gel regeneration, as has been done for DNA sequencing instruments. Sensitivity has not been fine tuned, as we expect the implementation of on-column sample preconcentration is possible in CGE¹⁶, and would likely further improve concentration LODs.

One hindrance to sensitivity in traditional Western Blotting is differential rates and efficiencies of differently sized proteins; entangled polymer gel may also be more amenable to transfer of large proteins. Fine control of translational stage velocity could also be used to improve either resolution or sensitivity. Finally, the small size of the capillary and resulting tracks indicate it is possible to store many electropherograms on a small membrane to reduce reagent consumption and improve throughput (many separations, one immunoassay) relative to a traditional slab gel Western blot.

CONCLUSIONS

CE has been recognized as a powerful method for protein separations; however, it can be argued that widespread acceptance of CE for protein analysis has been inhibited by lack of an analog to Western blotting. This report demonstrates a route to CE-Western blotting. Many of the improvements expected for migration from a slab gel to capillary format are demonstrated. The time of analysis is improved through faster separation, use of polymer solutions for sieving media, and elimination of the electro-blotting step. Mass detection limits of 10 pg are readily achieved with little optimization. The system has potential for substantial improvements in speed, throughput, reagent consumption, and sensitivity. Importantly, the method also retains the essential features of the traditional Western blot format by providing size (calibrated with standards) and immunoaffinity information in a semi-quantitative assay. Furthermore, immunoassay procedures are unchanged from traditional Western blotting, so that optimization of detection and development of assays for different proteins can follow well established procedures. This is exemplified by the fact that we were able to readily develop Western blots

for 3 different proteins in the course of this work. This approach may find use for sample limited analyses or in situations where better speed, throughput or automation is necessary.

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CHAPTER 3

IMPROVEMENTS TO CE-WESTERN BLOTTING FOR MULTIPLEXED OPERATION

INTRODUCTION

In the previous chapter CE-Westerns with sensitive semi-quantitative detection and about one hour assay time were demonstrated. Several routes for improvement were envisioned. As with all microscale methods, good control of preconcentration or sample handling is important for high sensitivity. In addition, tuning of sieving polymer formulation could lead to even faster separations and transfer. Lastly, advances in throughput could be achieved with multiplexed separations for simultaneous immunoassay. What follows is a review of preconcentration mechanisms in slab gels, with the hope they could be modified for this CGE application. Then concepts for choosing sieving media and the ways in which others have multiplexed protein separations are discussed. This chapter goes on to describe several improvements to the CE-Western method; in combination these enable, for the first time, multiplexed CE-Western analysis of complex mixtures.

Online preconcentration: in PAGE gels and CGE. In slab gels, there are four preconcentration or “stacking” mechanisms in play. First, there is discontinuity in the gel pore structure. Near the start of the gel (by the protein loading well) pore size is large, which does not greatly impede motion of sample from the injection site forward towards the anode. Protein exits this stacking gel and enters the resolving gel, which has much smaller pore sizes. A physical

stacking of SDS-protein occurs at the interface between the two pore sizes, and this concentrates sample into a narrow band. We hypothesize there is some stacking effect in CGE electrophoresis as well, between the free solution sample and entangled polymer gel.

The other three mechanisms are based on control of ionic strength, pH, and the nature of the ions (isoelectric points, mobility). That is to say, field amplified sample stacking, isotachopheresis, and pH junctions, respectively, are at work. For field amplified stacking differences in conductivity between the sample (or stacking) buffer and separation buffer cause sample ions to migrate more quickly and stack into a narrow band. With pH junctions a similar low conductivity region can be formed by sandwiching sample between strongly acidic and basic solutions, or by having ions change charge state when they pass from sample to separation buffer as is the case here. Isotachopheresis occurs when a sample is confined within the boundaries of a fast moving leading electrolyte and slow moving terminating electrolyte, rather than diffusing out from the initial sample band.¹ For each parameter, there is a dramatic buffer composition change between the sample loading region and the resolving gel. For instance, in a classical separation ²:

- Stacking gel: large pores, low IS buffer, lower pH
 - e.g. 0.125 M Tris-glycine, pH 6.8
- Resolving gel: small pores, high IS buffer, high pH
 - e.g. 0.375 M Tris-HCl, pH 8.6

With regard to CGE preconcentration, manipulation of mobilities at the inlet would seem to be of the most interest. In slab gels transient isotachopheresis (tITP) is employed to stack

proteins according to their intermediate mobilities relative to a fast moving leading electrolyte (LE) and a slow moving terminating electrolyte (TE). In particular, a small molecule, glycine (pI 6.7), starts in cathode buffer reservoir, with chloride ion in the gel buffer. Glycine moves slowly through the protein zone that stacks at the pore size barrier (proteins, which are much bigger experience greater increased friction). Glycine then experiences the pH junction, becomes more highly charged in the new high pH environment, and the glycine/Cl⁻ front moves ahead of the protein separation. So called “transient isotachopheresis” is in effect because the proteins initially move slowly with the glycine terminating electrolyte, stacking at the boundary of the two pore sizes. As pH increases glycine no longer acts as a terminating electrolyte, and size-separation proceeds.^{3,4} To implement this in capillaries, a leading electrolyte is injected, followed by sample injection, followed by terminating electrolyte injection. There are reports of tITP for preconcentration in capillary electrophoresis, but there are no size-based CGE reports.^{5,6,7}

Sieving Media Choices for Increased Speed and Tunability. In the prototype instrument and initial testing described in Chapter 2, it was practical to use a commercial sieving media (“gel”). Beckman-Coulter sieving media is formulated for use with Beckman-Coulter capillary electrophoresis platforms (P/ACE MDQ and others). In initial experiments Beckman-Coulter gel was chosen for its high resolving power and stability over the course of several hours of separations. However, this came with somewhat slow separations (40 minutes to separate 100 kDa protein at 300 V/cm). Most importantly, rational choices for sample buffer preparation and sheath gel were hindered by the proprietary sieving gel formulation. Ionic species, ionic strength, presence and amounts of additives (SDS, glycerol, etc.) were unknown. Additionally, measured properties were unfavorable: viscosity was extremely high at 78 cP, and pH was lower

than ideal for protein separations, at approximately 6.5. Aims of faster separation, greater leeway with sheath gel formulation, control of additives and reduced cost motivated experimentation with different sieving media for the CE-Western instrumentation.

Research on CGE sieving media has moved from covalently-attached crosslinked polymers to entangled polymer solutions. Capillary gel electrophoresis was first implemented by using the same crosslinked polyacrylamide chemistry of traditional SDS-PAGE, this time in a capillary. That is to say, crosslinked polyacrylamide gels were prepared *in situ* within the capillary, with covalent attachment to the inner silica surface.^{8,9} This method (and crosslinked gels in general) suffered from inhomogeneity, limited column lifetime and poor reproducibility. Instead, replaceable visco-elastic fluids, i.e. non-crosslinked entangled polymer mixtures came into use.¹⁰ Replaceable sieving media is pumped into capillaries and can be pumped out and replaced after prolonged use. With replaceable sieving media wall modification is still required to suppress electroosmotic flow and reduce sample adsorption. This can be achieved with a covalent “static” coating¹¹, or a “dynamic” coating in which the wall surface is intermittently modified by adsorbed species from the background electrolyte (BGE)¹². An intermediate condition, an adsorbed polymer that is semi-permanently attached to the capillary surface and not included in the BGE, has been described as either a dynamic or static coating.^{13,14} Here, it is considered to be a static coating, because it need not be frequently regenerated or included in the sieving media. While dynamic coatings are easily implemented by including the coating species in the sieving media, efficiency is poor relative to static coatings. Amongst static coatings, adsorbed coatings offer simplicity, possibility of regeneration and a lessened dependence on capillary surface chemistry. Covalent coatings have a limited lifetime, and reliable coverage of the capillary surface is both difficult and crucial for high efficiency separations. There are

several commercially available capillary coatings intended for static adsorption, including Ultratrol, which consists of a linear polyacrylamide, N-substituted acrylamide copolymer.

For the sieving media, several hydrophilic homopolymers have been reported. These include linear polyacrylamide (LPA)¹⁵, poly-ethylene oxide (PEO)¹⁶, poly-ethylene oxaline (PEOX)¹⁷, and cellulosic derivatives¹⁸, among others. LPA is acknowledged to have the best resolution, but this comes at a cost of a highly viscous sieving media and long analysis times. Copolymer or semi-crosslinked polyacrylamide medias have also been designed that can combine high resolution with lower viscosity or self-coating properties.^{19,20} Current options in sieving media are detailed in a recent review.²¹ With this body of work in mind, priorities when choosing a gel include good sieving ability, dynamic coating ability or a static coating strategy, and low viscosity.

High viscosity media results in more difficult replacement of media in the capillary. Additionally, there is a trade-off between speed and resolution that is approximated by the solution viscosity. With a separation media of linear polymer chains, the polymers entangle and thereby impart a mesh-like characteristic to the separation media. The pore-size of such an entangled mesh is dynamic such that the size and persistence time of the mesh in a linear polymer is related to both the length of each linear polymer unit, and the number of chains in the region.²² Thus, both polymer molecular weight and polymer concentration will impact the separation capabilities of the media for different sample components. Resolution of smaller sample molecules is more effective with a more closely-knit mesh, requiring lower molecular weight chains and/or higher polymer concentrations. Higher molecular weight sieving polymers, and lower polymer concentrations are preferred for larger molecules as they create a larger mesh (or effective pore size).²³ Interestingly, it has been reported that the intrinsic nature of the

sieving polymer has no effect on peak spacing or peak timing. Instead only mesh size is determinative. Polymer choice does influence peak width, thus contributing to efficiency.²² For best separation, the least viscous media that still resolves the sample with good peak shape is desired.

Cellulosic-based polymers such as pullulan and dextran are attractive sieving polymers because of relatively low viscosities and high hydrophilicity. Several groups have reported SDS-CGE with dextrans ranging in molecular weight from 7.5 kDa to 2 MDa and concentrations between 0.5 and 20%.^{24, 25, 26} Dextran, naturally derived from *Leuconostoc mesenteroides*, is also readily available and cost effective.

In addition to buffering salt and polymer species and concentrations, sieving media performance can also be tuned with additives.^{27, 28} SDS is added to the media to help maintain denatured protein analyte chains. Glycerol has been used to increase resolution^{24, 28}, and has also been reported to alter the mesh size of polymer solutions, via complexation with borate buffer, that in turn complexes with available electrophilic groups on the polymer chains.²⁹

Methods to monitor CE-Westerns throughout the experiment. In Chapter Two, inclusion of ladder proteins in the sample allowed for easy size calibration of the analyte. The initial “ladder scan” revealed fluorescent signal of pre-labeled standards that eluted for the duration of the separation. In addition to size calibration, the predictable pattern of ladder proteins allowed for quick assessment that the separation proceeded as expected. Good separation was confirmed before immunoassay resources were committed to identify the analyte.

Another metric of performance (long before the ultimate immunoassay output) is to monitor current during the course of the separation. All electrophoretic processes are regulated by Ohm’s Law:

$$V = IR$$

Where V = potential, I = current, and R = resistance. In PAGE separations it is reported reproducibility is best maintained by holding current constant.⁴ In capillary electrophoresis practice voltage is held constant, and so voltage was constant for these experiments. Resistance is determined by factors such as capillary dimensions and buffer composition. When a new sample buffer is introduced, the voltage is ramped up in a controlled sequence. The point at which current gives a non-linear response indicates when Joule heating occurs. In subsequent experiments applied field can be kept at a level where Joule heating is not observed. Should Joule heating occur, it is made obvious by observation of an initial drop in resistance (rise in current) as buffer viscosity reduces with increased temperature. In short order (a few seconds) bubbles form in the capillary, which impede the separation and eventually cause a discontinuity in the circuit, i.e. complete current failure. To prevent this, the applied electric field is kept at a level where the risk of Joule heating is minimal. With the Beckman-Coulter sieving media this was found experimentally to be 300 V/cm on the membrane capture apparatus. Temperature is a factor in all capillary electrophoresis modes, but especially in CGE, where a change of a few degrees lowers the barrier for detrimental Joule heating at low applied fields.

Multiplexed Western Blotting. As discussed in Chapter One, microscale CGE has long been of interest for high throughput separations. In recent years, several groups have developed different methodologies driving for more rapid separation and throughput for Western blotting. One strategy is to electrophoretically separate a sample with specific target capture by immobilized antibodies on-chip. This has been reported with 2D microfluidic chips in which all proteins were size-separated and then exposed to immobilized antibody with application of a perpendicular field.^{30,31} Additionally, for very sensitive measurements (80 pM), smaller cross-

linked sieving pores were utilized for a pore limit electrophoresis method that allowed for ample sample loading. In this case, the entire sample passed over immobilized antibody sites which captured the target protein, and then unbound analyte and other sample proteins were separated and accumulated in a size based manner for size calibration.³² With this level of sensitivity, the method is attractive for very dilute samples as it is automated and could be multiplexed with parallel channels. Yet fabrication is complex, and to achieve this sensitivity does require lengthy operation (40 min). Microfluidics technology has been used to create flow channels for applying blocking and antibody solutions over the surface of a blot. This is advantageous in reducing reagent consumption and optimizing conditions. The authors also make the point that each flow channel is isolated and could apply a different antibody for multiplexed detection.³³ A strong demonstration of the potential for multiplexed Western blotting is a so-called “microwestern array” in which a non-contact microarray printer was used to deposit many cell lysate samples into a thin layer slab gel cast in the pattern of a 96 well plate. After separation of the many protein spots, the proteins were electro-blotted onto one membrane. With use of a 96 well gasket many different antibodies could be applied to the samples. Resolution was poor and the variable transfer efficiency typical of electro-blottting was not addressed.³⁴ Lastly, another group reported a CGE separation coupled to a polytetrafluoroethylene (PTFE) substrate with an emphasis on matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) detection. Though the authors did not focus on low limits of detection or multiplexing, mass spectrometry does have the potential for automated, high throughput operation. This work demonstrates that our method of interfacing a CGE capillary to a moving substrate surface is transferrable to other laboratories.³⁵

To increase throughput with CE-Western blotting, as in CE generally, one strategy is to operate multiple capillaries in parallel. Slab gel electrophoresis is operated with about 10 lanes run in parallel, followed by one electro-blotting transfer step where proteins bind a PVDF membrane. Size-based calibration is achieved with inclusion of standard proteins in one gel lane. For CE-Westerns, parallel separation capillaries would simultaneously separate and deposit many traces on a single blotting membrane for streamlined immunoanalysis.

In previous work, detailed Chapter 2, we reported a capillary electrophoresis-based Western blotting method.³⁶ Using a commercial sieving gel a single separation capillary was grounded to a polyvinyl difluoride (PVDF) membrane mounted on an X-Y translational stage. The stage was moved at a steady rate to transfer and preserve the size-based separation. A sheath capillary surrounded the separation capillary outlet and contacted the membrane, providing a region of lower electric field that allowed for stable operation. With this instrument sensitive detection limits of 10s of pg were achieved, with separation and immunoassay in ~1 h. The commercial sieving gel demonstrated very good resolution, but was highly viscous. Some band broadening was observed as sample protein left the separation capillary and entered the sheath gel region.

Ultimately high speed highly parallel separations, with membrane capture and a single bulk immunoassay, are envisioned as a way to make Western blotting more accessible to high throughput screening or clinical applications. Blots of modest dimensions (perhaps 7 cm x 8 cm) are capable of storing hundreds of parallel separations, so a shorter separation and bulk immunoassay could allow for 100s of Westerns/day. We report here several improvements to the initial CE-Western instrumentation, which bring us closer to that goal. To increase speed, an inexpensive dextran sieving media was formulated, which allowed separation of 15-100 kDa

proteins in about 15 min. For the first time multiplexed separations in CE-Westerns are demonstrated with three capillaries operated in parallel. Repeated injections on each capillary are demonstrated, which also serve to increase throughput. Finally, these CE-Western blots are performed with a realistic sample matrix, NIH-3T3 cell lysate, and the semi-quantitative results typical of traditional Western blotting are maintained.

EXPERIMENTAL METHODS

Materials and Reagents. ECL Plus chemifluorescence kit and polyvinylidene fluoride (PVDF) membranes were purchased from GE Healthcare (Piscataway, NJ) and GE Osmonics (Minnetonka, MN) respectively. Filter paper was from Fisher Scientific (Pittsburgh, PA). Rabbit anti-carbonic anhydrase was from Genway Biotech (San Diego, CA). Actin from rabbit muscle, FITC-labeled bovine serum albumin (BSA), anti-actin from rabbit and mouse, and secondary antibodies: horseradish peroxidase-conjugated anti-rabbit IgG produced in goat, peroxidase-conjugated and AlexaFluor 633 and 488 anti-mouse in goat were purchased from Sigma (St. Louis, MO). Denatured NIH 3T3 cell lysate was purchased from Santa Cruz Biotechnology (part number 2210, Santa Cruz, CA). INS-1 cells were also lysed using RIPA buffer and protease inhibitor cocktail purchased from Pierce Biotechnology (Rockford, IL). AlexaFluor 488-conjugated ladder protein mixture was purchased from Invitrogen (Grand Island, NY). UltraTrol LN dynamic capillary coating formula was purchased from Target Discovery (Palo Alto, CA). All other chemicals, including dextran polymers, were purchased from Sigma. Fused silica capillaries were from Molex Inc. (Lisle, IL). All solutions were made using Milli-Q (Millipore) 18 M Ω deionized water. Phosphate buffer solution (PBS) was 100 mM Na₂HPO₄ adjusted to pH 7.5 with 100 mM NaH₂PO₄. For sieving gel comparisons, a proprietary solution of entangled polymers designed for resolution of proteins from 10 kDa to 225 kDa was used

(part number 390953 from Beckman-Coulter, Brea, CA). This media had a kinematic viscosity of 78 cP at 40 °C as measured using a glass semi-micro viscometer (Cannon Instrument, State College, PA).

Sample Preparation. Exogenous unlabeled proteins were denatured by heating at 70 °C for 5 min in denaturation buffer consisting of PBS, 3% sodium dodecyl sulfate (SDS) and 5% β -mercaptoethanol (BME). Purchased ladders and cell lysates were heated but required no added chemicals. In some instances, protein samples were then dialyzed against PBS using mini-dialysis cups from Pierce. Alternatively, a polyacrylamide buffer exchange column loaded with 10 mM tris-HCl pH 7.4 buffer was used predominantly with cell lysate samples (BioRad, Hercules, CA).

Apparatus and Procedure for Multiplexed CE-Western Blot. A diagram of the apparatus used to couple multiple separation capillaries to the blotting membrane is shown in Figure 3.1. High voltage was applied at the separation capillary inlet reservoir and grounded at the stage. Each separation capillary outlet was surrounded by a gel-filled sheath capillary, which made contact with a PVDF membrane secured to the X-Y translational stage. During injection and separation the sheath capillaries, yoked to each other and fixed at an angle of approximately 45°, were positioned to make light contact with the membrane. The stage moved the blot past the capillaries at a rate of 3 mm/min, which was the slowest setting possible for the motorized stage. A LabVIEW program controlled stage motion and application of high voltage. The maximum distance traveled by the stage, as constrained by the stepper motor track length, was 13 cm. Typically, a 5 x 7 cm blotting membrane was mounted on the stage, though these proportions are generous and separations may require a shorter length. A 20 min separation (traversing 6 cm) was completed, and then the stage was rapidly moved approximately 1 mm

perpendicular to the capillary traces, so that a subsequent set of parallel separations could be performed on a blank section of membrane. As each capillary was fixed ~1 cm apart from the next, up to 9 separations could be performed before separations overlapped. The footprint of the apparatus, including the stage, stepper motors, and capillary positioners was about 40 x 40 cm.

Polyimide-coated fused silica capillaries were used for both the separation and sheath capillaries. The separation capillary (20 cm in length, typically 50 μm i.d., 150 μm o.d.) was threaded through a sheath capillary (3.5 cm long, typically 180 μm i.d., 360 μm o.d.) and fixed in place using a PEEK tee (VICI Valco Instruments, Houston, TX). The sheath capillary extended past the separation capillary approximately 400 μm and was shaped into a cone at the outlet so that a narrow diameter made membrane contact. During separations electrophoresis gel was pumped through the sheath capillary at 10 nL/min using a syringe pump (Fusion 400, Chemyx, Stafford, TX). With Beckman-Coulter gel, separation capillaries were conditioned by sequential rinsing with 0.1 M NaOH, 0.1 M HCl, and water for 10, 5, and 2 min, respectively, followed by pumping gel through the capillary (10 min) as recommended by the manufacturer. For dextran gel separations, conditioning required sequential rinses of 1 M HCl, 1 M NaOH, water, UltraTrol dynamic coating and dextran gel, as recommended for UltraTrol usage. Gel-filled capillaries were used for 3-5 injections before regeneration was required to maintain performance.

The PVDF membrane mounted on the stage was kept wet by a layer of filter paper underneath the membrane. Both the membrane and filter paper were wetted with 50:50 (v:v) methanol and 200 mM tris HCl pH 8.4 buffer. After mounting the paper and membrane on the translation stage, three to five minutes passed before exposure to analyte to avoid diluted protein traces.

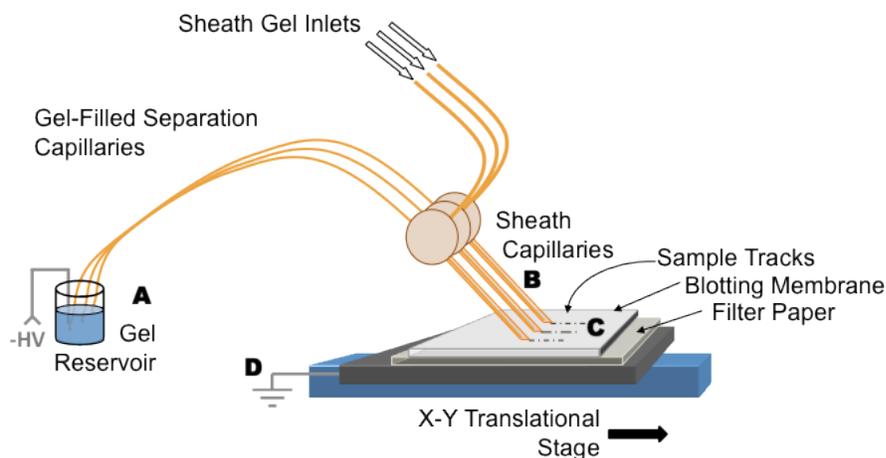


Figure 3.1: Instrument overview. Sample is injected at the inlet of each separation capillary (A). The protein mixture migrates the gel-filled capillary under an electric field that is generated by the application of negative high voltage (A) and ground (D). Proteins exit each capillary as it drags over the surface, and deposit on the blotting membrane (C). A translational stage moves the blot past the end of the capillaries to preserve the protein separations on the membrane. Gel is pumped through a sheath capillary (B) that surrounds the latter portion of the separation capillary and makes direct contact with the blotting membrane. Underneath the blotting membrane is one layer of filter paper, and both are moistened with 50:50 (v:v) methanol: 200 mM tris HCl pH 8.4 buffer.

Detection on Membrane. Fluorescent proteins were detected on the membrane by direct imaging using a Typhoon 9410 variable mode imager (GE Healthcare) in fluorescence mode. For Western blot analyses, immunoassays were performed per instructions of the manufacturer using a SNAP i.d. unit (Millipore), which partially automates antibody incubation and wash steps. In this system, 10 mL of blocking solution, 1 mL volume of antibody solutions, and tens of mL of rinse buffer, are poured over the surface of the blot (28 cm² in area in this case) in the normal sequence. After incubation (10 min for each antibody step) solution is forced through the PVDF membrane using a vacuum pump. Design of the unit, including a cartridge that holds the blot stationary, prevents membrane drying. The primary antibody sera to each target protein was diluted to 1:1700 and the secondary antibody was diluted to 1:33000. All dilutions were made with electrophoresis buffer. 0.1% nonfat dry milk and 0.1% Tween 20 were used in the blocking and rinse steps, respectively, per manufacturer suggestions. Signal generation, using hydrogen

peroxide and acridan ester substrates in a chemifluorescence kit (ECL Plus, GE Healthcare), was catalyzed by horseradish peroxidase-conjugated secondary antibody. Alternatively, some secondary antibodies were directly conjugated to a fluorescent tag, in which case secondary antibody dilutions were 1:250.

Capillary Gel Electrophoresis. For comparison to the X-Y translational stage apparatus and trials of sieving media formulations, experiments were conducted using a P/ACE MDQ capillary electrophoresis unit equipped with an LIF detector (Beckman-Coulter, Fullerton, CA). The detector used 20 mW of 488 nm light from an optically-pumped semiconductor laser for excitation (model: Sapphire LP; Coherent, Santa Clara, CA). Emission was detected after passing through a 488 nm notch filter and a 520 nm (10 nm band-pass) filter. Capillaries had an effective length of 20 cm, 50 μm i.d., and 360 μm o.d.. Separation capillaries were treated as described above for the membrane capture experiments. Fluorescence imaging of proteins migrating from separation capillary outlet into the sheath capillary region was performed using an inverted epi-fluorescence microscope (IX71, OlympusAmerica, Inc., Melville, NY) and CCD camera (C9100-13, Hamamatsu Photonic Systems, Bridgewater, NJ) as described in detail elsewhere.³⁷ In these experiments the capillaries were of similar dimensions to those of the CE-Western blot, except that the sheath capillary extended 5 cm beyond the separation capillary and was grounded in a gel reservoir rather than to a moving surface. Some preconcentration experiments requiring leading and terminating electrolyte (LE and TE) solutions were conducted. Separate LE, sample, and TE injections were conducted, and each injection was 30 s unless otherwise noted. LE was 20 mM NaH_2PO_4 -tris, 0.1% SDS, pH 8.3, the sample was in 25 mM tris-HCl pH 8.3, and terminating electrolyte was 20 mM tris, 200 mM γ -aminobutyric acid (GABA), pH 8.3.

RESULTS AND DISCUSSION

CGE separations of complex mixtures: test “real world” function. To assess robustness of the prototype instrument it was first necessary to test protein standards against a background mixture of cell lysate proteins. This was the first instance in which a complex mixture with different salt components was analyzed. The biological sample chosen to generally represent a biological mixture of interest was lysed clonal INS-1 cell line, used to study pancreatic beta-cell function. Different levels of carbonic anhydrase were spiked into a sample that included the cell lysate and commercial fluorescent ladder. In this manner the sample could be separated, scanned for fluorescent signal from the ladder, and then immunoassayed to reveal a different fluorophore specific for carbonic anhydrase.

The first injections of carbonic anhydrase spiked cell lysate made clear significant Joule heating was observed in the capillary. With each injection current would spike and then drop to zero, ending the separation within minutes of injection. The relatively high ionic strength (IS) of the sample buffer created a localized increase in field strength that quickly boiled the buffer and impeded separations. Lower applied fields for injection did allow for separation (Figure 3.2), but showed a great disparity in signal for the cell lysate samples. In a similar experiment on a commercial CGE instrument, peak heights for ladder in cell lysate were on average 88% smaller than ladder in buffer. Dialysis of the cell lysate mixture against low IS buffer was sufficient to return peak heights to the same order of magnitude, and separations continued much as with a ladder standard in buffer. With a dialyzed sample of fluorescent ladder in cell lysate, the protein ladder traces were remarkably similar between commercial CGE instrument (Beckman P/ACE MDQ) and the membrane capture instrumentation (Figure 3.3). When the mobility of the immunoassayed “unknown” peak of carbonic anhydrase was plotted on the size calibration

graph, a molecular weight value of 29.0 kDa was returned for that protein. This agrees well with the manufacturers' estimation of CA molecular weight equal to 29 kDa.

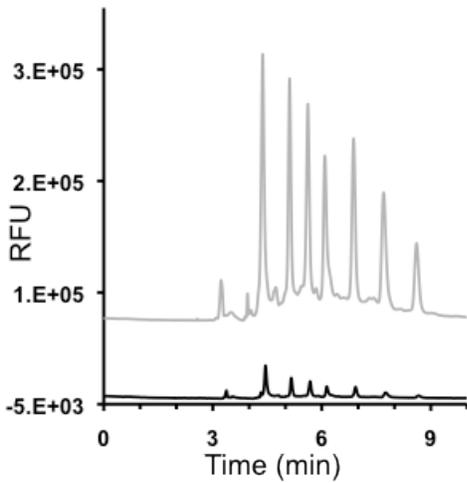


Figure 3.2. Separation of fluorescent ladder in different background buffers. Sample dissolved in cell lysate sample (black) or in 25 mM tris HCl buffer (grey). Separation performed on commercial CGE platform with commercial sieving media.

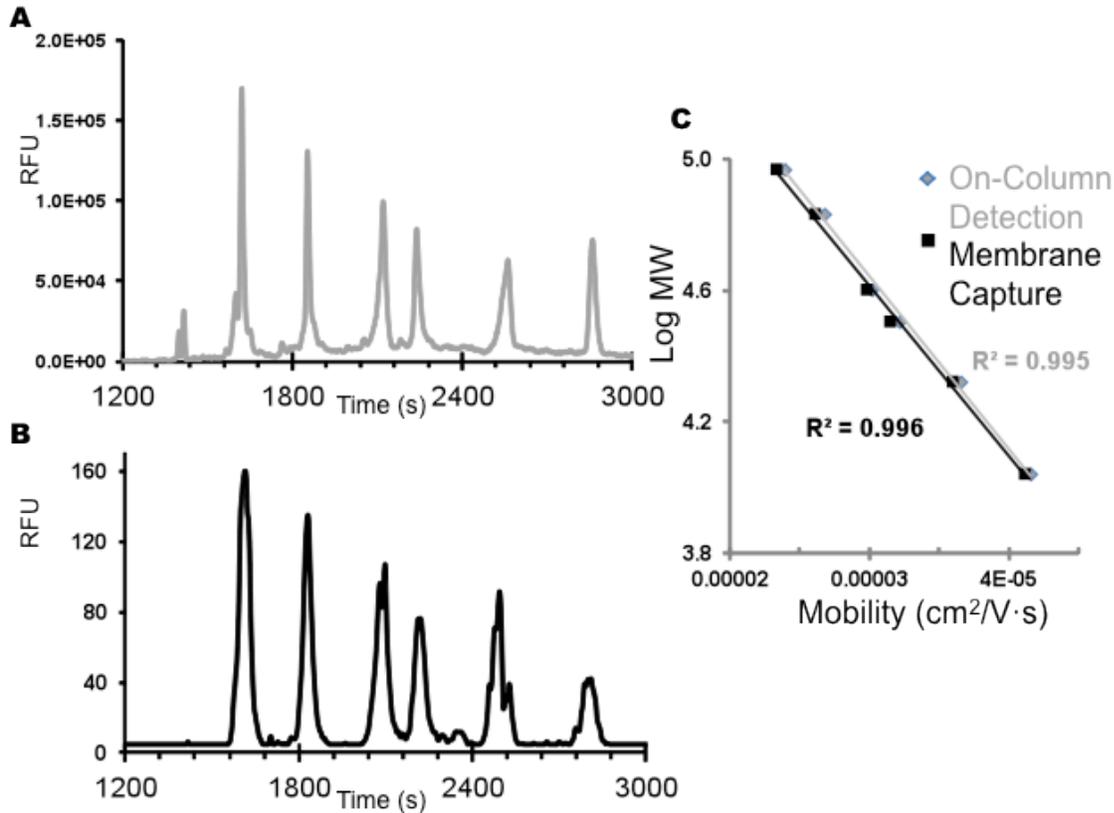


Figure 3.3. Commercial sieving media separations of protein standards in cell lysate after dialysis. Similar electrophoretic performance is observed with the on-column detection (A) and with membrane capture (B). Size calibration is also similar (C).

Initial trials with commercial gel: detection of endogenous proteins with membrane capture. After achieving separations with spiked cell lysate samples, immunoanalysis of actin proceeded. Actin is a ~42 kDa, highly conserved, cytoskeletal protein found in nearly all eukaryotic cells. In Western blotting, actin is commonly used as a positive or loading control against which to compare fluctuations in a protein of interest. It typically comprises 2-3 % of total protein content for cultured mammalian cells. Some is lost in centrifugal cell lysate preparation as fibrous actin pellets out as cell debris. Thus in a cell lysate sample, actin may comprise ~1% of total cell lysate proteins.³⁸ If total protein content in the sample is adjusted to 2

mg/mL (a reasonable value for slab gel Western blotting), the concentration expected for actin would be 20 $\mu\text{g/mL}$ or 500 nM.

With actin standards dissolved in low ionic strength buffer, 75 nM samples were easily detected with a limit of detection approximated as 15 nM. However, when endogenous actin was probed within cell lysate samples (total protein concentration ~ 1.5 mg/mL) signal was only intermittently detected. That is to say, over the course of many replicate runs, immunoassay signal for actin was observed in about 50% of the separations. Possible rationales for this troublesome response include that it is due to some irregularity in injection, differing immunoassay response to endogenous actin and actin standards, or actin levels that are nearer the LOD than expected. Actin was detected in these samples by dot blot with 0.1 μL dots. It was unclear how this signal would compare to the small injected volumes with CGE separations (low nL). In any case, this motivated careful study of preconcentration for SDS-protein capillary gel electrophoresis.

Preconcentration in CGE by transient ITP. To investigate preconcentration potential, we characterized transient isotachopheresis with SDS-proteins in CGE. Xu *et al.* performed CGE separations using the separation buffer as leading electrolyte (LE) and were able to obtain about 30-fold improvement in sensitivity.³⁹ This paper and others report on strategies for LE and TE ion choice, from which suggestions for appropriate electrolytes were chosen.^{6,7} Using phosphate ion and GABA as leading and terminating electrolytes respectively, sample injection alone was compared to sequential injections of LE, sample, and TE. Long sample injections (on the order of 30 s) are needed to observe good signal on the blotting membrane. With or without tITP there is at least a 10x increase in peak heights for the longer injections (compare 4A to 4B). With 5 s injections of sample (30 s LE and TE injections), peak height was shown to increase by

at least an order of magnitude using the isotachophoretic method. However, with 30 s sample injections the isotachophoretic concentrating effect was not pronounced (Figure 3.4). For the types of injections necessary with CE-Westerns, tITP will not improve peak heights significantly. It was also observed that dilute samples exhibited a greater degree of enrichment than did concentrated samples (Figure 3.5). This phenomena has been noted by others employing ITP concentrators.⁴⁰ We concluded that while this technique can concentrate samples online for size-based CGE, variability from to sample to sample, disappointing preconcentration with long injections, and differential concentrating effects make it undesirable for our purposes.

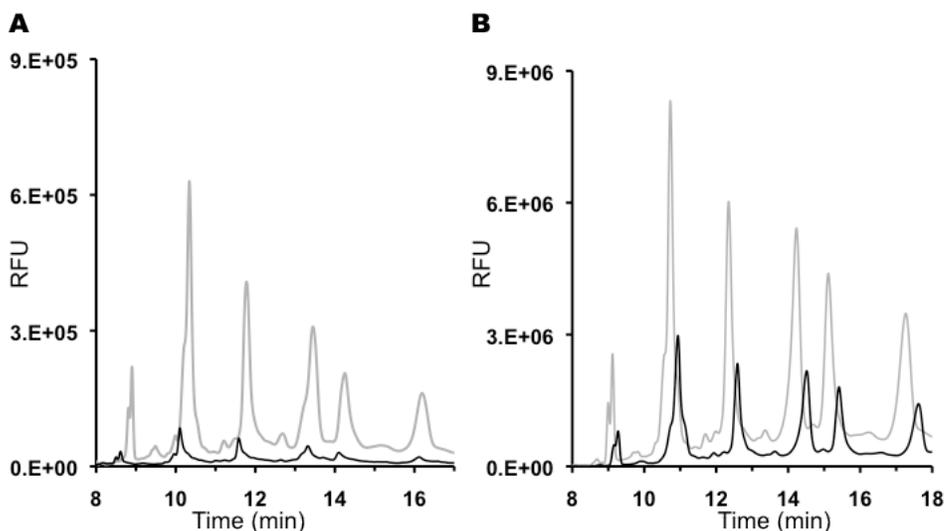


Figure 3.4. Effect of t-ITP pre-concentration on ladder separations conducted with online detection. (A) 5 s injections and (B) 30s injections with (grey) and without (black) accompanying LE/TE injections. (A) and (B) were measured on the same detector, but here the axis labels are an order of magnitude higher for (B).

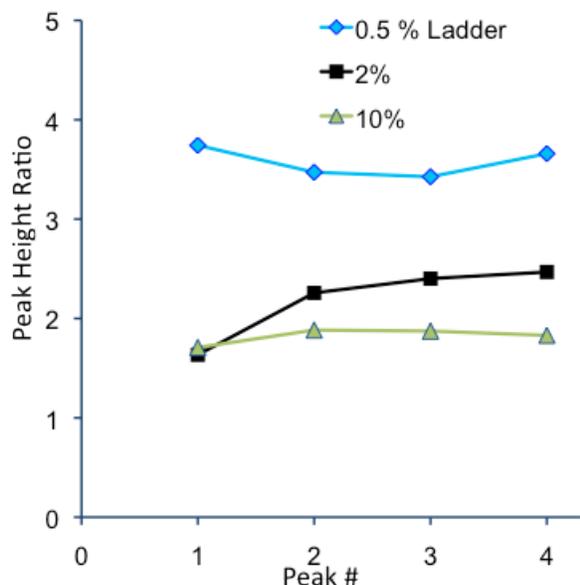


Figure 3.5. Preconcentration factor for tITP injections of different ladder concentrations. Ratio of peak heights for the first four ladder peaks with/without LE and TE injections for different percentages (v/v) of protein ladder in buffer.

Dextran-glycerol sieving media for faster CE-Westerns. For higher speed separations, relative to commercially available sieving media, we studied several entangled polymer formulations with dextran. There are wide-ranging reports of dextran CGE experiments with dextran molecular weights varying from 5000 to 2 M Daltons for separations of nucleic acids, peptides and proteins. Pilot experiments for separations of protein ladder (10-150 kDa) indicated high molecular weights were necessary to approximate the resolution of commercially available protein sieving gels (Figure 3.6). In a comparison of 500 kDa dextran to 2 MDa dextran, resolution between the two closest peaks (peaks 3 and 4) increased from 1.7 to 2.6. Peak capacity also increased for the larger MW, from 36 to 46. Commercial gel had the highest resolution and peak capacity, 3.0 and 62. Again, to achieve goals of faster separation and low current, the intermediate dextran percentage, 10% was chosen.

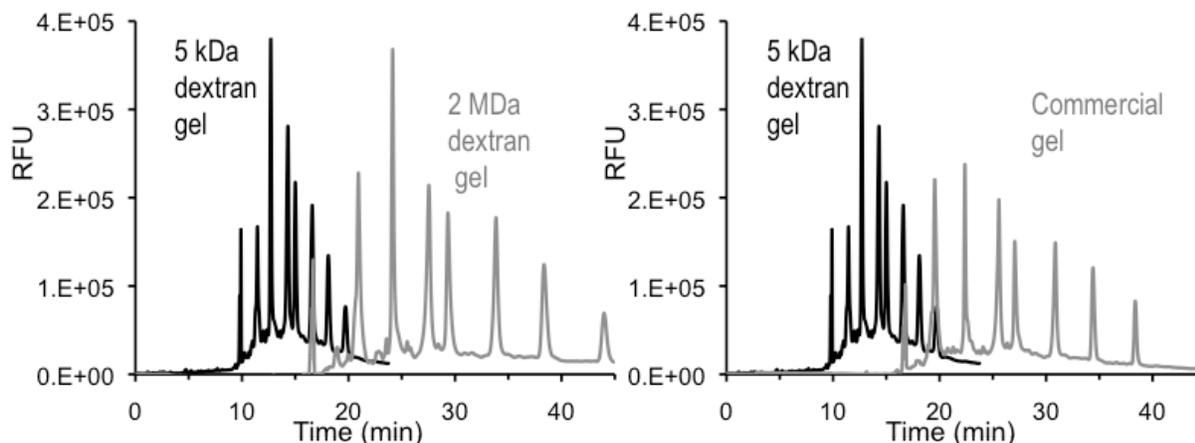


Figure 3.6. Effect of polymer MW on CGE separations. Lower MW dextran showed poor peak capacity relative to either high MW dextran or commercial sieving media. Dextran gels were operated at 600 V/cm, while commercial gel was operated at 300 V/cm.

For multiplexed CE-Westerns a favorable gel formulation would have good resolution and efficient separation in a short period of time. The separation capillary length was 20 cm, and was dictated by the geometry of the translational stage and fixtures (~15 cm would be the lower limit) and so this limiting in terms of speed. Other groups working with CGE and online detection report separations of between 10-30 minutes for similar protein ladders and conditions, though many seem to compromise either resolution or speed.^{41,42,25,17} Therefore a 20 min separation on a 20 cm capillary was a goal. Low current is also a requirement because many gels that operated well on commercial instrumentation did not operate well at reasonable applied fields (400-600 V/cm) when used in the membrane capture apparatus (Figure 3.7). This could be because of subtle differences in capillary conditioning (automated hydraulic vs. bomb-driven rinsing), or fluctuations in temperature, which was not controlled with the membrane capture instrument. Another possibility is that the sheath gel may not be enough to compensate for electrolysis products, causing more Joule heating and bubble formation than in the commercial gel platform. For dextran gel specifically, an advantage in speed (over commercial sieving media) was mainly possible at applied fields above 400 V/cm. A gel that showed evidence of

Joule heating (with a non-linear Ohm plot) at such fields would be eliminated in initial testing on the membrane capture instrument. It was observed that low current for a given dextran media, as measured on the commercial CGE platform, correlated well with linear Ohm plots and eventual success on the membrane capture platform. With parameters of high resolution, high speed and low current, the variables of a gel formulation (ionic strength, constituent concentrations, additive species) were systematically evaluated on the commercial CGE platform, for eventual use with the multiplexed membrane capture instrument.

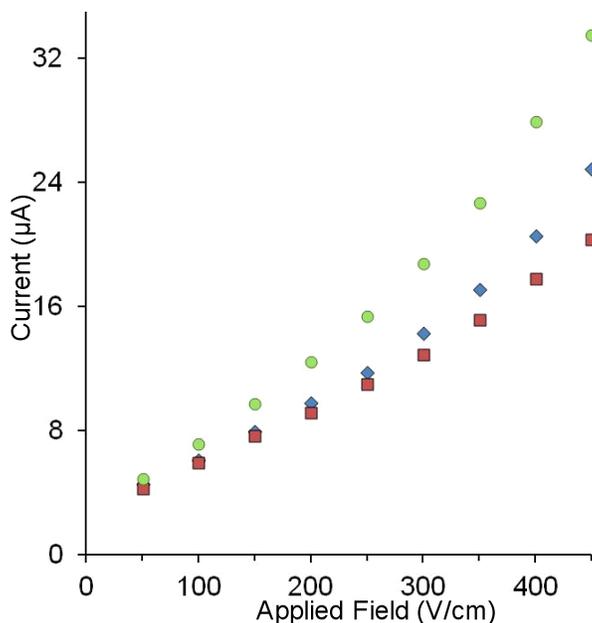


Figure 3.7. Ohm plots for commercial and two dextran gels as measured with the membrane capture instrument. 8% dextran, 10% glycerol, 400 mM tris-borate gel (circles) and commercial gel (diamonds) were linear through 350 V/cm. 8% dextran, 10% glycerol, 200 mM tris-borate gel (squares) was linear through 450 V/cm. On the commercial CGE instrument, at 600 V/cm applied field, current was approximately 33, 30, and 26 μA , respectively.

As expected, gel buffers of increasing ionic strength exhibited higher current (Fig 3.8-B). Unexpectedly, buffer ionic strength also increased protein mobilities (Fig 3.8-A). This is an indication that there is significant electroosmotic flow in this separation. Typically in capillary

gel electrophoresis this is problematic because size-based separation is compromised. Proteins experience an electrophoretic force proportional to shape and charge (or size for SDS-proteins). With electroosmotic flow they experience a net flow towards the outlet that may deteriorate separation resolution. However, mobility plots showed a linear relationship for these dextran gel separations (Fig 3.8-C). Among the three options, resolution between the close eluting peaks was highest in the lowest ionic strength buffer (Table 3.1). Peak capacity was similar in each. With this in mind an ionic strength that minimized current and maximized resolution, 100 mM tris-borate buffer, was chosen.

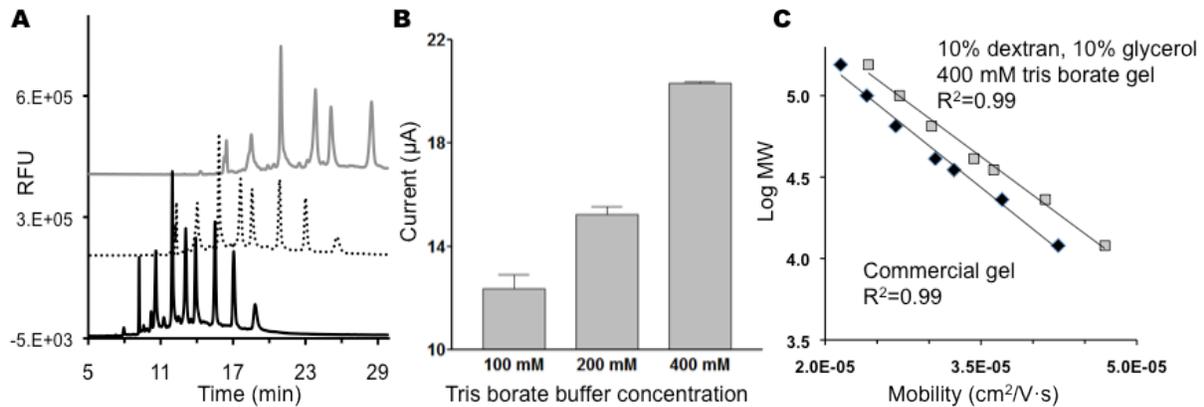


Figure 3.8. Effect of ionic strength on ladder separations and dextran gel size-based separation performance. Electropherograms (A) of 100 mM (black), 200 mM (dotted), and 400 mM (grey) tris borate, 10% dextran, 10% glycerol gel. Observed current with an applied field of 500 V/cm (B), and a representative plot (C) of mobility versus the logarithm of ladder protein molecular weights for dextran gel (squares) and commercial gel (diamonds).

Table 3.1. Resolution and peak capacity results for gel formulations. Buffer ionic strength, dextran percentage (w/w), and glycerol percentage (v/v) effects were measured in at least three levels, as described in the text. Bolded values are for the chosen conditions.

Variable	R_s	n_c
Ionic Strength		
low	3.0	53
med	2.6	56
high	2.6	52
Dextran %		
low	2.0	43
med	2.4	44
high	2.7	49
Glycerol %		
low	0.6	16
med	2.0	31
high	2.0	29

Varying weight percentages of 2 MDa dextran were also tested (Figure 3.9). More polymer resulted in both a more viscous sieving media (data not shown) as well as a much slower separation, e.g. 29 min for 100 kDa with 12% dextran instead of 18 min with 8% dextran. Interestingly, higher dextran percentages resulted in lower current. A concentration that was intermediate in speed, resolution and peak capacity, 10% dextran, was adopted.

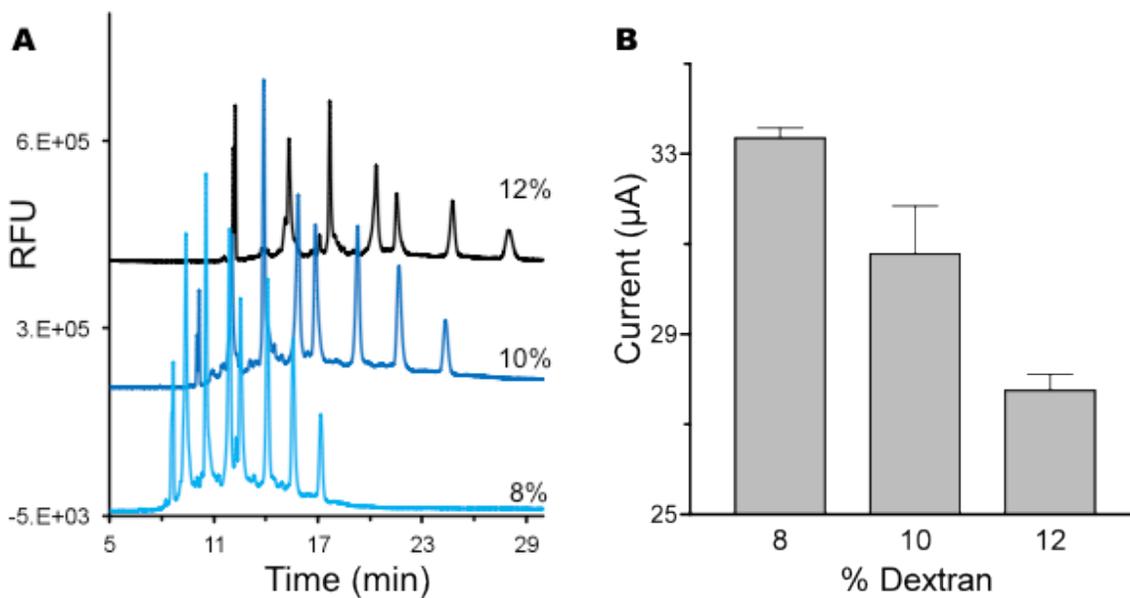


Figure 3.9. Effect of dextran levels on CGE separations. Varying weight percent 2 MDa MW dextran in 400 mM tris-borate, 10% v/v glycerol sieving media. Representative traces of ladder separations (A) and observed currents (B) for 8, 10 and 12% w/w dextran.

Perhaps the most dramatic change was seen in varying percentages of glycerol. At zero or low levels of glycerol, resolution of ladder separations was very poor (0.6 for 2.5% glycerol gel) and current was low (Figure 3.10 and Table 3.1). With increasing glycerol concentration, efficiency drastically improved, and migration slowed. Current increased with increasing glycerol contribution up to a threshold value (around 5%) at which additional glycerol did not significantly increase current (Figure 3.10). This supports the hypothesis of Mitchelson, *et al.* who offered that glycerol may complex with hydrophilic groups on a polymer backbone and borate ions in solution.⁴³ This complexation affects net mesh size and can result in a gel that efficiently sieves proteins without a drastic increase in polymer density or concentration.

To examine the mechanism of glycerol action, separations with and without glycerol and borate ions were conducted (Figure 3.11). When both glycerol and borate ions were absent

(dextran in tris-HCl buffer) separation efficiency was poor. The addition of glycerol without borate ions shifted the separation to longer times. Thus glycerol does change the sieving media viscosity, but this does not explain its full contribution to the improvement in resolution. Dextran in borate buffer showed slightly increased peak heights, even as resolution remained poor. Borate ions, as is well documented in CE literature, complex with polar species in solution.⁴³ Lastly, the best scenario with gel comprised of glycerol, dextran, and borate buffer showed efficient separation of all the ladder proteins. With 10% dextran and 100 mM tris borate buffer, 5% glycerol was sufficient to increase peak capacity and resolution, beyond which there was no additional benefit.

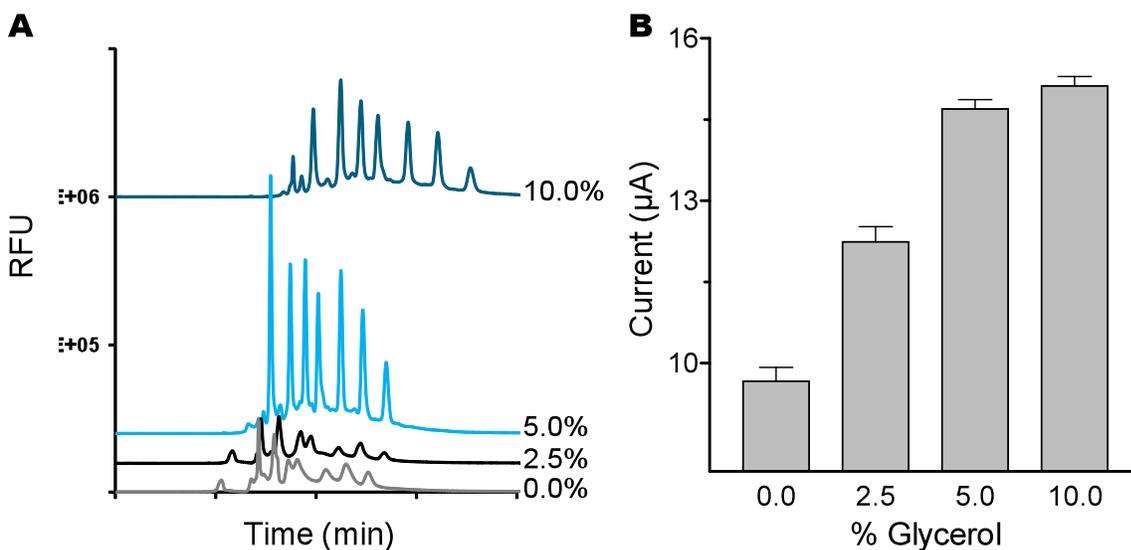


Figure 3.10. Effect of varied percentages of glycerol on ladder separations. Electropherograms (A) and current (B) shown for different gels, all with 8% dextran and 100 mM tris-borate buffer.

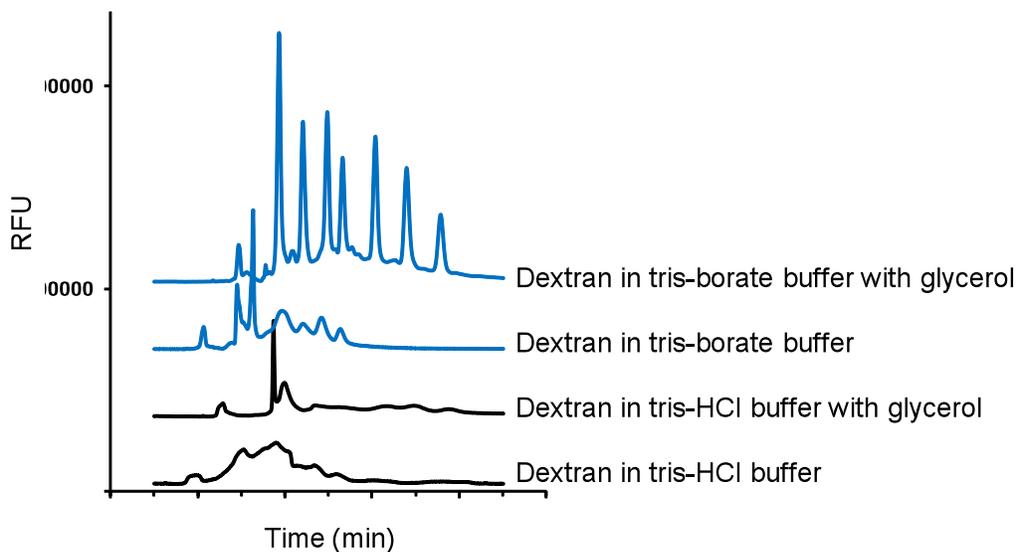


Figure 3.11. Variations in buffers and additives to probe complexation mechanism.

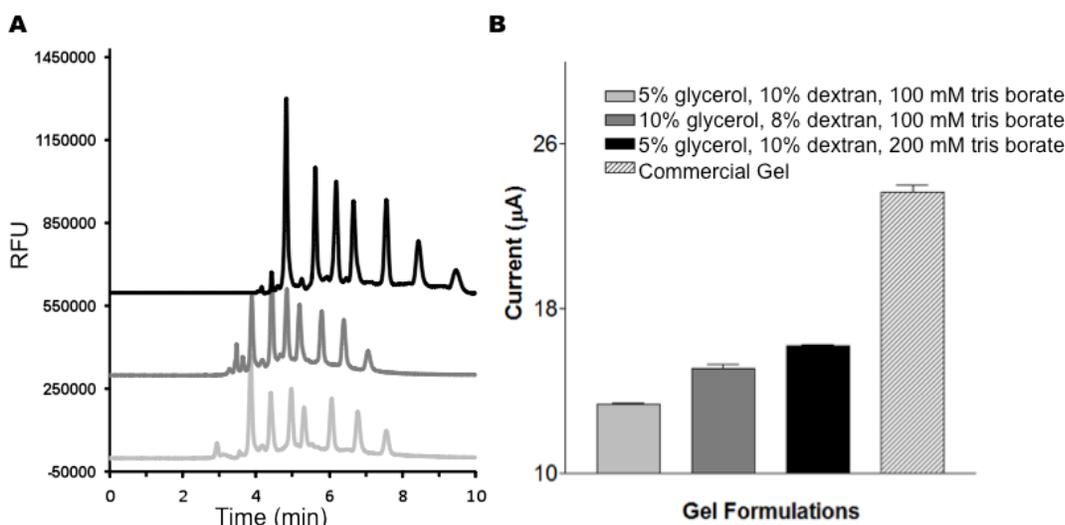


Figure 3.12. Summary of the best separation conditions for dextran gels. (A) Electropherograms and (B) observed current for 5% glycerol, 10% dextran, 100 mM tris-borate (light gray), 10% glycerol, 8% dextran, 100 mM tris-borate (dark gray), 5% glycerol, 10% dextran, 200 mM tris-borate (black), and for comparison commercial gel (striped bar).

After consideration of several combinations of glycerol, dextran and ionic strength with respect to efficiency, time of analysis and current, three options were of particular interest because they were judged to be of reasonable resolution and had relatively short separations

(under 10 minutes with a 10 cm effective separation length). When the current for these runs was compared, 10% dextran, 5% glycerol, 100 mM tris-borate buffer was chosen for its separation performance and low current (Figure 3.12). In experiments conducted on the commercial CGE instrument (P/ACE MDQ) this gel showed a 45% reduction in current at 500 V/cm when compared to commercial gel. For Beckman gel operated at 400 V/cm (slightly above membrane capture limits) and a 20 cm capillary resolution was 3.3 and peak capacity 73. Dextran gel at 500 V/cm (slightly below membrane capture limits) and a 10 cm capillary resolution was 3.55 and peak capacity 29. With a 20 cm capillary peak capacity would likely improve. Though Beckman gel has a clear advantage in that there is a large separation window, dextran gel is faster and much more reliable with the membrane capture instrument.

After consideration of several combinations of glycerol, dextran and ionic strength with respect to efficiency, time of analysis and current, three options were of particular interest because they were judged to be of reasonable resolution and had relatively short separations (under 10 minutes with a 10 cm effective separation length). When the current for these runs was compared, 10% dextran, 5% glycerol, 100 mM tris-borate buffer was chosen for its separation performance and reduced Joule heating (Figure 3.12). In experiments conducted on a commercial CGE instrument (P/ACE MDQ) this gel showed a 45% reduction in current at 500 V/cm when compared to commercial gel. For Beckman gel operated at 400 V/cm (slightly above membrane capture limits) and a 20 cm capillary resolution for the two closest peaks (peaks 3 and 4 representing MWs of 32 and 40 kDa) was 3.3 and peak capacity 73. Dextran gel at 500 V/cm (slightly below membrane capture limits) and a 10 cm capillary resolution was 3.55 and peak capacity 29. Though Beckman gel has an advantage in that there is a larger separation

window, dextran gel is faster and less viscous, which helps to alleviate band broadening in the sheath gel region.

This gel was then put in use for multiplexed capillary gel separations. In practice dextran gel allowed for higher applied fields (Figure 3.13-A) and band broadening as imaged in the sheath capillary. Above 300 V/cm commercial gel was seen to experience irreproducible separations and Joule heating (as indicated by non-linear current response to increasing applied field). Dextran gel was operational up to 600 V/cm. The dextran gel allowed for separation of 100 kDa proteins in about 15 min, relative to about 40 min with commercial media (Figure 3.13-B). Measurement of broadening on the membrane, as assessed by the width of the protein track improved from commercial sieving media (Figure 3.14), with a width of 300 μm , i.e. 150 μm broader than the capillary outlet. Previously broadening was 250 μm for an overall track width of approximately 450 μm (Figure 2.4).

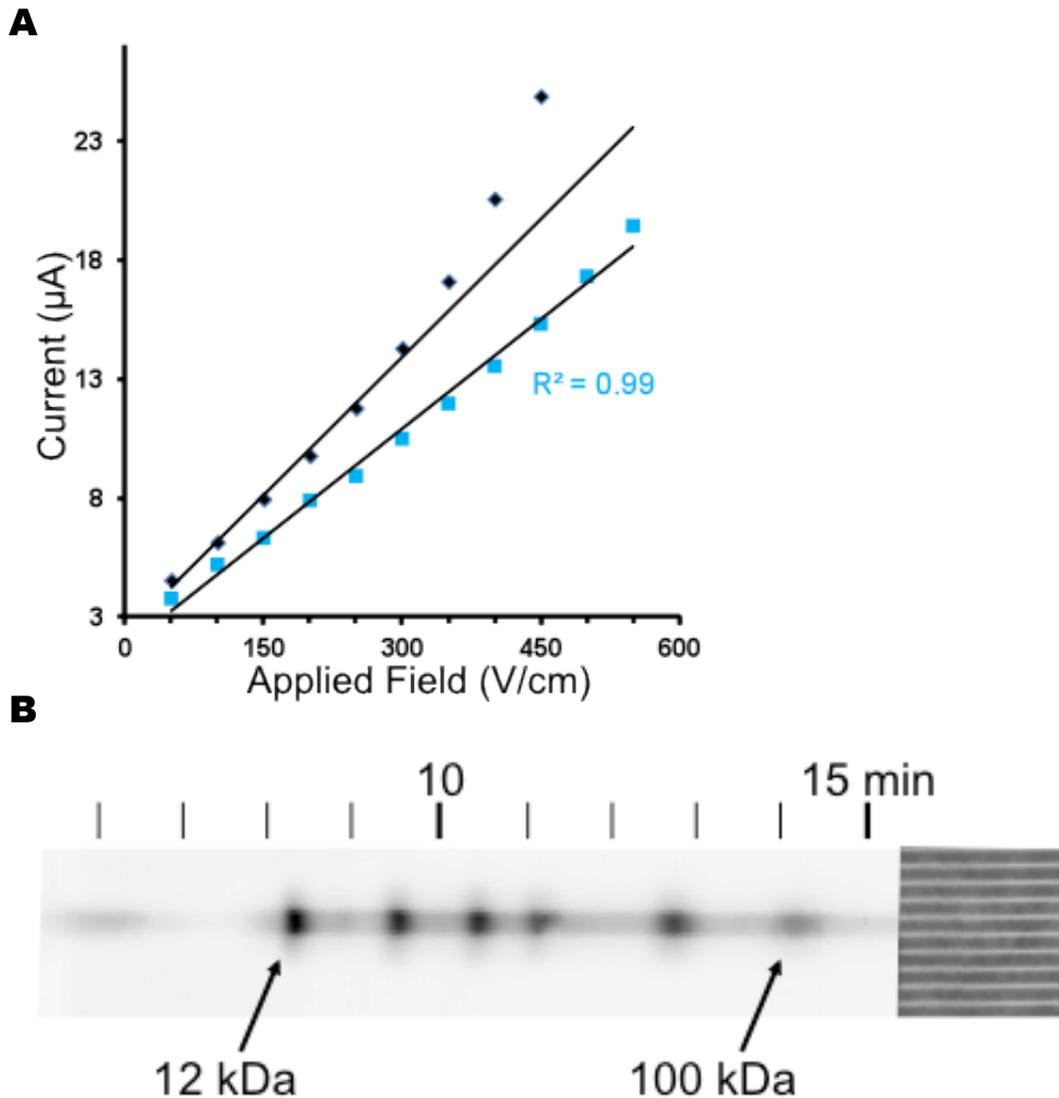


Figure 3.13. Ohm plot testing and trial separation with new sieving formula. (A) Ohm plots of commercial gel (diamonds) and 10% dextran, 5% glycerol, 100 mM tris-borate gel (squares) with the CE-Western instrument. (B) Ladder separation with membrane capture using dextran sieving media at 500 V/cm shows size-based separation of 100 kDa protein in 15 min. The image is stretched 3x on the vertical axis to better show the track width (with a 200 μ m ruler at the right of the image).

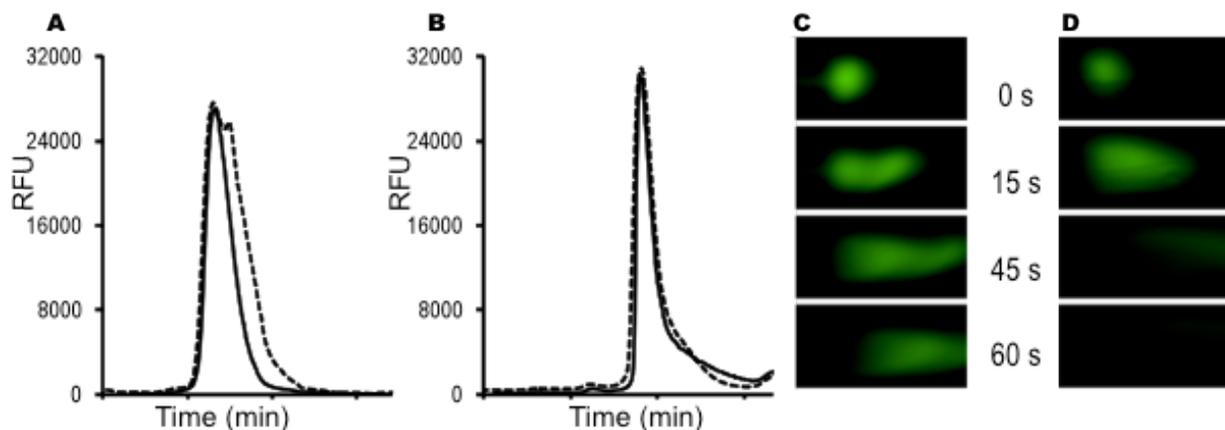


Figure 3.14. Imaging sheath gel region to investigate band broadening. Commercial (A) and dextran (B) sieving media are compared with FITC-BSA zones at the end of the separation capillary (black line), and 350 μm downstream in the sheath gel (dotted line). CCD images of protein in the sheath gel with commercial sieving media (C) shows protein lingering at the separation capillary outlet. By comparison, FITC-BSA moves rapidly through dextran sieving media sheath capillary (D).

Development of this dextran sieving media may seem outside the mainstream of CGE research, as most of the literature has focused on a certain set of water soluble polymers without much progress in recent years.²¹ Any further reports of borate-polymer complexes with glycerol after the initial studies in 1994 have not been published, and reports of protein CGE separations that use glycerol attribute resolution improvements to changes in viscosity.^{5,44} Glycerol has been favored as an additive at high concentrations (30-50%) for nucleic acid sample stacking⁴⁵ and protein capillary isoelectric focusing (CIEF)⁴⁶. For CIEF, glycerol is sought to solubilize hydrophobic proteins and reduce electroosmotic flow.^{47,48} While this work may not single-handedly revive interest in sieving matrix design, it does demonstrate that an assays detection limits are only as good as its separation. For multiplexed operation of CGE capillaries and membrane capture, a gel that did not exhibit Joule heating and provided faster separations was required. We found a variety of small changes in buffer composition can make a big difference in speed and resolution, particularly in exploitation of dextran-glycerol-borate complexation to

form sieving pores. Interestingly, in the literature separation speed has been found to correlate to effective pore sizes, i.e. polymer MW and concentration, rather than the molecular species of the sieving polymers. Peak width did vary with polymer choice.²³ With a good understanding of the factors in play (conductivity, polymer and additive composition and concentration) a gel can be quickly designed to adapt to particular protein samples. Most CGE experiments (including these) focus on proteins in the 15-100 kDa regime. It would be interesting to further develop gels that would quickly separate proteins outside this size range, as we would expect CGE to reduce separation time of large proteins as well.

Sample Preparation with reduced times. In initial experiments with commercial sieving media, dialysis to exchange higher ionic strength sample buffers was shown to allow for stable injection and separation of cell lysate mixtures in CE-Western blotting. However, dialysis is disadvantageous because it adds about 1 h of sample processing, and sample recovery from the dialysis membrane reservoirs is poor. Instead, buffer exchange via centrifugation of polyacrylamide size exclusion columns was found to be a rapid solution (about 5 min). Preparative size exclusion chromatography also was shown by electrophoresis to return peak heights of ladder in cell lysate samples to the same order of magnitude as ladder in buffer alone. One indication the size-exclusion preparation is effective is that a flat baseline is observed at the start of the separation in those runs, presumably where removal of smaller protein fragments and/or excess dye is successful. In buffer-exchanged samples, a changed mobility of the ladder proteins was also observed, perhaps because SDS was diluted below the critical micelle concentration with size-exclusion preparation (Figure 3.15). Smaller MW proteins in particular had lower mobility. With the addition of as little as 0.1% SDS to the sample size-based calibration performance returns. The addition of up to 5% SDS does slightly alter protein

migration but overall linearity between protein mobility and the logarithm of the molecular weight does not change (Figure 3.16). The sample was adjusted to contain 0.1% SDS (assuming complete depletion by size exclusion chromatography).

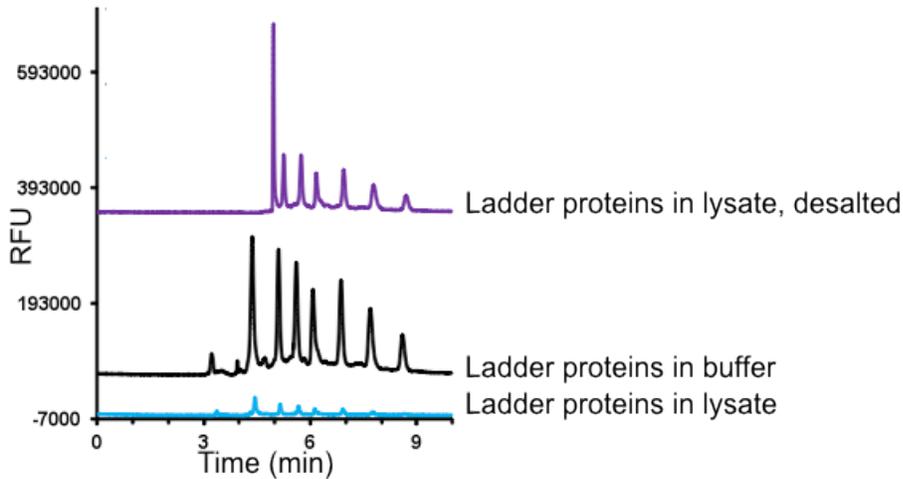


Figure 3.15. Effects of preparatory size-exclusion chromatography for buffer exchange of complex protein samples. CGE separation of ladder proteins added to buffer or cell lysate sample, some of which was desalted prior to analysis.

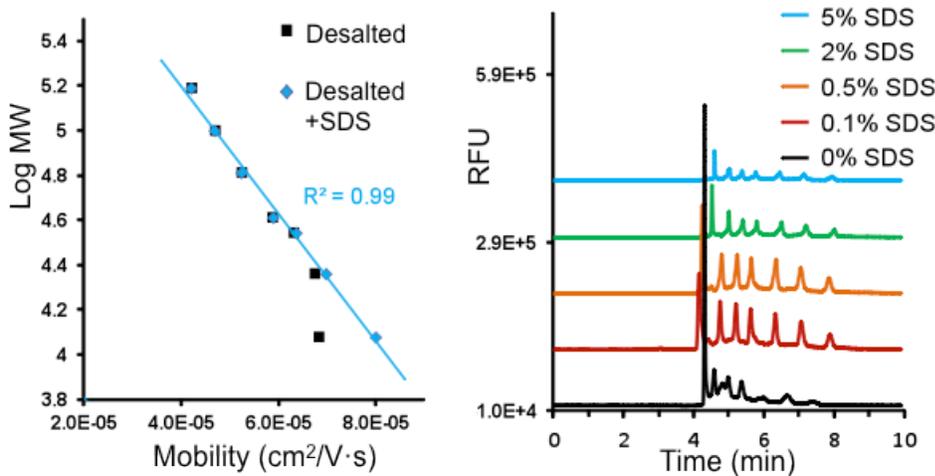


Figure 3.16. Addition of SDS to cell lysate sample after desalting with preparative size exclusion chromatography. At left, 0.1% SDS returns size-based separation performance.

Multiplexed Capillary Westerns with Dextran Gel. Dextran CGE separations of NIH-3T3 cell lysate, fluorescent ladder and, in some cases, exogenous actin protein were performed

with a homebuilt multiplexed CE-Western apparatus (Figure 3.1). Separations were conducted in experiments with two to four capillaries operated simultaneously. A common inlet gel reservoir and constant applied field for both injection and separation were found to reduce capillary-to-capillary and run-to-run variability.

In Figure 3.17, four capillaries with one separation each represent the potential for multiplexing CE-Westerns. Taking into account one half hour for immunoassay, four capillaries could return size-based information and affinity-based identification for four samples in under 1 h. In Figure 3.18, two capillaries with three separations of 15 min each, six samples were analyzed in 75 min. With the instrument currently constructed, 4 capillaries completing three 20 min separations each, could complete 12 CE-Westerns in 1.5 h. The closest result to this work is a membrane capture CGE instrument reported last year.³⁵ In that work, the capillary outlet is dragged across a membrane submerged in a buffer reservoir (in lieu of a sheath). An 100 kDa protein ladder was separated in 22 minutes, with an applied field of 175 V/cm. A large diameter capillary, 150 μm was used, presumably to inject large volumes, thus aiding detection and counteracting the effects of dilution at the capillary outlet. 500 $\mu\text{g/mL}$ AcrA protein was detected by MALDI-MS and immunoassay. While the CGE performance was very good, sensitivity was poor. Multiplexing was not investigated but presumably could be demonstrated in the future. In other research, Herr's work with microfluidic immunoassays has delved into a variety of analytical problems for protein analysis. This work is very exciting in that it clearly demonstrates the sensitive detection possible with microfluidic separations. Multiplexing has been demonstrated with immobilization of antibodies in sequence on chip to capture analytes after separation. In general microfabrication steps such as immobilization of antibodies or introduction of photoreactive hydrogels limit widespread adoption at this time.^{32, 49} Parallel

separations for bulk immunoassay has been reported with very poorly resolved proteins samples, yet this may have the easiest real-world operation. The adaptation of a traditional microarrayer, multiwell plates, and transfer apparatus provides for easier exchange of the method between laboratories, even as microarray delivery to the gel is an expensive strategy. With this method many samples can be analyzed in parallel, the authors give an example of probing separated samples with 192 antibodies for one membrane.³⁴ A great contribution of this work is a return to Westerns performed in parallel. This has not been demonstrated extensively on chip or capillary (CGE+immunoassay) until now.

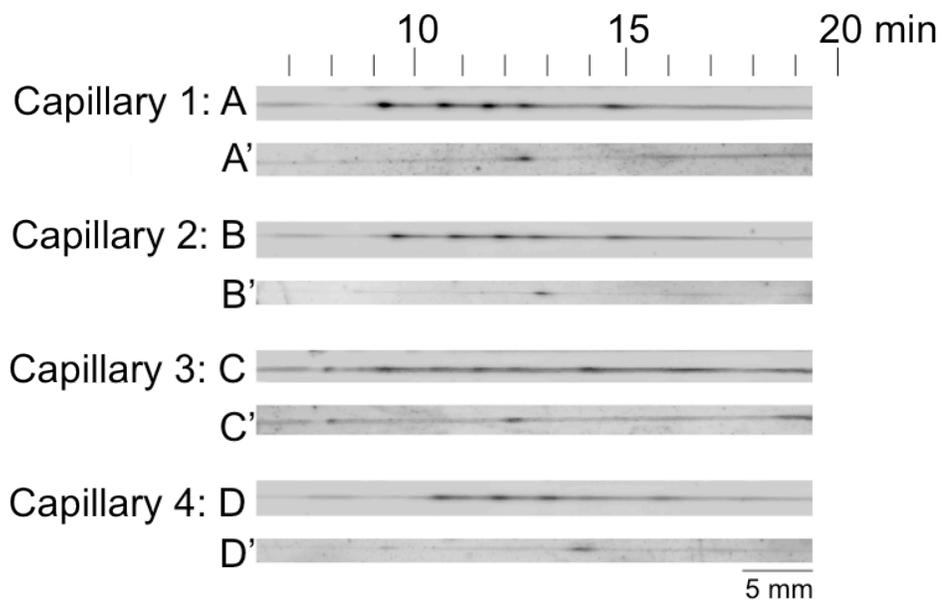


Figure 3.17. Multiplexed CE-Westerns. Four capillaries operated in parallel with separations of 0.825 mg/mL cell lysate protein and 0.25 μ g/mL exogenous actin. Traces A-D are fluorescent signal from the protein ladder (excitation and emission filters appropriate for FITC label). A'-D' traces are fluorescent signal from immunoassay for actin (excitation and emission filters appropriate for Alexafluor 546-conjugated secondary antibody).

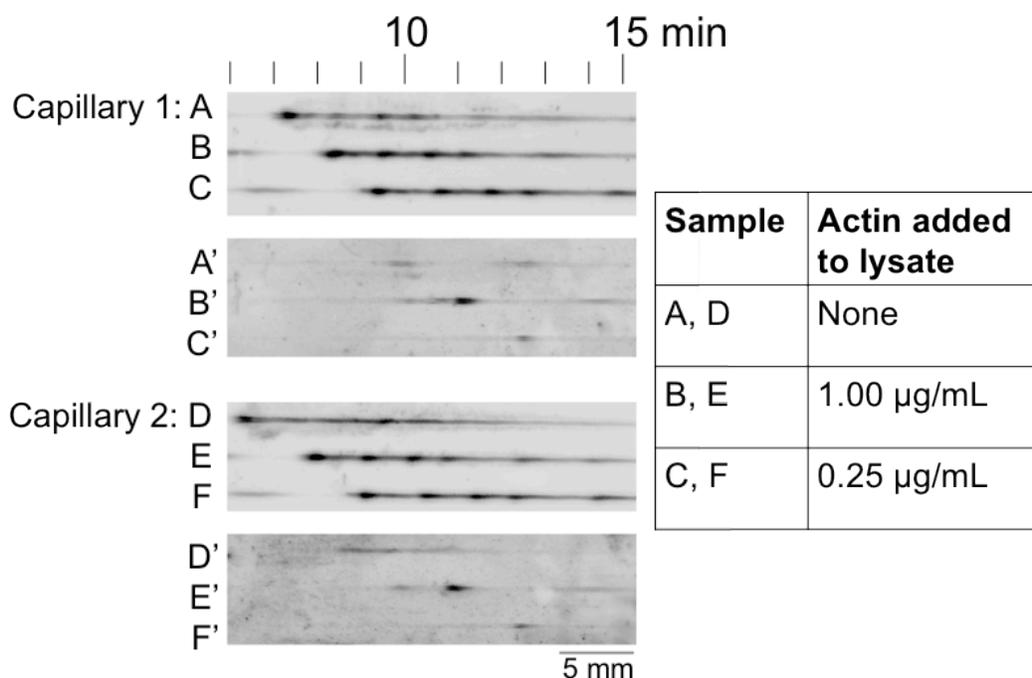


Figure 3.18. Two capillaries in parallel, with three separations performed sequentially. All samples contained fluorescent ladder standards and 1 mg/mL cell lysate proteins, with exogenous actin as noted in the table. Traces A-F are fluorescent signal from the protein ladder, while A'-F' are fluorescent signal after actin immunoassay.

Each capillary (and each injection) is independent and can be used for a different sample. This is demonstrated in Figure 3.19, where the separation capillaries were of two different lengths, yet experienced the same applied voltage. For capillary 2 the separation capillary was 17 cm instead of 20 cm, so that the separations were performed with 590 V/cm instead of 500 V/cm, as in capillaries 1 and 3. As expected, elution of both ladder and actin zones was faster with this higher applied field in capillary 2. Yet, the inclusion of ladder in each sample allows for size calibration in both scenarios (Fig 3.19-B). Mobility, as expected, was not changed with separation length.

Size-based calibration plots show that mobility was more closely correlated with run order than separation capillary. From injection to injection the separation window (time between earliest and late eluting peaks) would typically drift slightly by perhaps one or two minutes, but

it was observed to drift in all parallel capillaries in a similar fashion. This may be due to variations in ambient temperature over the course of several sequential separations. Attempts were made to eliminate any run-to-run variability due to slight changes in applied field by maintaining constant voltage after initial voltage ramping to start an experiment (conditioning).

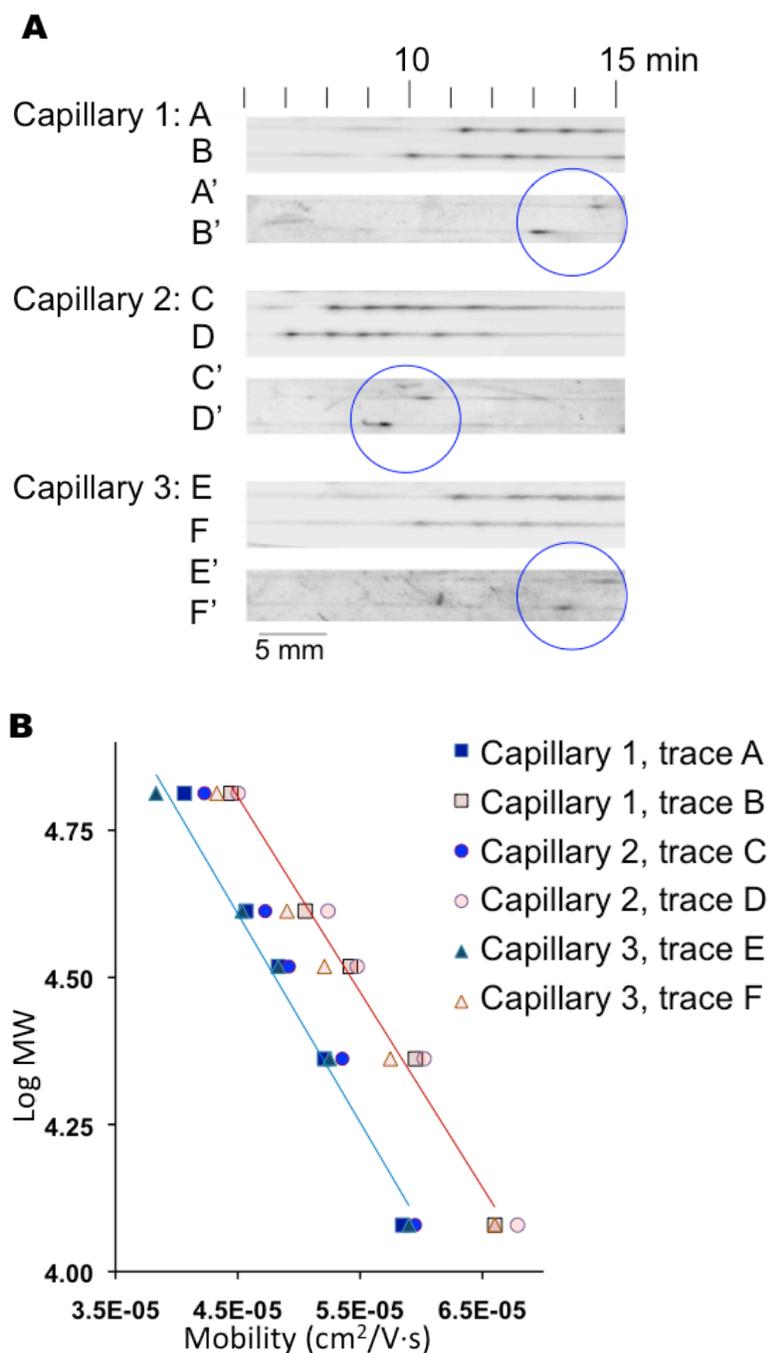


Figure 3.19. Capillaries of different lengths operated simultaneously. Capillaries 1 and 3 were 20 cm long. Capillary 2 was 17 cm. Voltage applied was 10 kV. (A) Immunoassays for actin (A'-F') show signal at about 14 min for capillaries 1 and 3, and 10 minutes for capillary 2. Each sample contains protein ladder and 0.825 mg/mL cell lysate. A', C', E' are the actin traces lysate with 0.25 $\mu\text{g}/\text{mL}$ exogenous actin. B', D' and F' are the actin traces for lysate proteins with 1 $\mu\text{g}/\text{mL}$ exogenous actin. (B) Calibration plots for molecular weight determination based on molecular weight standards. Actin molecular weight was estimated (on average over 6 runs) as $41,700 \pm 1100$ daltons.

Sensitivity with dextran CGE and buffer exchange sample preparation was improved relative to earlier trials with commercial gel and dialysis sample preparation. In slab gels endogenous actin is detected from complex samples with a total protein content on the order of mg/mL. With cell lysate samples of 1 mg/mL or 0.825 mg/mL total protein, endogenous actin was detected in every run where the ladder separation was well behaved (Figures 3.17 and 3.18). With cell lysate samples of lower overall total protein concentration (e.g., 0.4 mg/mL) endogenous actin was not observed and presumed to be at levels below the LOD (data not shown). As seen in slab gels and single capillary CE-Westerns, some quantitative information could be gleaned, especially at actin levels well above the LOD. For instance in Figure 3.20, cell lysate with an additional 1 $\mu\text{g/mL}$ actin displayed a dark zone upon immunoassay, relative to cell lysate alone or cell lysate with 0.25 $\mu\text{g/mL}$ actin. For ladder separations on the membrane capture instrument, the resolution of the 2 closest peaks, peaks three and four, was 1.5 and 1.2 for these separations. The peaks were not always baseline resolved (defined as $R_s=1.5$), but could be distinguished from each other for size calibration purposes. Dark regions in between protein spots on the membrane were thought to be labeled ladder proteins of intermediate MWs, possibly degradation products. With intermittent cuts in applied field, as the capillary continued to move across the stage, no signal was seen without voltage, and signal returned as voltage was again applied. This indicates that the fluorescent signal is not due to the capillary dragging excess protein along the capillary track, but instead the signal is due to the electromigration of fluorescent species. Further study of the regions between ladder peaks with MALDI would definitively answer this question.

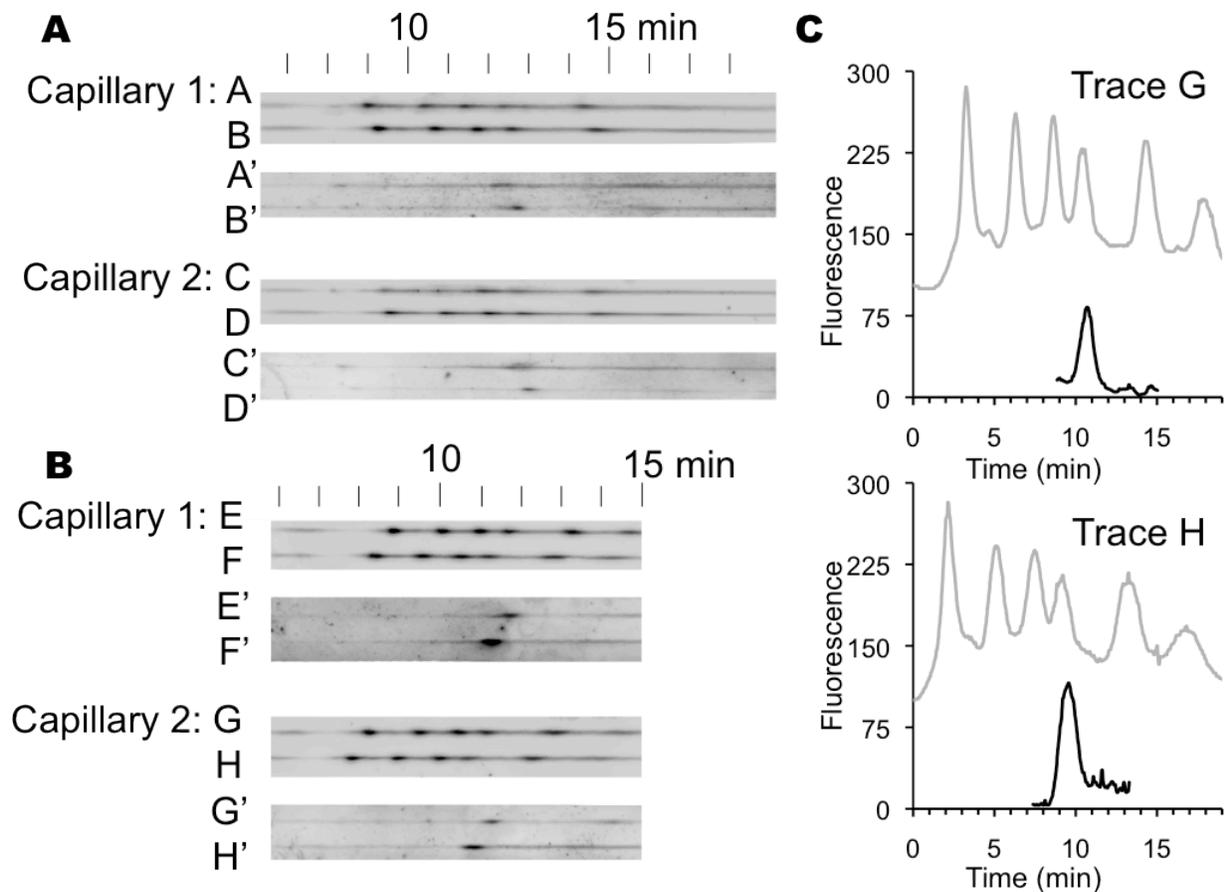


Figure 3.20. Endogenous actin detected in cell lysate, and semi-quantitation with varying levels of actin. (A) Traces A' and C' are the actin immunoassay signal for 0.825 mg/mL total cell lysate protein. Traces B' and D' are the actin immunoassay signal the same cell lysate sample and an addition 0.25 $\mu\text{g/mL}$ exogenous actin. (B) In capillary 1, E' and F' represent 0.825 mg/mL with 0.25 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ exogenous actin respectively. Capillary 2 is a replicate of capillary 1, run in parallel. (C) Reconstructed electropherograms of separations G and H in grey, with actin signal (G' and H') in black.

Several minor adjustments to the CE-Western apparatus were made to improve performance with multiplexed CGE separations. First, filter paper was placed under the PVDF membrane, rather than using a wick dragged across the surface. For sequential runs on the same membrane, the filter paper was sometimes rewetted with methanol/buffer between each run; a few (3-5) minutes passed before the next exposure to analyte to avoid diluted protein traces. In

conditions of higher humidity, rewetting was found to be unnecessary for, and was indeed detrimental to protein binding.

With single capillary CE-Westerns, the outlet of the sheath capillary was cut straight across. Thus when the capillary was held at a 45° angle only one edge made contact to the PVDF membrane (and electrical ground). In order to make better contact to the stage both 45° angled and coned capillaries were assessed (Figure 3.21). Angled capillaries do make a lot of contact to the membrane, but this comes with a strict requirement that they be correctly oriented to the stage. If there is a slight tilt or twist of the angled sheath capillary contact is poor, and the tip of the capillary can catch and rip the PVDF membrane. As more separation capillaries were run in parallel, coned capillaries were found to be easier to implement. Additionally, less surface area of the coned sheath capillary makes contact to the stage, reducing chances to smear the protein track. Several different placements of separation capillary inside the sheath capillary were evaluated. With the outlet separation capillary 400 µm upstream from the coned region of the sheath capillary, rates of success were improved. During separations with high applied fields, when the separation capillary was placed nearer the end of the sheath capillary bubbles were more likely to form in the separation capillary and cause current failure. With the separation capillary further inside the sheath capillary (>400 µm) band broadening would be expected to worsen.

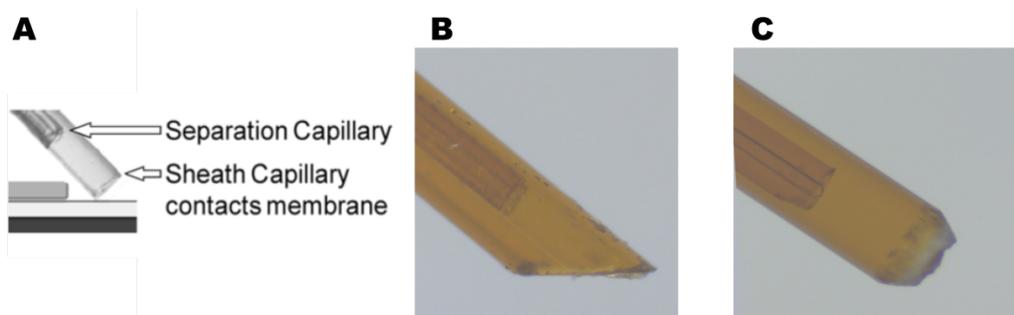


Figure 3.21. Different sheath capillary profiles. (A) Flat fronted sheath capillary used in single capillary CE-Westerns. (B) Angled and (C) coned sheath capillaries.

At this stage, no attempts were made to streamline injection with multiplexed CE-Westerns. We envision in the future that instead of manual injection onto one capillary at a time, all parallel capillaries could be moved between multiwell plates for injection and separation reservoirs, much as with bundled capillaries developed for DNA sequencing. Carryover due to sample adhesion on the outside of the separation capillary inlet was not observed in these experiments, but a capillary wash step (dipping capillaries in water) is a common practice to mitigate this issue.

One major challenge to successful multiplexed CE-Westerns was design of an apparatus that would hold the separation capillaries in place and allow for even contact of the sheath capillaries to the membrane. The apparatus was also raised several mm from time to time to allow for movement of the membrane and capture of the next separation. To improve positional control of the separation capillaries the apparatus was placed on an optics table. Coarse adjustment knobs of micropositioners were used to raise and lower the capillaries, which were all mounted to a single horizontal rod (Figure 3.22). The apparatus worked well in that each PEEK tee holding a sheath and separation capillary could easily be removed and replaced as needed for new experiments. Voltage ramping of parallel capillaries before sample injection was generally successful, with success rates as high as 90%. Sheath gel assembly and capillary conditioning

were well controlled. Additionally, success rates of single capillary separations with dextran gel and buffer exchange sample preparation were good (over 80%) provided ambient temperatures were below 25 °C. However, in the course of three to four separations with three or more capillaries, consistent performance was rare.

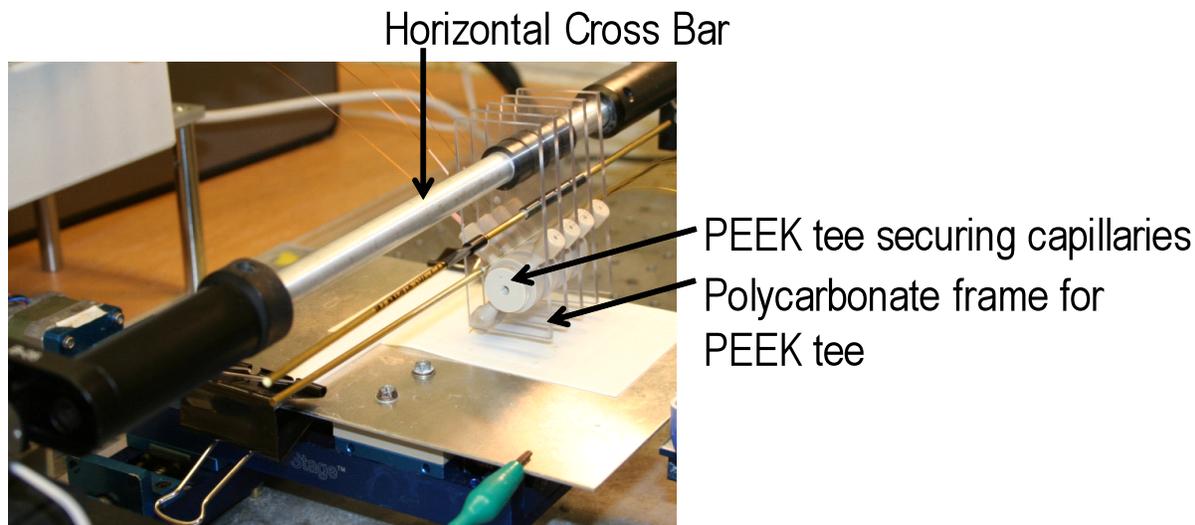


Figure 3.22. Multiplexed CE-Western apparatus photograph. PEEK tees surrounding the separation and sheath capillaries were held in place by polycarbonate frames mounted to a horizontal cross bar. The bar could be raised and lowered on both sides, and the frames could be rotated on that axis, so as to make contact of all sheath capillaries to the PVDF membrane on the translational stage.

When traces were found to diminish or not appear on the PVDF membrane this nearly always corresponded to a decline in current and appearance of bubbles in that separation capillary. While lack of contact of the sheath capillary to the membrane was not observed to be a direct cause of run failure (the separation was not absent because the capillary made no contact), it is posited that minute differences in sheath/blot interfacing from one capillary to the next is the root cause of variation between capillaries operated in parallel. Fine control of the height of each capillary was difficult to implement, especially as after each separation the whole apparatus needed to raise several mm to allow the translation stage to move the membrane for

capture of the next separation. Further engineering to place multiple sheath capillaries at fixed (but not permanent) positions in three dimensions would be anticipated to provide for rugged operation of this method.

CONCLUSIONS

Multiplexed operation of CGE capillaries, operated in parallel, demonstrated higher throughput than was demonstrated previously with one separation capillary. Changes in sieving media allowed for faster separations, and while isotachophoretic preconcentration was not successful, knowledge of the gel composition should aid in future efforts at manipulation of electrokinetic injections. This work brought up a curious result in that there was not an adequate way to measure sensitivity of the immunoassay component if troubleshooting was required. The normal method, dot blotting 100-1000 nL samples on a membrane in large spots did not provide an accurate response for what might be expected from injection of a few nL, separation and transfer: a relatively narrow peak on a PVDF membrane (300 x 1000 μm). Further discussion of solutions for this quandary is presented in Chapter 4.

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CHAPTER 4

CAPILLARY DOT BLOTTING

INTRODUCTION

Western blotting is a powerful bioanalytical tool for identification of target proteins in tissue or cultured cell samples. The separation component allows for a reduction of sample complexity, and signal is proportional to abundance. However, traditional Western blotting is laborious and requires large samples. In instances when a separation is not needed to ensure adequate immunoaffinity response, reverse phase assays have great utility. In a “reverse phase” assay, sample is bound to a substrate and then probed with antibodies directly, often without prior sample preparation. “Forward phase” assays feature immobilized capture agents (antibodies) that capture targets in a mixture.¹⁻³ Advantages for both forward and reverse assays include small volume requirements and straightforward multiplexing for the generation of protein arrays. Without a separation component, time and sample loss are reduced because protein arrays can be performed with much less manipulation. When performed manually, reverse protein arrays are called “dot blots”, while a more massively scaled analysis is termed a “protein microarray”.¹ With use of capillaries to deliver discrete volumes to a substrate we demonstrate a route to fast, inexpensive, automated dot blotting for use when microarrays are not feasible or greater sensitivity (relative to dot blotting) is required. There is also an exploration of

large volume accumulations for immunoassay, with the aim of detecting minor constituents of dilute solutions by immunoassay.

Microarrays are in use for high throughput screening and diagnostic assays in several formats. Planar microarrays are the immobilization of protein sample or antibodies to a surface for probing. Fabrication of planar microarrays can be performed in many different variations of pins, stamps and spray nozzles.⁴ Ideally, a dense matrix of spots, each spot 10^2 - 10^3 μm in diameter, is created while minimizing volume requirements, sample denaturation and cross-contamination.² Contact printing with solid or split pins (where sample sticks to the end of the pin or enters the interior shaft of a pin) is the most common format for planar arrays. Pins move between wells in a multiwell plate and then contact a substrate, sequentially moving sample from liquid reservoirs to a substrate in an automated fashion. Reproducibility is dependent upon sample loading and discharge, which in turn can change with many factors such as solution viscosity, uniformity of pin surfaces, pin cleaning between samples, etc.⁵

In the design of a protein array factors such as reproducibility, infrastructure costs, and assay sensitivity, among others, must be considered. Contact printing is the most prevalent protein array format in use in clinical and research settings. Limitations of the method mostly relate to the high maintenance costs of the pin array, which must not clog or deform when contacting a substrate. Inherent in the pin design there is little variation of solution volume delivered to the membrane, except in deleterious cases where solution viscosity or adsorption hampers delivery of a discrete volume. Non-contact methods such as ink-jet or electrospray may reduce sample contamination and design costs, but irregular spot size and sample denaturation (due to imparted charge with application of an electric field) are significant disadvantages.⁴ Variability is quite high, with a coefficient of variation (CV) typically $\leq 15\%$. At protein levels

near the lower limit of quantitation, a CV of $\leq 20\%$ and 80-120% accuracy is acceptable for FDA regulatory purposes, in pharmacological and pre-clinical studies.¹

Many substrates have been evaluated and implemented for use with protein arrays. PVDF and nitrocellulose, as in Western blotting, have excellent properties for protein attachment via adsorption and absorption. Covalent cross-linking with aldehyde or epoxy-activated surfaces is in commercial use, and can provide for high density, well resolved arrays. Attachment using affinity binding requires a labeled target, but then may give selective information. In addition to immobilization of antibodies, common affinity array strategies involve avidin or Ni-NTA coated surfaces, which bind biotinylated or Hisx6 tagged proteins, respectively.⁵

Control of spot size and volume are critical for good limits of detection. In the late 1980s, Ekins theorized that with dense spots of capture molecules (a small dense spot of antibody) the antigens captured from solution will not change the bulk solution concentration even if the overall concentration is low.⁶ A conceptual diagram of spots with different concentrations of antibody is shown in Figure 4.1. With dense microarray spots, signal intensity is not dependent on the bulk solution, but rather is concentration sensitive. It follows that, in the reverse phase assay case, if the dense spots contain protein sample instead of antibodies, the antibody solution used in incubation does not differentially alter the signal and therefore the signal is related to the protein concentration of the sample deposited to that spot. Small spots are important not only for sample-limited analyses, but because sensitivity is enhanced when the same volume is confined to a smaller region and signal density is optimal.

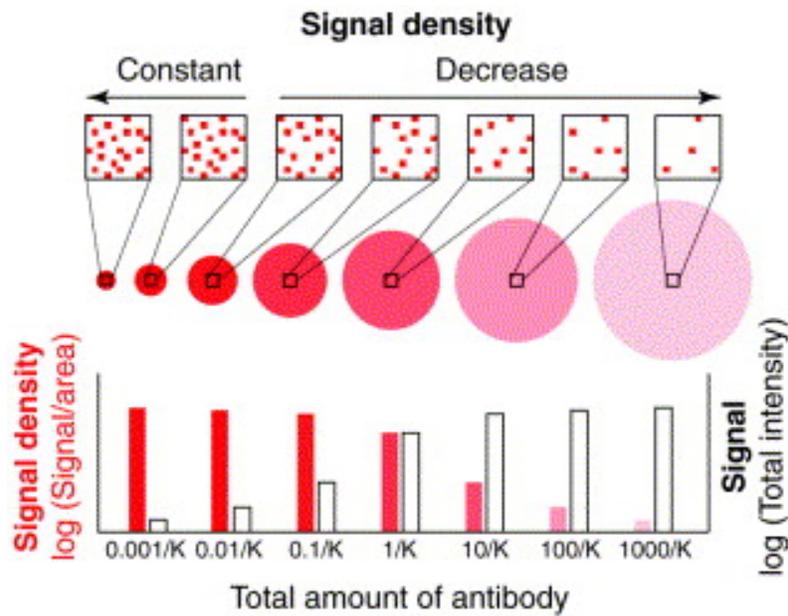


Figure 4.1. Microarray theory predicting concentration sensitivity. Representation of signal intensity (white bars) and density (dark bars) with varied dotted antibody concentrations. Signal density is uniform in small spots. Reprinted from Templin, M. F.; Stoll, D.; Schrenk, M.; Traub, P. C.; Vohringer, C. F.; Joos, T. O., *Trends in Biotechnology* **2002**, *20* (4), 160-166, with permission from Elsevier.

Contact printing takes advantage of this concentration dependence phenomenon by employing very narrow spot dimensions (around 50-300 μm). However, volume control is minimal and thus spot density (or sensitivity) is not as high as is theoretically possible.² Compared to DNA arrays, protein arrays face many challenges. Protein tertiary structure can be important for function or recognition, but proteins are relatively fragile. Next, proteins are complex molecules with varied hydrophobicity or charge states, so design of universal substrates is complex. Lastly, proteins lack an amplification scheme, such as polymerase chain reaction, which revolutionized the study of DNA.² For these reasons sensitivity is of the utmost importance in protein array biotechnology.

Microscale fluid handling with capillaries offers an opportunity for low cost protein arrays. Materials (pump, translation stage, tubing) are relatively inexpensive and robust. Importantly,

sensitivity may be improved when necessary because the amount of sample deposited in one spot can be controlled and varied by the volume of a sample deposited. In this method the substrate may take on a role as an extraction (stationary) phase, whereby sample is both deposited and concentrated in a narrow region of the substrate. In this chapter we demonstrate excellent sensitivity (pg/mL-ng/mL) for capillary dot blotting with low to mid range volume (5-500 nL) samples confined to 100s μm dots. Further efforts to increase sensitivity with large volume accumulations (500 nL-5 μL) show mixed results, dilute protein solutions show irregular spot densities, but when target analyte is a major component (>1%) dramatic concentration is observed.

EXPERIMENTAL METHODS

Lysates, antibodies and PVDF Immobilon-FL membranes were described in Chapter 3. Fluorescent signal was measured, as before, by a Typhoon 9410 scanner. Additionally, confocal fluorescence microscopy was performed with an Olympus instrument. For capillary dot blotting, some assays were conducted with samples provided to the researchers: proteins secreted from tissue cultures into EpiLife media, as well as with blank EpiLife not exposed to culture dishes. EpiLife media and EpiLife growth supplement (added to media) were from Life Technologies (Grand Island, NY).

In the capillary dot blotting apparatus (Figure 4.2), several capillaries were ground into a cone shape and then secured to each other in parallel (Figure 4.3). The end of each capillary was held rigid and a blotting membrane was moved past the tip, much as with the capillary Western blotting apparatus described in Chapters 2 and 3. Solution was pumped through the 18 cm capillaries at a constant rate using a syringe pump (Chemyx, Stafford, TX). Solution was

allowed to accumulate for a period of time, and then the stage was moved, leaving behind parallel sample dots. Sample flow was continuous, so that a small amount of sample did elute as the stage moved between accumulation spots. A LabVIEW program directed stage motion at a rapid rate (300 mm/min). Flow rates, accumulation times, and capillary dimensions were varied as described in the text.

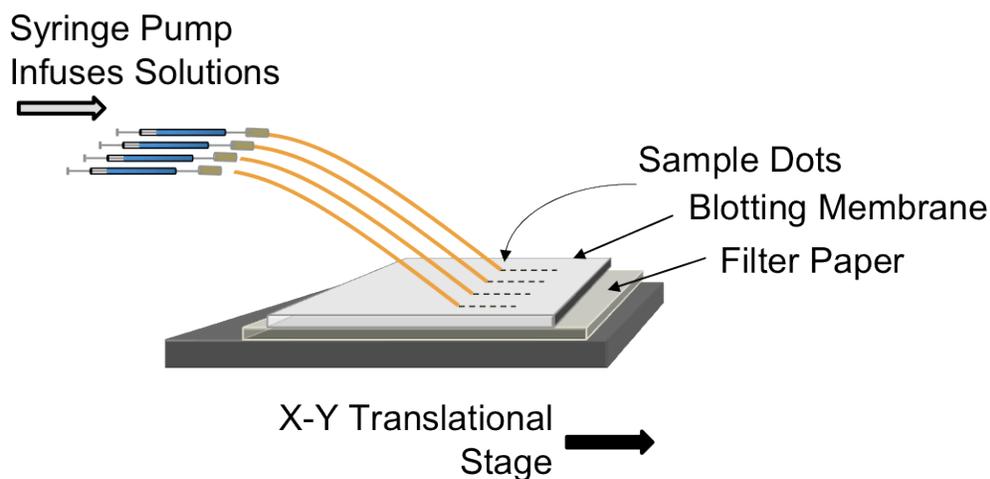


Figure 4.2. Capillary dot blotting apparatus. Sample pumped through capillaries directly interfaced to a PVDF blotting membrane mounted on a translational stage.

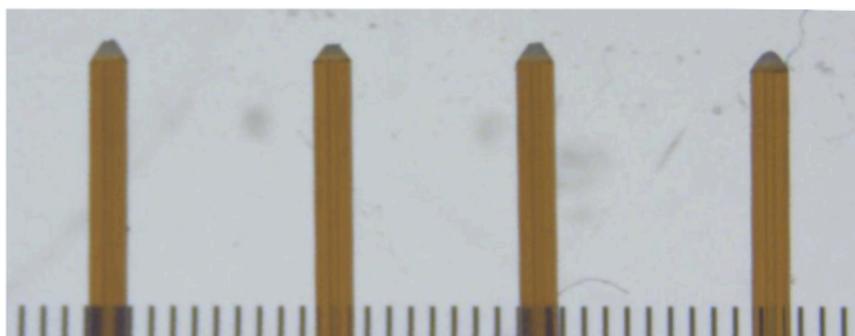


Figure 4.3. Close view of parallel dot blotting capillaries. 75 μm i.d., 360 μm o.d. coned capillaries secured to each other in parallel. A 200 μm increment ruler is included at the bottom for scale.

Treatment of membranes was identical to Western blotting. For pipetted and capillary dot blots there was a brief rinse in methanol, equilibration in water (2 min), and then equilibration with 50:50 (v:v) methanol and 200 mM tris HCl pH 8.4 buffer (5 min).

RESULTS AND DISCUSSION

Capillary dot blotting was first evaluated relative to the simplest form of a protein array, a dot blot. For the traditional dot blot, several concentrations of actin in buffer were pipetted at the smallest volume possible with the manual pipette, 0.1 μ L. The lower concentrations of the actin dilution series were also flowed from a capillary and allowed to accumulate on a PVDF membrane into either 5 or 100 nL dots (Figure 4.4). With immunoassay for actin, signal for the next-to-lowest concentration, 1 μ g/mL (25 nM) actin, was significantly higher for the 5 nL capillary dot blot than the pipette dot blot, even though capillary dots were 20x smaller in volume. Though replicates were not performed for 100 nL capillary dots, they were observed to be easily detected, especially compared to faint pipette dots. Time of analysis was similar between the methods. Solution was flowed through the capillaries at a rate of 50 nL/min so that the capillary dot blot was completed in the time it took to assemble syringe/capillary connections plus < 1 min to deposit four sets of 5 nL drops. At these low volumes membrane equilibration in transfer buffer is the slowest step for both manual pipetting and capillary dot blotting (~7 min).

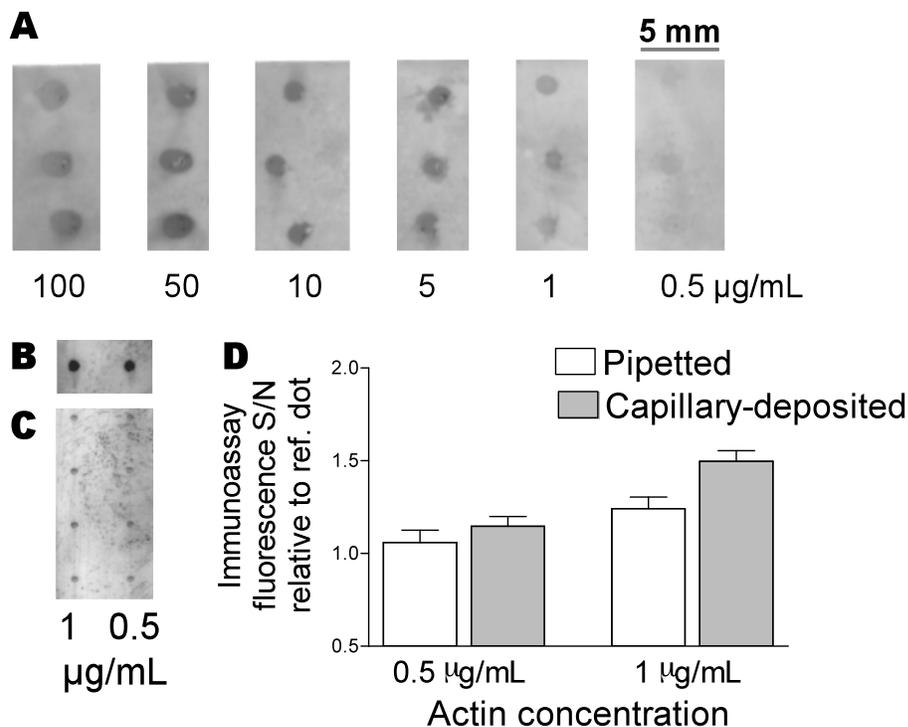


Figure 4.4. Comparison of immunoassay signal for hand-pipetted and capillary-deposited dot blots. (A) 100 nL pipetted dots of actin at various concentrations. (B) 100 nL dots of actin at low levels deposited with parallel capillaries. (C) 5 nL dots of actin at low levels with capillary deposition. (D) Comparison of signal from pipette dots and capillary dots, with signal for that dot and a neighboring blank region relative to S/N for a reference dot. All dot images are on the same size scale.

Compared to manual dot blotting, capillary dot blotting offers some advantages in maintaining uniformity throughout the experiment. In capillary dot blotting four capillaries may be operated in parallel with one syringe pump. More capillaries could be aligned, using tees so that one syringe might infuse solutions into two capillaries. With simultaneous deposition any variability in conditions during the assay (such as membrane drying) occurs across all dots in the series. Regular placement of dots at intervals controlled by automated motion of the translation stage also aids interpretation of the blot as the pattern makes obvious where signal would be expected if present.

To evaluate working conditions for capillary dot blotting, fluorescently labeled bovine serum albumin (FITC-BSA) was flowed through a capillary, deposited onto a membrane, and scanned without need for immunoassay. To simulate a complex mixture, unlabeled BSA was included in the sample, typically 1 mg/mL to approximate total protein content in a growth media or cell lysate scenario. Unlabeled BSA was added to simulate the so-called “matrix effect” or decreased binding observed in protein microarrays when many proteins in a complex mixture saturate a particular substrate region.¹ Flow rates and capillary diameters were varied to start.

Effect of flow rate. For a specific volume, 50 nL, dot size did not significantly differ with different flow rates. However, flow rate did have an effect on signal intensity (Figure 4.5). It was found that very low flow rates such as 10 or 25 nL/min gave higher signal than flow rates above 50 nL/min. Among faster flow rates (50-500 nL/min) there was not a significance difference (Figure 4.6). This was also true at larger volumes. With 500 nL 0.5 $\mu\text{g/mL}$ FITC-BSA drops, flowed at 100 nL/min, fluorescent signal/noise for replicate dots was 5.1 ± 1.1 . With the same sample volume flowed at 500 nL/min signal was not significantly different, 4.66 ± 0.64 (data not pictured). If low volume dots are appropriate for the assay, low flow rates may be preferable for highest sensitivity. Described below are capillary dots with volumes as high as 5 μL , wherein depositing dots at a rate of 10 nL/min is impractical. For medium or high flow rates signal is diminished by up to 30%, but significant accumulation is still achieved. For good sensitivity, which is related to sample concentration, dot volume, and dot dimensions, high flow rates may be used to quickly deposit proteins for immunoassay.

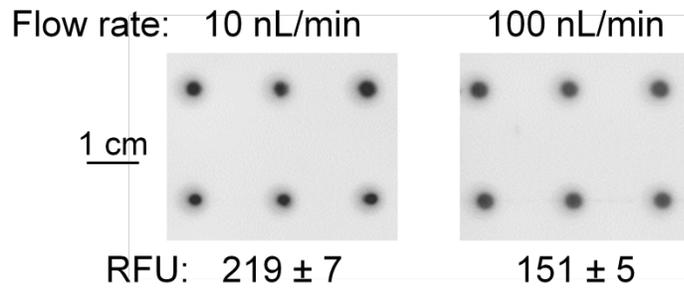


Figure 4.5. Fluorescence intensities of FITC-BSA at different flow rates. Values are indicated below the dot blot images, and dot represents 50 nL of 6 $\mu\text{g/mL}$ FITC-BSA and 1 mg/mL unlabeled BSA.

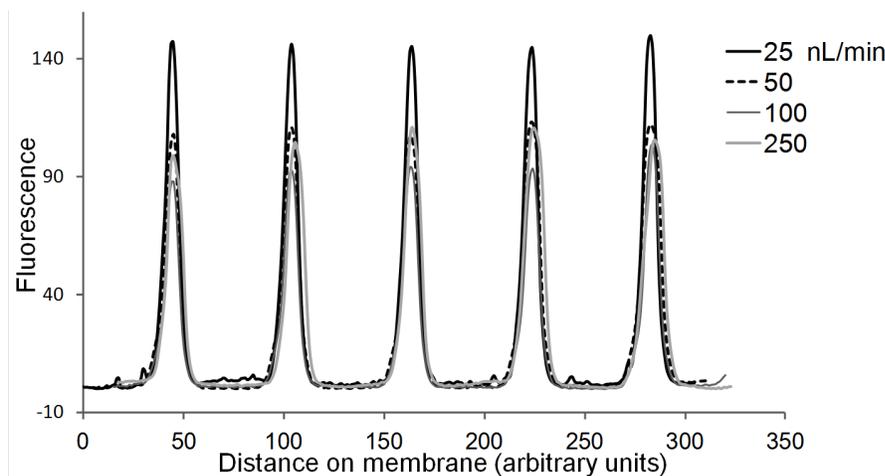


Figure 4.6. Line scans of FITC-BSA protein deposited at different flow rates. Flow rates were 25, 50, 100, and 250 nL/min. In each case five 50 nL drops of 6 $\mu\text{g/mL}$ FITC-BSA with 1 mg/mL unlabeled BSA were deposited.

Effect of capillary diameter. It was thought that the diameter of the capillary delivering solution to the membrane might influence the dot dimensions and density. For instance, a 20 μm i.d. capillary would provide a narrower outlet so that as the sample left the capillary it might remain in a narrower region overall. No trend was observed over a range of common capillary inner diameters (Figure 4.7). 360 μm o.d. capillaries were tapered to reduce the amount of glass contacting the membrane (Figure 4.3). In general 75 μm i.d. capillaries were used, because they had an intermediate backpressure that was desirable for ease of use.

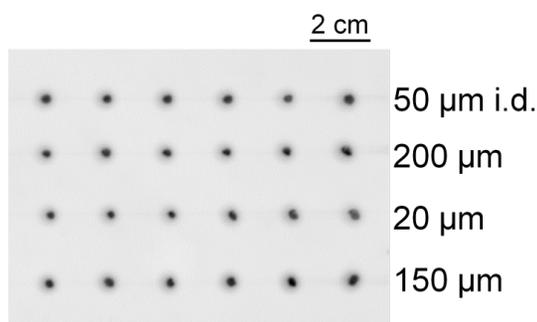


Figure 4.7 Fluorescence intensities FITC-BSA dots flowed from different capillary diameters. With 25 nL FITC-BSA dots delivered at a rate of 50 nL/min from capillaries with 360 μm o.d. and i.d. ranging from 20 to 200 μm .

Sensitivity and Quantitation. To assess capillary dot blotting for quantitative ability three concentrations of vascular endothelial growth factor (VEGF) were tested (Figure 4.8). VEGF is a 42 kDa signaling protein found in many human tissues. Among other functions, it promotes angiogenesis during embryonic development and in wound healing.¹¹ In this VEGF assay a fluorescently-labeled secondary antibody was used to specifically detect varying analyte levels with fluorescence scanning. For comparison, a small molecule fluorophore, rhodamine B (RhB), was added at a constant level to each solution. This was added to assure that all capillaries were making contact to the membrane and flowing as expected. Rhodamine B would be expected to wash away during immunoassay, so dots of VEGF and RhB were first scanned for rhodamine signal, then immunoassayed and scanned for a different fluorophore bound to VEGF. Dot-to-dot variability in the rhodamine signal was computed with S/N for each dot and a neighboring blank region. For this experiment the RSD was 8% for 12 replicates. In general, replicates of fluorescently labeled proteins ranged in RSD 5-15%. While small molecule fluorophores can confirm that syringes are operating well, for complex mixtures a positive control such as actin would be a more helpful metric to account for both deposition and immunoassay. In terms of quantitation, differences in signal were seen for different concentrations of VEGF. For instance 500 ng/mL had a normalized fluorescence signal of 0.49 ± 0.76 which differed significantly from

that of 5 ng/mL at 0.274 ± 0.051 . However the response was not linear. This is expected for microarray data, which are typically calibrated with a log-log plot¹ or curve fit to four or five parameter plots.⁷ For reliable calibration use of highly purified natural or recombinant proteins is advantageous, though resource intensive.⁸

For capillary dot blotting, care was taken to accurately measure fluorescence response, but the results were not automated as with microarray software in commercial and clinical use. Fluorescence data was adjusted so that the most intense dot was on scale, then signal was measured as the average signal of the area of the dot, divided by the signal for a similar blank area, in between the dots on the capillary track. Lastly the least intense dot was taken as the reference, against which all other dots were measure. This was suggested by other dot blot authors.⁹ A more sophisticated data interpretation might take into account the area of the spot or algorithmically improve discernment of signal from noise. The values reflected here are expected to be very conservative measures, as in most cases S/N is very low before incorporation of a reference dot, even for visually obvious depositions.

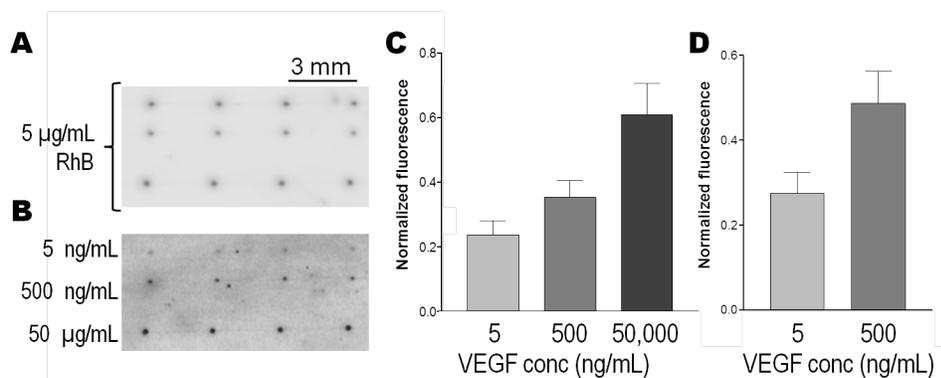


Figure 4.8. VEGF calibration curve with 500 nL dots deposited at 250 nL/min. Each dot contained some amount of VEGF and a constant level of RhB, 5 µg/mL. (A) Scan for RhB with 532 nm excitation, 588 nm emission filters. (B) Immunoassay signal for the specified concentrations of VEGF. The secondary antibody was conjugated with Alexafluor 488 dye, and fluorescence was measured using 488 nm excitation, 526 nm emission filters. (C) Signal/noise for each VEGF dot was normalized using the S/N of corresponding RhB dots. (D) Adjusted response for scans of lower concentrations only, where 50,000 ng/mL is off scale.

Performance of capillary dot blotting with more complex mixtures was also evaluated. One application of this method is detection of VEGF secreted from tissues into culture media. Specifically, performance of analytes in EPILIFE media, which contains about 40 µg/mL total protein (BSA, bovine transferrin, growth factors), was studied. Though normal levels of VEGF in human serum are in the low nanomolar range¹¹, VEGF levels for this experiment, secreted from tissue samples into media, were expected to be 0.1-15 pM (4-630 pg/mL). To start, blank culture media with no secreted proteins was spiked with VEGF standards (Figure 4.9). For comparison the lowest concentration of VEGF (10 pg/mL) was also prepared in tris buffer rather than EPILIFE media. This provided a direct measure of the interference caused by other proteins in the media. Signal for 10 pg/mL VEGF in tris buffer was significantly higher than 10 pg/mL in the media, on par with 200 pg/mL VEGF in media. Though this result is to be expected, as it is well documented that microarray sensitivity can suffer due to a so called “matrix effect” or diffuse signal due to competitive binding or non-specific antibody recognition of other proteins in the biofluid.¹ The assay may sometimes have sufficient sensitivity to overcome matrix effects, or

sample can be diluted before deposition to lessen the impact of other proteins. To achieve this low pg/mL detection of VEGF standard in buffer a large volume dot was deposited (5 nL). With this result (sensitive detection somewhat hindered by matrix effects) further capillary dot blotting with large volume accumulations of dilute and concentrated protein species were investigated.

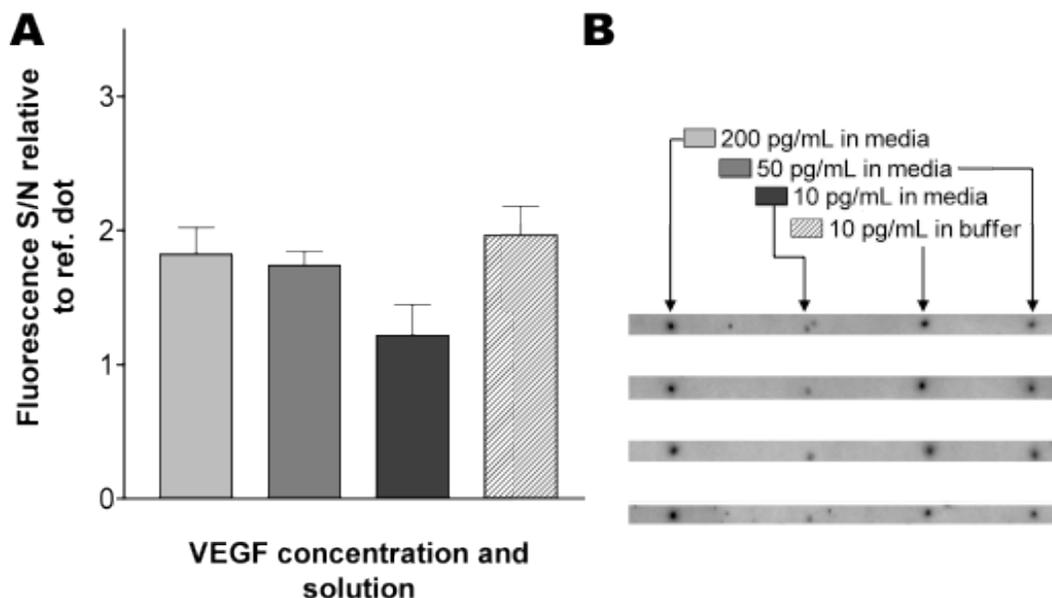


Figure 4.9. Low levels of VEGF added to media. 5 μ L dots deposited at a rate of 500 nL/min.

Dot Blotting with Large Volume Accumulations. One exciting possibility with capillary dot blotting is the accumulation of large volumes of sample on a single dot. This is not a typical work flow for commercial protein microarrays, but there may be an opportunity for extraordinary sensitivity. In instances where a complex solution has minute quantities of a given protein, a simple method to confirm presence of the analyte would be quite valuable.

For these capillary dot blotting experiments the substrate was PVDF, which binds protein via adsorption and absorption. The manufacturer reports a protein binding capacity of 200 μ g/cm² for the 150 μ m thick PVDF membrane with 0.45 μ m diameter pores. With exposure of

flowing protein solutions to a small area for extended periods of time (several minutes) one would expect significant absorption. In fact, for a 250 μm diameter spot and 150 μm deep membrane the volume that that region encompasses (without accounting for inaccessible solid regions) is $7.4 \times 10^{-6} \text{ cm}^3$, or 7.4 nL, orders of magnitude lesser than the 10^2 - 10^3 nL sample deposited on the surface of the porous membrane. Not only would absorption be expected, significant concentration would result. To evaluate the binding behavior of protein solutions accumulated for long times (large volumes) both fluorescence scanning on the surface of the dot and confocal microscopy were used.

Large Volume Capillary Dot Blot: concentrated sample. In samples where target protein was a somewhat large part of the total protein content, perhaps $\sim 1\%$, large volume accumulations were quite successful (Figure 4.10). In the confocal microscopy data the exact slice that represents the surface of the PVDF membrane is unspecified, but it is clear that with both the 0.1 μL and 1 μL drops fluorescent protein extended 50-150 μm into the membrane. Additionally, the 1 μL series extends further (has more signal in more frames) than the 0.1 μL series. Even though these dots represent the same concentration, the larger volume dots have a greater signal/noise in both confocal and planar spectroscopy.

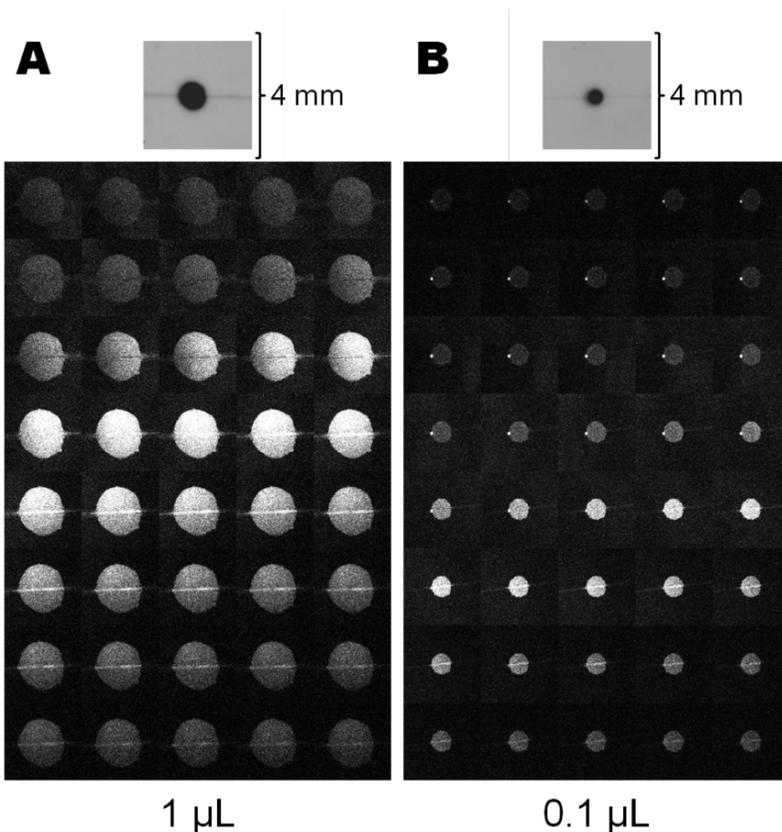


Figure 4.10. Planar and confocal fluorescence microscopy of capillary dots. (A) 1 μL dot of 0.5 $\mu\text{g}/\text{mL}$ FITC-BSA, 0.5 mg/mL unlabeled BSA, deposited at a rate of 500 nL/min . (B) 0.1 μL dot of the same sample, deposited at a rate of 100 nL/min . The planar fluorescence scan is shown at top, with S/N values of 5.5 ± 0.3 for (A) and 4.7 ± 0.1 for (B). The confocal microscopy data are presented as a montage of 10X slices on the z-axis of the dot. Each small square represents a 2.5 μm slice, so that as the images move from left to right the focus moves down into the membrane. Confocal data from (A) and (B) are not on the same scale for dimensions or intensity.

Large Volume Capillary Dot Blot: dilute sample. Large volume capillary dot blotting was somewhat less successful when the protein of interest represented a smaller percentage of total protein content. Though signal was still detected there was often a dark region in the center of the dot and then a bulls-eye pattern extending radially out from that point (Figure 4.11). This was especially apparent when the membrane was more moist at the start an experiment, but was always observed to some extent in dots where the protein of interest was a small component of the whole, $\sim 0.01\%$ or less. A ring of fluorescent signal was observed for large volume dots when the membrane was relatively dry (perhaps 40 minutes after exit from buffer equilibration).

These effects were most pronounced with large accumulations, $\geq 1 \mu\text{L}$. We envision that they could be partially counteracted by diluting protein samples and then accumulating large volumes, as is indirectly probed in Fig 4.11-B, but this was not extensively studied, in part because at these long time frames membrane drying was the dominant consideration. After the hydrophobic PVDF membrane dries aqueous samples will not penetrate into the pores, rather sample will bead on the surface. As discussed in Chapter 5, other substrates may be better suited to large volume accumulations.

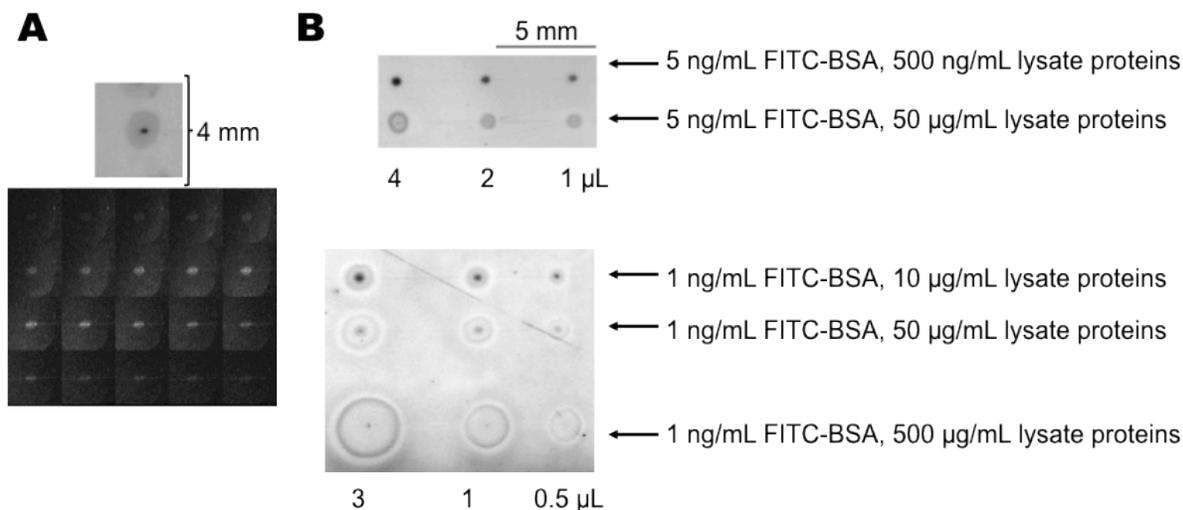


Figure 4.11. Effects of cell lysate on detection of minor constituents in large volume accumulations. (A) Planar fluorescence and confocal images of 5 μL dot of 5 ng/mL FITC-BSA with 0.5 mg/mL unlabeled BSA. The frames of the confocal image each represent a 5 μm slice on the z-axis traveling down into the membrane (B) Planar fluorescence of FITC-BSA in samples with different quantities of cell lysate proteins. In the top row there is 100x more cell lysate protein than FITC-BSA, while with the extreme case in the bottom row there is 5×10^5 more cell lysate protein than FITC-BSA.

In studies of large volume accumulations it became apparent that even before immunoassay large volume dots had fluorescent signal (Figure 4.12). At least, signal was detected with the fluorescence scanner. Whether it was native fluorescence, contrast of the

sample against the background of the ultra-low background membrane or light scattering was not known. This was not anticipated, and furthermore might be expected to give misleading signal in the final immunoassay. However upon immunoassay, signal for the protein of interest was seen but no signal was observed in dots without lysate or target protein, or in dots with a level of target protein below the LOD for that immunoassay. While it is disconcerting to have detected more dots before immunoassay than after, experiments with large volume accumulations are still feasible with the inclusion of proper controls. Possible controls include dots of: water, buffer, an unlabeled protein (BSA), and cell lysate. Water and buffer controls provide assurances all the instrumentation was cleaned of previous deposition experiments. Unlabeled protein and blank cell lysate help to identify non specific immunoassay signal.

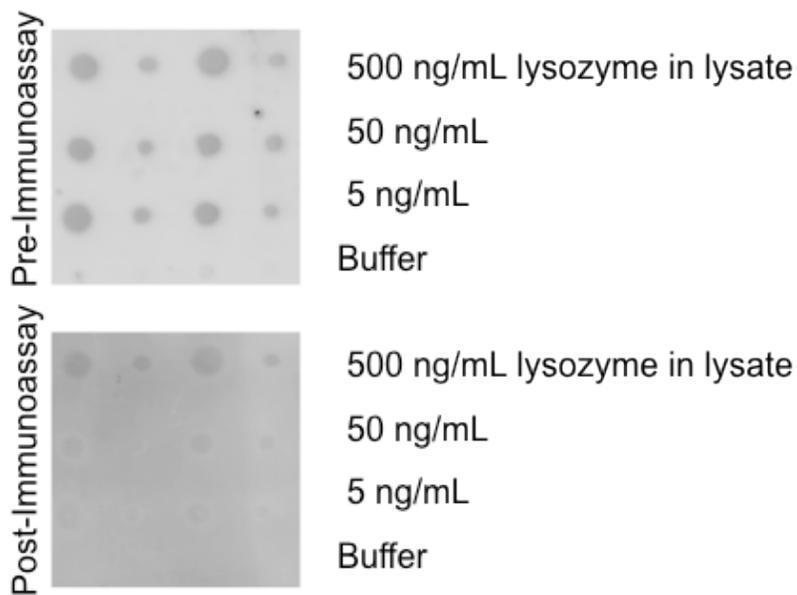


Figure 4.12. Fluorescent scans of PVDF before and after lysozyme immunoassay. For both images the laser, filters, PMT voltage and imaging parameters were matched. After immunoassay only signal from the greatest concentration of lysozyme is plainly visible. Large dots are 1 μ L while the alternating small dots are 0.4 μ L.

Multiplexing. One hallmark of protein microarrays is the ability to multiplex, screening many samples simultaneously to confirm presence of a protein, quantify the target, evaluate protein activity with specific substrates, etc. Microarrays can be multiplexed with different samples in each dot, which is implemented in capillary dot blotting by having different samples in each parallel capillary. Arrays can also be multiplexed by immunoassaying for more than one protein with antibodies conjugated with different antibodies, which is discussed in this work.

Two fluorescent signals (one from immunoassay, and a protein standard directly labeled with a different fluorophore) were tested for two proteins in solution (Figure 4.13). Signal for each fluorophore corresponded to the relative concentrations of the associated proteins. The most intense dot in the initial scan of the membrane had the lowest signal upon lysozyme immunoassay, which matched the sample that had the largest amount of FITC-BSA and least amount of lysozyme. Accompanying pipetted dot blots with one protein or the other confirmed that the two fluorophore signals were independent of each other (data not shown). Yet in this case there were fewer opportunities for cross-reactivity because only one primary/secondary antibody pair was introduced for detection. The concentrations for these proteins were also greater than would arise in most biochemical research.

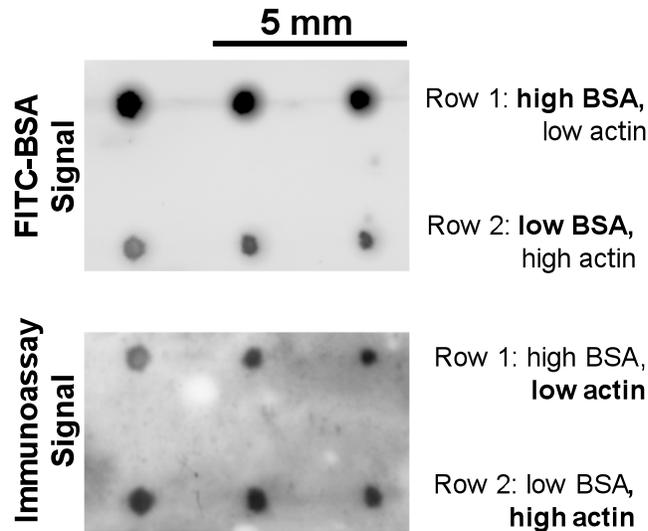


Figure 4.13. Dot blotting and detection with a mixture of two proteins. Sample A (row 1) contained 5 $\mu\text{g/mL}$ FITC-BSA and 1 $\mu\text{g/mL}$ actin. Sample B (row 2) was 1 $\mu\text{g/mL}$ FITC-BSA with 10 $\mu\text{g/mL}$ actin. With scan for FITC-BSA, fluorescent signal was 157 ± 15 and 75.6 ± 4.4 for samples A and B. After immunoassay for actin, chemifluorescent signal from the same blot was 151 ± 17 and 181.2 ± 9.6 for samples A and B. Each dot was 50 nL, deposited at a rate of 100 nL/min.

In a more realistic experiment, two proteins were measured after an exogenous protein was added to cell lysate (Figure 4.14). Actin was detected at the same level for each of the cell lysate samples as is expected. Actin was not detected in the unlabeled BSA control where there were no cell lysate proteins. VEGF, which was added exogenously to cell lysate, was found as expected at high levels with the highest concentration sample. However there was VEGF signal in dots where VEGF would not be expected, i.e. in the cell lysate with no added VEGF and in the BSA-only sample. It is possible that there would be endogenous VEGF in the cell lysate, but signal for the BSA-only sample indicates there is either carryover from earlier experiments or cross-reactivity of the VEGF antibody. Without the confirmatory data provided by a separation component, careful controls to account for immunoassay cross-reactivity are required. One can also observe different background levels between the actin and VEGF immunoassays. The membrane inherently has different background fluorescence for different excitation/emission

filter pairs. Additionally, the different primary or secondary antibodies for each protein may have different propensities for non-specific binding. Obviously, absolute values from one immunoassay to the next cannot be directly compared, but actin can act as a positive control by which to monitor VEGF sample-to-sample variations.

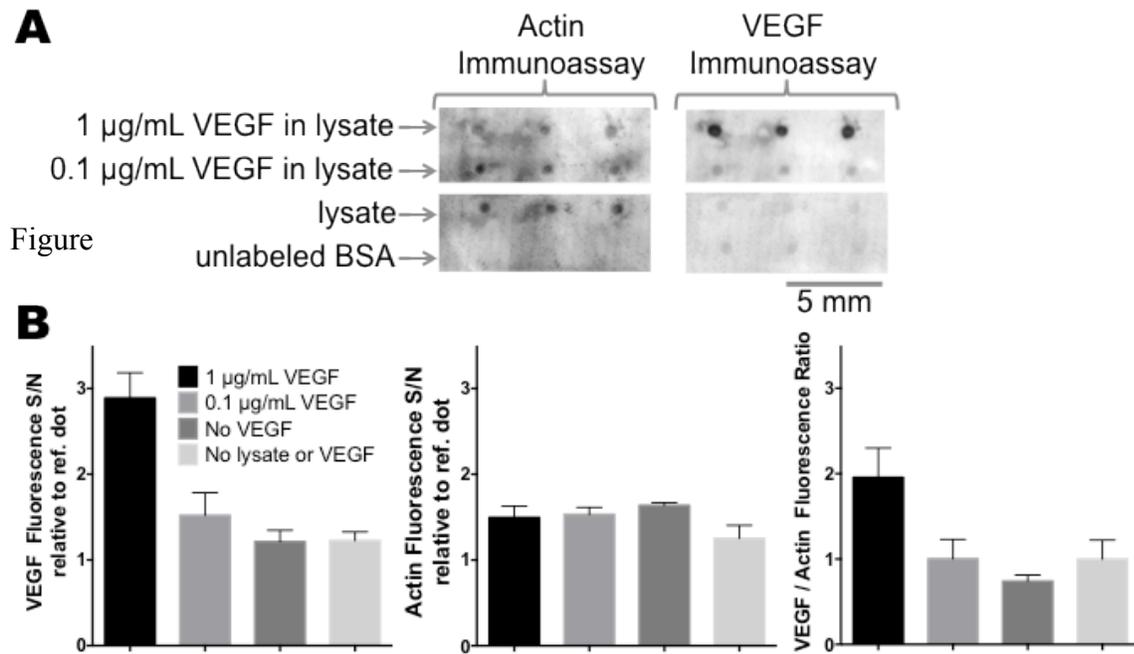


Figure 4.14. Simultaneous immunoassay detection of two proteins. 1.43 mg/mL cell lysate was spiked with two different concentrations of VEGF. 200 nL drops were deposited at a rate of 100 nL/min. S/N for VEGF (B), actin (C) and the ratio of VEGF to actin (D) are represented in the charts. In the cell lysate actin would be expected to stay constant. VEGF signal was highest with the greatest concentration, 1 µg/mL.

This experiment was replicated many times where VEG-F signal for cell lysate, blank culture media or unlabeled BSA controls was present, including trials with new syringes, fittings and capillaries. While the method could certainly find utility with another protein-antibody pair for detection in complex mixtures, detection of secreted VEGF in tissue culture media was stymied. Overall, this assay took about 45 minutes, 10 minutes of membrane equilibration and syringe assembly, 5 minutes to deposit spots and 30 minutes for immunoassay. Thus, this is a

clear illustration of both the strengths and weaknesses of protein arrays, i.e. a speedy multiplexed immunoassay was performed but interpretation was hindered because there was no separation to reduce sample complexity.

CONCLUSIONS

Capillary dot blotting shows promise as a bridge between manual pipetting for optimization of antibody titre and protein microarray for large-scale screens. Initially conceived as a way to calibrate signal for the small injection volumes of capillary Western blotting, the method quickly showed promise for a wider range of applications. The method boasts excellent sensitivity without investment in and maintenance of specialty equipment such as microscale pin arrays.

Confocal microscopy confirmed that protein does penetrate into the porous membrane. It would be interesting to repeat this with unlabeled proteins and fluorescently tagged antibodies to see if the antibodies can traverse the membrane to the same depth. In neighboring dots, samples 1000x larger in volume were deposited onto spots roughly 7x larger in diameter, representing a dramatic concentration on a small region of membrane. Large accumulations on this porous structure did display a signal with planar fluorescence scanning before immunoassay. After immunoassay it appears that the subsequent fluorescent signal does correlate either to the protein of interest or cross-reactivity from antibody interactions, rather than non-specific fluorescence. In preliminary testing no signal was seen for unlabeled protein in confocal microscopy (data not shown), however careful implementation of controls is important for this method in routine use.

A review of the microarray literature (and limited dot blotting literature) reveals that there does not appear to be much published on protein arrays on this modest scale. One dot blotting

paper reports the use of five 10 μm -thick membranes stacked on top of each other, instead of the more typical 100-150 μm membrane.¹⁰ After placing this stack of membrane proximal to a 96 well plate and applying vacuum, protein was delivered from all the wells to all the membranes for parallel downstream analyses. While this is certainly a straightforward way to multiplex, sensitivity was low with reported sensitivities of about 0.5 μg , perhaps because the thin membranes did not have a high binding capacity and proteins were moved rapidly through the membranes. Some groups have advanced planar protein microarray sensitivity to fM levels with use of silicon substrates engineered to be very low background.¹¹ Sensitivity for protein microarrays in widespread use is said to be sub pg/mL-pg/mL, with a dynamic range of pg/mL-ng/mL. Multiplexing for planar microarrays has been reported to be 10-24 analytes probed (up to 100 for bead based assays).¹ Capillary dot blotting is at or near this level of sensitivity for the large volume accumulations, but multiplexing beyond two labels has not been attempted.

Going forward, use of glass slides with activated surfaces that bind proteins are reported to enable greater density spots, so it would be valuable to see if glass slides would support large volume accumulations in capillary dot blotting.⁵ For the initial application of capillary dot blotting in direct support of capillary Western blotting the high speed SNAP i.d. immunoassay platform, which works with membranes, was valuable for throughput. However, with larger scale experiments with many spots on glass slides, a slower immunoassay step would still allow for higher net throughput. Additionally, the PVDF membrane was disadvantageous in that for best results the surface needed to remain uniformly wetted for long stretches of time (1 h). With glass slides there is no such requirement, which may help reduce spot to spot variability.

In the current apparatus multiplexing samples is limited to the number of capillaries in operation. A syringe pump might hold between 4 and 10 syringes to infuse fluid through

capillaries. Additionally, tees could be used to increase the number of capillaries serviced by each syringe. In a compromise between capillary methods and microarray pins one could withdraw a set volume with four capillaries from four samples at a very fast rate, move them to a substrate and then expel them at an appropriate flow rate. With rinse steps this could occur iteratively, either with hydrophobically modified capillaries or intrinsically hydrophobic tubing (Teflon). Lastly, segmented flow could prove a useful way to sample many different reservoirs and deliver many unique sample dots to the substrate for immunoassay. This last idea is developed further in Chapter 5.

Overall, capillary dot blotting is a fast and inexpensive way to generate a large number of dots for analysis. With PVDF membranes a long series of replicate dots can be cut into smaller pieces and exposed to different antibody solutions (optimization of conditions) or different substrates (enzyme activity experiment). Conversely, many replicate dots of antibody could be divided and exposed to different sample solutions, reducing the quantities of antibody solutions needed for immunoassay. It is hoped this approach may find use for immunoassay of dilute solutions, or more generally in situations where more sensitive, automated dot blotting is advantageous in basic research.

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CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation, analytical instrumentation for microscale Western blotting and dot blotting was developed. Goals of increasing both sensitivity and throughput with low sample volumes were achieved. As seen in the timeline (Figure 5.1), manual operation steps and time in CE-Westerns were reduced when compared to traditional Western blotting, which may help to reduce overall costs of analyses.

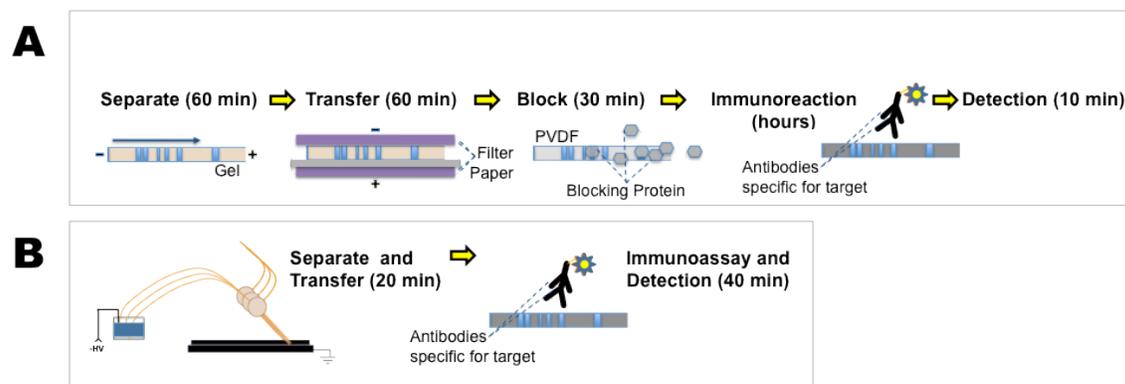


Figure 5.1. Timelines of Western blotting. With a typical Western protocol analysis requires several (A) compared to microscale Western blotting of about one hour (B). Microscale Western throughput may be further increased by depositing many separations onto one membrane, thereby reducing time/per sample devoted to immunoassay.

Future Directions in CE-Westerns. In addition to capillary Western blotting, microfluidic fabrication may serve to increase separation speed and automation for

streamlined performance. Injection, separation and transfer can be achieved by etching narrow ($\sim 50 \mu\text{m}$) channels in glass, filling reservoirs and channels with sample and sieving media, applying high voltage and interfacing the microfluidic chip to a membrane much as with CE-Western. Work by a researcher in our lab confirms that fabrication of a narrow sheath gel channel on the chip reduces the band broadening seen with commercial sieving matrices.¹ While the short separation distance on the chip (3 cm) allows for very fast separation, capillaries still have some advantages. Capillaries can be easily regenerated by flushing the sieving polymer out of the capillary, reconditioning, and refilling with gel. Though not part of the research presented herein, implementation of *in situ* pressure driven flow at the inlet of the multiplexed CE-Western apparatus would allow for regeneration of the separation capillaries after prolonged use ($> 2 \text{ h}$). Microfluidic chips can also be regenerated, but capillaries are more straightforward in this regard because of tedious flushing of microfluidic chips. Capillaries are also easier for sample injection as microfluidic chips typically require a reservoir for each sample, only to be cleaned and refilled after the experiment is complete. Capillary injections with different samples simply require moving the capillary and electrode from one vial to the next.

Another avenue of research that has not yet been pursued with CE-Westerns is their possible advantage for efficient protein transfer. Transfer from slab gels to membrane is often hindered for both small and large proteins ($\leq 15 \text{ kDa}$ and $> 150 \text{ kDa}$), though they remain of biological interest. Small proteins are documented to undergo “blow-through”, in which electro-transferred small ($< 15 \text{ kDa}$) proteins actually travel through the PVDF membrane and thus are not revealed by immunoassay. Conversely,

large proteins (>150 kDa) may not transfer out of the gel efficiently.² Many researchers have worked to optimize conditions for particular subsets of proteins³, but perhaps entangled polymer solutions and membrane capture via CE-Westerns could provide a route to more efficient universal transfer. Sieving media can also be adjusted (with polymer species, molecular weight, concentration) to improve separation resolution of a particular analyte size range.⁴ These concepts have not yet been explicitly interrogated in this instrumentation but may further expand practical use of CE-Westerns for analysis of biological mixtures.

Multi-Sample Capillary Dot Blotting. One way to increase utility of the capillary dot blot apparatus would be to introduce many different samples to the membrane for simultaneous immunoassay. Creation of sample plugs (or “droplets”) separated by an immiscible oil phase allows manipulation small discrete volumes. Recently published work provides a protocol to alternately sip sample and oil from multiwell reservoirs (Figure 5.2).⁵ Using this method, multiple small fractions of many different samples can be stored in a length of capillary, each physically and chemically isolated from its well-plate neighbor by an oil segment. Importantly, damage to the sample is unlikely, as the flow rates within the tubing are low enough to prevent shearing or chaotic mixing. Segmented flow typically creates sample plugs on the order of 10^{-13} - 10^{-8} L ; however, with larger tubing (10^2 μm i.d.) 10^{-6} L plugs are possible. This would be very exciting to implement with capillary dot blotting, because a fraction of each sample in the multiwell plate could be used for protein microarrays, reserving the bulk of the solution for other experiments or characterization.

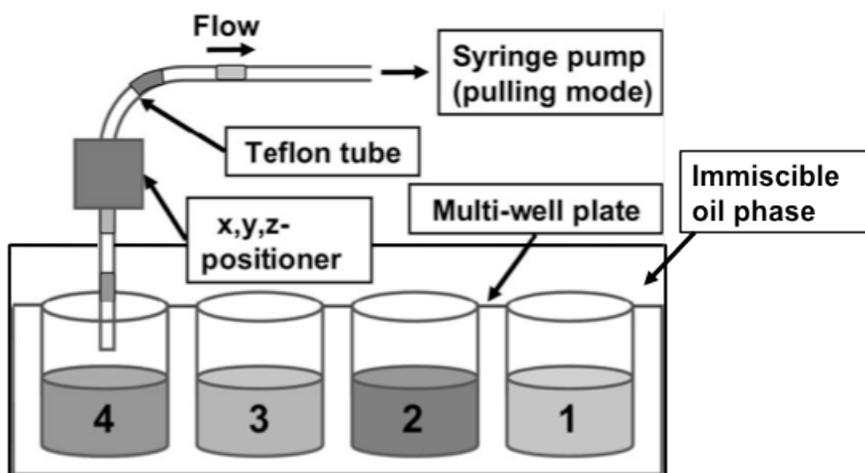


Figure 5.2. Droplet generation from a multiwell plate. Multiwell plate reservoirs are sampled by a Teflon tube connected to a syringe pump. The syringe pump pulls sample into the tube. With movement between wells the tube passes through the oil phase and oil enters the tube to separate one aqueous sample from the next. Reproduced with permission from Pei, J. A.; Li, Q. A.; Kennedy, R. T., *Journal of the American Society for Mass Spectrometry* **2010**, 21 (7), 1107-1113.

To test droplet generation for use in multiplexed capillary dot blotting, 150 μm i.d. Teflon tubing with a 500 nL/min withdraw rate was used to sample FITC-BSA and rhodamine solutions (Figure BB). In this case FC-770, a fluorinated hydrocarbon, was used. With these parameters 20 nL plugs were formed. As seen in Fig BB-A, some trial-and-error was required to create regular plugs. Importantly, the transfer from Teflon to membrane was very good, with discrete dots formed on the membrane throughout the deposition. Even with a high intensity off-scale image in Fig BB-A there is no fluorescent track observed between dots and no apparent effects of the immiscible oil phase. At these volumes the oil is presumably volatile enough to evaporate and dissipate quickly, rather than dilute the dots.

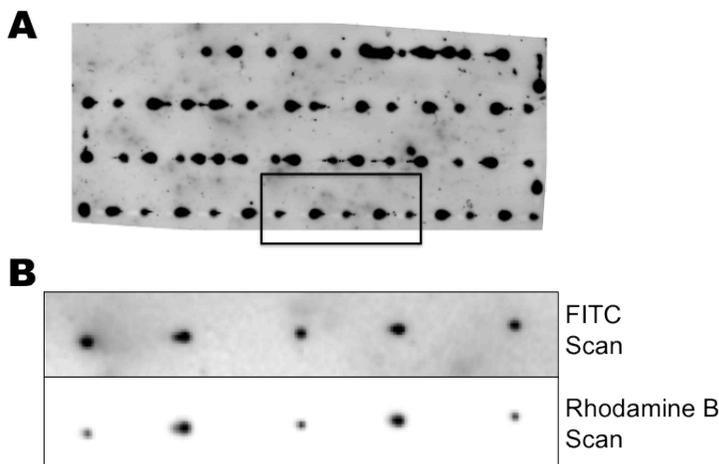


Figure 5.3. Multiplexed capillary dot blotting using segmented flow. Alternating dots of 5 $\mu\text{g}/\text{mL}$ FITC-BSA or 5 $\mu\text{g}/\text{mL}$ FITC-BSA with 4 $\mu\text{g}/\text{mL}$ rhodamine B in tris buffer. FITC-BSA scan of the entire membrane (A) shows a serpentine path of sample plugs flowed from the Teflon capillary at a rate of 50 nL/min onto a membrane moving at rate of 3 mm/min. A closer view of the inset region (B) with scans for FITC and rhodamine B.

Pilot experiments with larger i.d. tubing did provide a means to create large plugs (Figure CC). With 500 μm i.d. tubing surface tension prevents entry of aqueous solutions into the hydrophobic tubing, but this is easily circumvented by forcing a 4 cm, 125 μm i.d., 800 μm o.d. segment of tubing inside the larger tubing and allowing this segment to interface with the multiwell plate. Though the small tubing o.d. is larger than the interior of the larger tubing, the larger tubing is flexible and can mold around the segment. In a preliminary experiment with sequential sampling 14 different solutions, 600 nL drops were obtained (Fig CC-A). Four of the 14 solutions contained FITC-BSA. Plug formation was straightforward, however deposition onto a membrane was problematic. With large volumes of sample came larger volumes of oil pooling on the membrane. A bulls-eye pattern was observed for initial drops, much as with dilute drops discussed in Chapter 4. Additionally,

multiple drops fused together at the outlet of the Teflon tubing before adsorption to the PVDF. Further experimentation might allow for direct deposition, as there is a large body of literature on manipulating droplets with surfaces of varying hydrophobicities.⁶ Perhaps large volume droplet deposition would work better with a hydrophilic (glass) surface as mentioned in Chapter 4. Additionally, air-segmented droplet generation is possible and could be assessed for capillary dot blotting.⁵

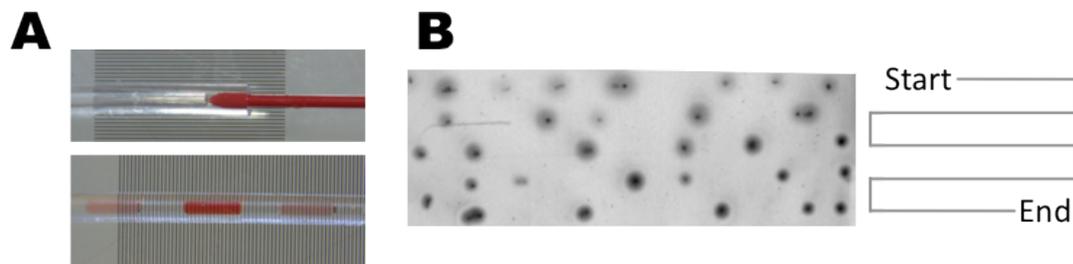


Figure 5.4. Deposition of large volume plugs onto PVDF membrane. (A) 150 μm i.d. FEP tubing was inserted inside 500 μm i.d. Teflon tubing for generation of 600 nL. Drops entered the capillary at a pull rate of 2 $\mu\text{L}/\text{min}$. A 200 μm interval ruler is included in the images for scale. (B) Plugs were flowed from the Teflon onto the membrane at a rate of 2 $\mu\text{L}/\text{min}$ with the translational stage moving at a rate of 12 mm/min. The distance between each long trace is 5 mm. Each dot observed represents at least two fused sample plugs from the Teflon capillary.

This work represents a demonstration of great potential for improvement in the sensitivity, throughput, and time required to perform Western blots and dot blotting. Further improvements could be made by following the suggestions outlined above, including work with multiplexed CGE microfluidics, characterization of protein transfer efficiency, and segmented flow capillary dot blotting with large volume accumulations. In sum, this work has the potential to impact researchers at

the academic or industry level by decreasing both volumes of precious samples and time required for analysis, thus reducing costs.

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