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Microfluidic Bioanalytical Device and Assay Development for
High-Throughput Electrophoretic Protein Analysis

By

Todd Andrew Duncombe

A dissertation submitted in partial satisfaction of the
requirements for the degree of

Joint Doctor of Philosophy

with University of California, San Francisco

in

Bioengineering

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Amy Herr, Chair

Professor Joseph DeRisi

Professor Susan Muller

Professor Dorian Liepmann

Spring 2015

Microfluidic Bioanalytical Device and Assay Development for
High-Throughput Electrophoretic Protein Analysis

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Abstract

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

University of California, Berkeley

Dissertation Mentor:
Professor Amy Herr

Seminal bioanalytical technologies for high-throughput analysis, such as flow cytometry and capillary electrophoresis, were leveraging microfluidic physical phenomena long before the advent of the term “microfluidics”. Transitioning from the initial solid-state micro-electronic fabrication approaches, microfluidic fabrication has moved towards polymer based technologies that are amenable to a rapid design, prototype, and test development cycle. In my dissertation, I took advantage of these features to create new tools for performing electrophoresis-based protein assays over a range of applications, including, rapid low-power electrophoretic immunoassays, open-microfluidic ‘soft-MEMS’ platform for high-throughput protein analysis, and spatially & temporally controlled separation media for enhanced single-cell western blotting assays.

Rapid low-power electrophoretic immunoassay: To reduce the power requirements for a portable electrophoresis platform, we developed a new assay format that minimized the separation length to 1 mm for an electrophoretic immunoassay. The polyacrylamide gel moving boundary electrophoresis (PAGMBE) assay consumed just 3 μ W and was completed in less than 30 seconds using only a 9 V battery - the lowest voltage reported for an electrophoretic protein separation.

Open-microfluidic ‘soft-MEMS’ platform: We developed an open-channel hydrogel architecture for rapid protein analysis. Directly photo-patterned free-standing polyacrylamide gel (*fsPAG*) microstructures support electrophoretic performance rivalling that of microfluidic platforms while maintaining easy interfacing with automated robotic controllers and downstream

processing (e.g., sample spotters, immunological probing). We demonstrated 96 concurrent SDS protein *fs*PAGE separations in under 5 minutes.

Spatially varied separation media: Grayscale mask photo-patterning of gel density realized periodic 1 mm gradient gels arrays for single cell analysis. These dense arrays with a spatially varied sieving medium enabled concurrent protein sizing for 1000s of single cells in parallel. We demonstrated the single cell gradient gel western blotting to analyze multiple targets in the Her2 signaling pathway in a primary breast cancer tissue that varied by an order of magnitude in molecular weight. This broad-range protein sizing 1 mm gradient gel condition corresponds to the molecular weight range for 78% of the human proteome.

Pore-expansion for enhanced intra-gel assays: Protein separations in dense sieving media followed by in-gel immobilization enable high performance separations and minimal protein loss. This assay integration is necessary for processing low abundance samples – for example the protein content of a single cell. Unfortunately, for dense sieving medium prevents easy access for downstream assays on the immobilized proteins. We developed an active hydrogel matrix that could maintain a dense form to achieve a high-resolution protein separation and immobilization, then after a simulation, could transition to a lower-density form that is more accessible to antibody probing and the delivery of other large-reagents.

In summary, this dissertation focused on high-throughput protein analysis platforms that are designed towards the goal of massively parallelized proteomics, a major unrealized goal from bioanalytical technology.

*To the pursuit of progress,
and its advocates throughout humanity.*

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Introduction

1.1. Motivation for High-Throughput Protein Separations

Proteins are intricate biomolecules that embody an enormous range of structural and functional diversity. Large interacting networks of proteins and other biomolecules determine biological outcomes. For researchers to deconstruct and make sense of the complexity present in biological systems there is a need for platforms that can conduct concurrent-analysis of multiple samples with multiplexed protein readouts.

Measurements to identify specific proteins are typically performed using either affinity reagents, for example antibodies or aptamers, or through mass spectroscopy based protein sequencing. While protein sequencing is capable of detecting thousands of proteins within a given sample, it is inherently limited in rapid and parallel processing of multiple samples due to the serial nature of the liquid chromatography and mass analyzers (up to eight samples at a time have been demonstrated with isobaric labeling¹). In contrast, protein microarrays – typically with immobilized antibodies – can analyze hundreds to thousands of samples with multiplexed protein measurements per sample^{2, 3}. While protein microarrays meet the massive data acquisition and multiplexing demands of large scale proteomic questions, they typically take hours to perform (due to multiple washing steps) and have low specificity, especially in complex sample matrices. Further, antibodies typically cannot distinguish between the protein of interest and isoforms or non-specific interactions, and their affinity can easily degrade over time. These shortcomings can lead to high false positive rates and embarrassing mistakes⁴.

Analytical protein separations are routinely used to fractionate complex mixtures of proteins (e.g. cell lysate or human serum) by physiochemical parameters – such as charge, size⁵, isoelectric point⁶, hydrophobicity⁷, or solubility⁸. Protein separations can be used to dramatically accelerate the detection of an affinity reagent⁹, for example by rapidly separating the bound and unbound affinity reagents to detect relative abundance (e.g. the homogenous immunoassay discussed in Chapter 2). Alternatively, fractionation can be performed to simplify the sample matrix so that the next stage analysis can be more specifically targeted to the protein of interest (Chapter 6). For example, off-target antibody binding can be eliminated from the on-target binding if the two protein species are fractionated from one other.

1.1.1. Benefits of Microfluidic Analysis

Over the past 60 years, biologists have measured, manipulated, and even mimicked biology using microscale physics and chemistry. Microfluidics is defined as a device that handles and manipulates fluids at length-scales from one to one thousand microns with typical internal volumes of microliters to picoliters. Indeed, biology's use of distinctly microfluidic physical phenomena precedes the advent of the term “microfluidics”. For example, the Coulter counter and the flow cytometer revolutionized single-cell analysis¹⁰; and DNA sequencing in small bore capillary tubes markedly accelerated the progress of the Human Genome Project¹¹. The advent of microfabrication techniques for the semiconductor industry (to route electrons) inspired micro-

scale tools for biological research (by routing cells and molecules). As micro/nanofabrication techniques matured, so have microfluidic tools specifically designed for biological investigation.

Once fabricated, microfluidic devices can efficiently and precisely route fluids. The sub-microliter microfluidic volumes reduce reagent usage¹², mitigate sample loss³, and offer high surface area-to-volume ratios as compared with macroscale containers and channels¹³. In a free-solution environment, precise flow stems from the fact that microfluidic flows lack turbulence: the random eddies and vortices that churn fluids at the human-scale¹³. Instead, microscale fluid flows are ordered, with adjacent fluid streams moving as well-defined “lamina” or layers over long distances (relatively speaking, of course). This laminar flow behavior is determined by flow velocities, channel dimensions and fluid properties (which are characterized with the non-dimensional number known as the Reynolds number¹⁴). Laminar flow has long played an important role in biological enquiry, as laminar sheath flows in flow cytometry¹⁵ focus a suspension of dispersed cells into a single-file procession past the photodetector – making interrogation of tens of thousands of single-cells per second possible.

The seminal micro-fabricated microfluidic devices¹⁶⁻¹⁸ introduced in the early 1990s used solid-state fabrication of glass microchips for electrophoretic separations of biomolecules. The high surface area to volume ratio at the microscale (compared to macroscale analogs, e.g. slab PAGE) enables rapid heat dissipation during electrophoresis. Thus, higher electric fields can be used in microfluidic electrophoresis - without deleterious effects of excessive diffusion or bubble formation - to realize faster and higher performing separations even over short distances. Due to the small form factor of microfluidic separations, sophisticated microstructures of integrated channel networks can be arrayed to achieve large-scale systems¹⁹. These arrays would be difficult and unwieldy to create and use with conventional tubes and chambers. Microfluidic electrophoresis platforms are automating existing assays²⁰⁻²³ and enabling new types of measurements not previously possible²⁴.

1.2. Electrophoretic Separations

This section provides a cursory introduction to the fundamentals of electrophoresis²⁵, typical considerations when optimizing separation performance, and commonly utilized sample stacking strategies²⁶ to improve performances. A detailed discussion of electrophoretic separations can be found in the cited sources.

1.2.1. Electrophoretic Transport

The velocity of a particle in the presence of an electric field (E) is dictated by the relative electrophoretic force, $F_{EP}=qE$, and the drag it experiences during its motion. For small sphere-like particles traveling in a solution we can assume the particle experiences a Stokes drag force, $F_{Drag}=6\pi\eta rU$. Where r is the particles hydrodynamic radius, U is the particle velocity, and η is the dynamic viscosity of the solution. By balancing those two forces we can solve for the particle velocity, $U= qE/6\pi\eta r$. The electrophoretic mobility of a particle (μ), is defined as the velocity normalized by the applied electric field, $\mu = q/6\pi\eta r$. In a solution of different particles the $1/6\pi\eta$ term is typically constant. Therefore electrophoresis fractionates particles by their charge and size, q/r . It bears mentioning that this is an oversimplification. Most notably, the effective charge

of a particle in solution is dictated by the interaction between the particle and the ions in the solution – specifically, the formation of an electrical double layer.

For small particles in a solution where a concentration gradient is present, diffusion acts to move particles down the gradient. The process of diffusion can be estimated by the Stokes-Einstein equation, which defines a diffusion constant as the ratio of thermal energy to Stokes Drag, $D = k_B T / 6\pi\eta r$. Where D is the diffusion constant, k_B is the Boltzmann's constant, and T is the absolute temperature. For a particle population with a known initial normal distribution (σ_0), diffusion constant, and period of time in which diffusion occurs (t), we can predict the final distribution (σ): $\sigma = \sqrt{2Dt + \sigma_0^2}$. Dispersion is defined as the square of a populations standard deviation distribution, $\sigma^2 = 2Dt + \sigma_0^2$, and is linearly related to time. Dispersion is typically the term used when describing the population distribution of a particle. For simplicity in this thesis we focus on dispersion resulting from time-based diffusion. In Appendix A, we show preliminary results demonstrating how anomalous diffusion (that is, non-time dependent diffusion) may play a critical role for dispersion through sieving matrices.

1.2.2. Separation Metrics

Electrophoretic fractionation assays at the most basic level consist of three phases, (1) injection, (2) separation, and (3) readout. Each phase plays an important role in the overall performance of the separation. Figure 1.1 depicts these phases for a seminal microfluidic electrophoresis format¹⁸ that is still in use today²⁷. We use this format as a way to introduce some of the integral concepts of separation science, including injection dispersion (σ_0^2), separation length (L), and separation resolution (R_s).

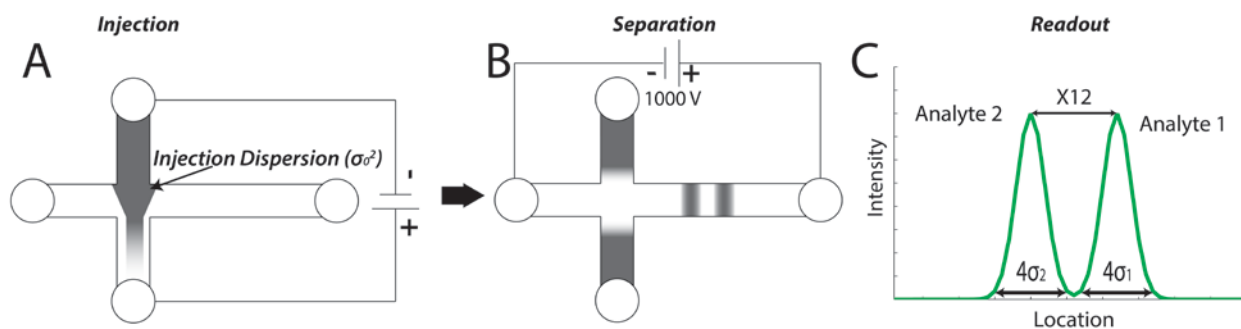


Figure 1.1: Injection, separation, and readout are the basic three steps performed in any analytical separation. We use a T-channel injector¹⁸ to represent a basic microfluidic electrophoretic (A) injection and (B) separation process. (C) A plot profile is a common readout after a protein separation. It can be acquired with epi-fluorescence and a CCD camera, to observe, in this example, the separation of two analytes with a separation distance of X_{12} and peak widths of (4σ) .

The T-channel consists of orthogonal channels, one for the injection and another for the separation. In-channel electric field profiles are controlled with electrodes at all four channel reservoirs. To inject the sample, an electric field profile is used to drive the current from one

injection reservoir to the opposite reservoir. This forms a small zone of sample in background electrolyte (depicted in figure 1.1A). The dispersion prior to electrophoresis along the separation axis is termed the injection dispersion (σ_0^2). The T-channel injector helps improve separation performance by limiting the injection dispersion to within the width of the injection channel. After injection, the electric field profile is changed to initiate separations along the separation channel. The two species depicted in figure 1.1B are separated by their differential electrophoretic velocities, $\Delta U = \Delta \mu E$, and their mean separation distance equal to the electrophoresis time, $\Delta X = \Delta U t$. The separation length (L) is defined as the length of the separation axis used for performing a separation, $L = \mu_1 E t$, where μ_1 is the mobility for the faster of the two species being separated. To detect the separation either a plot profile is taken to observe the species concentration over length, for example using epi-fluorescence and a CCD camera, or an electropherogram is taken to observe the species concentration over time at a single point along the separation axis.

To determine the resolution between two species in a separation, analytical chemists use the metric Separation Resolution (R_s), which is defined as the distance between the mean of two species normalized by the average peak width (4σ) of each species, $R_s = \Delta X_{12} / (2\sigma_1 + 2\sigma_2)$. Below we expand the equation for R_s to solve for it as a function of electrophoresis time, differential mobility, electric field, injection dispersion and diffusion constant (we assume injection dispersion and the diffusion constant are the same for both species).

$$\text{Eq. 1} \quad R_s = \frac{(\mu_1 - \mu_2) E t}{4\sqrt{2Dt + \sigma_0^2}}$$

From this simple equation for separation resolution, the rationale for most strategies to improve separation performance is apparent. As the separation time or differential mobility is increased so does the separation performance. Conversely, as the diffusion constant or injection dispersion is increased, the separation performance is reduced. As the applied electric field relates to both an increase in separation distance between species and an increase in the rate of diffusion (via joule heating) a balance must be reached between separation speed and heat dissipation. In microfluidics the high surface to volume ratio allows for the efficient heat dissipation and for the electric field to be increased to higher values before consequences in increased diffusion rates are observed.

A separation is considered baseline resolved when $R_s = 1.5$, which means there is no overlap between two separated samples, therefore they are 100% separated. Species are typically considered resolved when the $R_s = 1$, or 98% separated. Figure 1.2A displays a range of R_s values, their corresponding plot profiles for two species and their corresponding cumulative curve. Their dispersion held constant and their separation distance varied to alter R_s . Figure 1.2B shows the percent separated of two species as a function of the R_s value. The critical region between 0.6 and 0.9 R_s spans from a non-separation (only 66% separated) to nearly resolved (95% separated) and is shown in figure 1.2C.

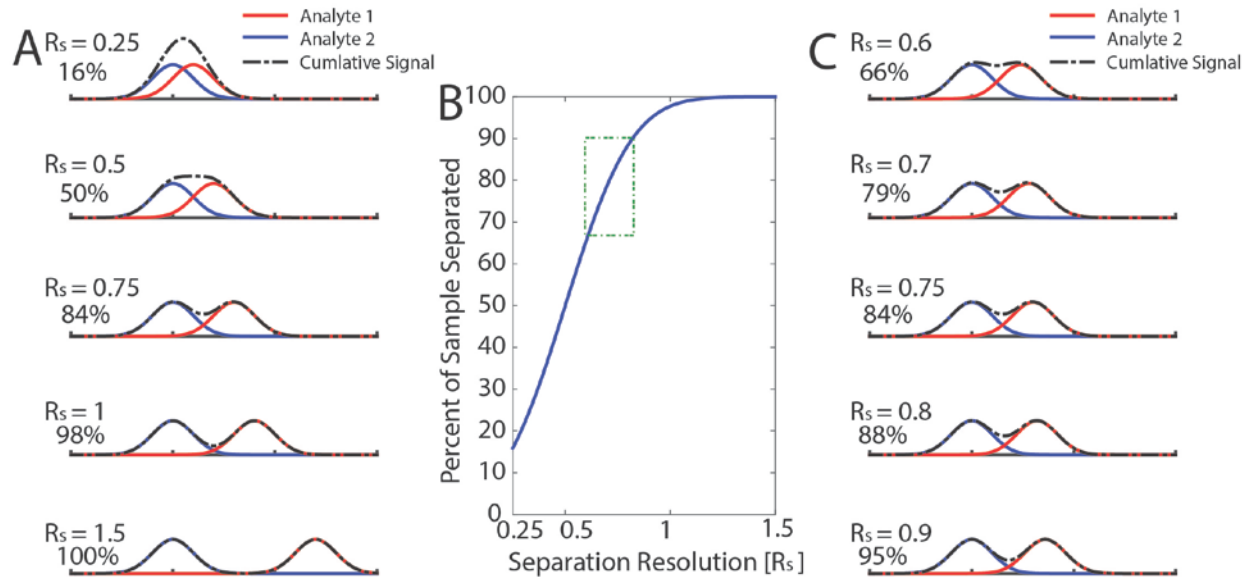


Figure 1.2: The corresponding analyte distributions for separation resolution (R_s) spanning 0.25 to 1.5 is displayed (A). Analyte 1 (red) and analyte 2 (blue) show the location of two species with the labeled separation resolution. The cumulative plot of both analytes (dashed black) show the resulting signal. This would be representative of the observed signal if an antibody bound to both analyte 1 and analyte 2. (B) The percent separated is shown as function of R_s . Ranging from 16% at an R_s value of 0.25 to 100% at an R_s value of 1.5. A critical region of this curve, from 0.6 to 0.9 R_s values, is high-lighted with a green box. (C) The corresponding curves of R_s in that critical region are shown.

1.2.3. Sample Stacking

Sample stacking was originally used and described by Ornstein²⁸ in his seminal work using discontinuous buffer systems for transient isotachopheresis. Sample stacking is defined as concentrating a sample into a small volume. Most protein slab PAGE separations today still utilize the discontinuous laemmli buffer system. Proteins are initially concentrated into a short isotachopheretic stack ($< 100 \mu\text{m}$) within the low density ‘stacking gel’. When the stack of proteins migrates into the denser ‘separation gel’ region, their mobility becomes too slow and they fall out of isotachopheretic stack and electrophoresis proceeds. The advantages of sample stacking are two-fold: high sample concentration to enhance assay sensitivity and sample stacking which reduces injection dispersion to improve separation resolution, see equation 2. Minimizing injection dispersion is especially important for achieving separations over short separation lengths.

In this thesis, while we did utilize transient isotachopheresis in Section 4.7, the majority of stacking was achieved using a discontinuous sieving matrix in homogenous buffers. At a sieving discontinuity there is a difference in mobility across the discontinuity, $\mu_{\text{solution}} > \mu_{\text{sieve}}$, such that when a species migrates from the solution into the sieving medium it will be stacked by the ratio $\mu_{\text{solution}}/\mu_{\text{sieve}}$. An increasing concentration results equal to $C_o * \mu_{\text{solution}}/\mu_{\text{sieve}}$, as well as a decreased population distribution equal to $\sigma_o * \mu_{\text{sieve}}/\mu_{\text{solution}}$. While the sieve discontinuity based stacking typically stacks less than other approaches, the ease in which sieving interfaces can be

fabricated and distributed throughout a device make them well suited for implementation in high-throughput protein electrophoresis applications.

1.2.4. Minimizing Separation Length

In the context of high-throughput separation platforms, the separation length (L) restricts the maximum density of devices that can be realized in a given device area. To consider how resolution is related to L , we solve equation 2 by substituting in separation length for electrophoresis time $t = L / (\mu_1 E)$.

$$\text{Eq. 2} \quad R_s = \frac{\frac{(\mu_1 - \mu_2) L}{\mu_1}}{4 \sqrt{\frac{2DL}{\mu_1 E} + \sigma_0^2}}$$

We assume perfect sample stacking such that injection dispersion is negligible ($\sigma_0=0$) for simplicity in equation 3.

$$\text{Eq. 3} \quad R_s = \sqrt{L} \frac{\mu_1 - \mu_2}{4 \sqrt{\frac{2D\mu_1}{E}}}$$

Reorganize in equation 4.

$$\text{Eq. 4} \quad \frac{R_s^2}{L} = \frac{(\mu_1 - \mu_2)^2 E}{32D\mu_1}$$

Solve for the separation length when the separation resolution is equal to one in equation 5.

$$\text{Eq. 5} \quad L = \frac{32D\mu_1}{(\mu_1 - \mu_2)^2 E} \quad (\text{at } R_s = 1)$$

Equation five highlights the key contributing factors to separation length at a separation resolution of one (excluding injection dispersion, discussed in Section 1.2.3). Similar to the positive factors for increasing separation resolution discussed in Section 1.2.2, increasing differential mobility and electric field while minimizing diffusion will enable short lengths to achieve a separation resolution of one. Notably, separation length is directly proportional to $\mu_1 / (\mu_1 - \mu_2)^2$. To minimize the separation length – for high density separation arrays – it is desirable to maximize the mobility difference between species without increasing the overall mobility. To accomplish this for particles that vary in size, a sieving matrix can be used which impedes the migration of a particle non-linearly with particle size. In this work to minimize separation length for both native and denatured protein separations we use a polyacrylamide gel sieving matrix.

1.3. Protein Polyacrylamide Gel Electrophoresis

The most ubiquitous example of protein fractionation is slab polyacrylamide gel electrophoresis (PAGE)⁵. Polyacrylamide gel (PAG) is a nano-porous hydrogel which acts as a sieve that impedes proteins on the basis of their size and structure. In this work, in addition to PAG sieving, we use the hydrogel material because of its compatibility with engineering new structures and functions.

Polyacrylamide gels $[(-CH_2CHCONH_2)_n]$ are polymerized through a free-radical reaction from a precursor solution containing an acrylamide monomer $[CH_2=CHCONH_2]$ and a crosslinker, typically methylene bisacrylamide $[(H_2C=CHCONH)_2CH_2]$. The nano-pore size of the gel and effective sieving properties, can be tuned by simply modifying the concentration of its monomers. To be covalently linked into the PAG network during polymerization, a chemical requires a carbon double bond to be accessible for free-radical polymerization. Thousands of commercially available chemicals (e.g. molecules containing acrylates, methacrylates, and allyls) can be integrated into the PAG network by simply adding it in the precursor solution prior to polymerization. Facile integration of functional components, such as photo-capture groups²³ or immobilized buffers²⁹, to engineer new assays makes the PAG backbone appealing.

The free-radical polymerization can be initiated using a number of different approaches, most commonly thermally, chemical, or photo initiated. Throughout this work we use photo-initiated PAG polymerization through photo-masks to spatially pattern the hydrogel structures. Photo-patterning facilitates rapid prototyping of new geometries and the realization of spatially complex structures.

1.3.1. Ferguson Relationship & Protein Sizing

Ferguson demonstrated in 1964³⁰ that the relative reduction in protein mobility from free-solution (μ_0) to the mobility through a sieving matrix (μ) can be described by a log-linear relationship with a retardation constant (k) and the density of the sieving matrix (T). The Ferguson relationship, as it is known, is shown in equation 6.

Eq. 6
$$\frac{\mu}{\mu_0} = 10^{-kT}$$

In native protein electrophoresis the retardation coefficient is related to protein size and shape, where larger proteins have a larger retardation coefficient. Migration shift assays take advantage of the large reduction shift in mobility to directly detect a binding affinity molecule³¹. In contrast, when proteins are reduced and denatured in sodium dodecyl sulfate (SDS) they generally form a rod-like conformation whose length is a function of molecular weight. The result is a retardation coefficient that scales with protein molecular weight³². Further, due to the conserved ratio of SDS binding to protein molecular weight (1.4 gram of SDS per gram of protein)³², the free-solution solution mobility is similar for most proteins (although not identical)³³. Sizing can be achieved with SDS denaturation in combination with the PAGE sieving³⁴. We demonstrate in figure 1.3A the mobility of two proteins with a 20% difference in

molecular weight as a function of gel density. In figure 1.3B, we show how the optimal gel density condition for maximizing separation resolution (eq. 1) and for the minimizing separation length needed to achieve an acceptable separation resolution (eq. 5) are different.

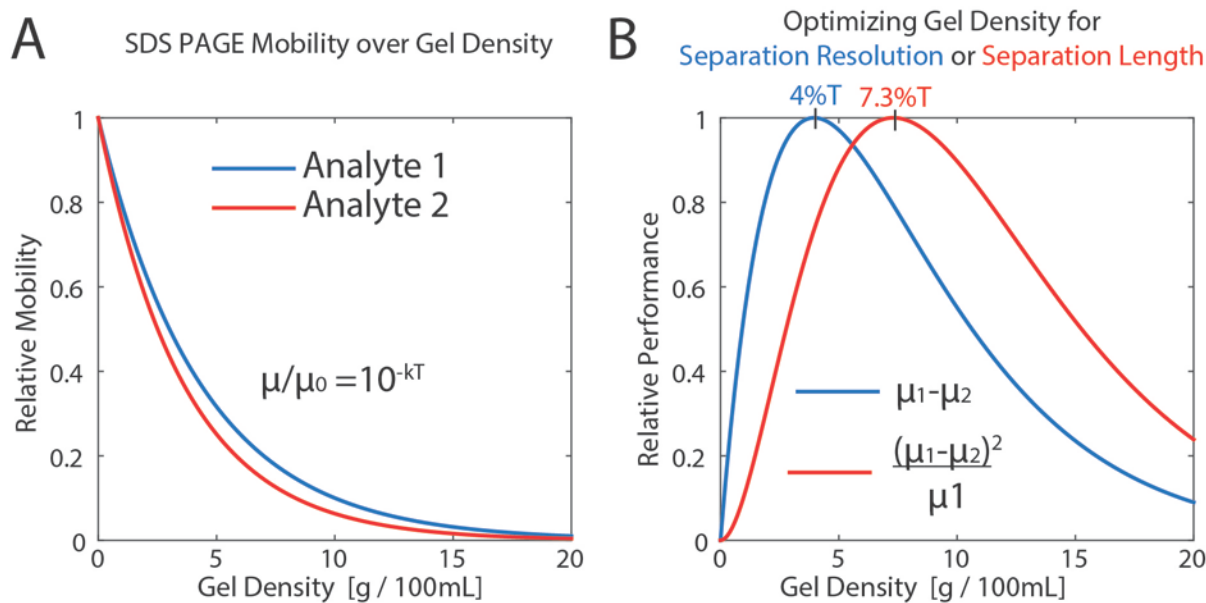


Figure 1.3: PAG density is non-linearly related to protein mobility. Density can be tuned to achieve assay objectives. (A) The relative electrophoretic mobility as a function of gel density, solved for by the Ferguson relationship, is shown for two theoretical proteins in ideal SDS PAGE that have a 20% difference in molecular weight. With SDS PAGE assumptions the retardation coefficients scale with mobility, $k_2 / k_1 = 1.2$, and the free-solution electrophoretic mobilities are the same. (B) Separation resolution, described in eq. 1, is optimized when we maximize the absolute difference mobility of the two species (blue line), shown here occurring in a 4% gel density. Alternatively, to perform high-throughput electrophoresis in a dense array, we want to minimize the separation length required to achieve a separation resolution of 1, described in eq. 5. Here, separation length is minimized at a 7.3% gel density. This demonstrates how gel density can be tuned to maximize protein separations and to achieve specific assay objectives, for example minimizing separation length. For simplicity here we ignore the impact of gel density on the diffusion constant.

1.4. Thesis Overview

This dissertation reports the design and development of PAGE protein separation platforms to enhance throughput. Distinct approaches were taken to accomplish this at various throughput scales throughout my dissertation (figure 1.4).

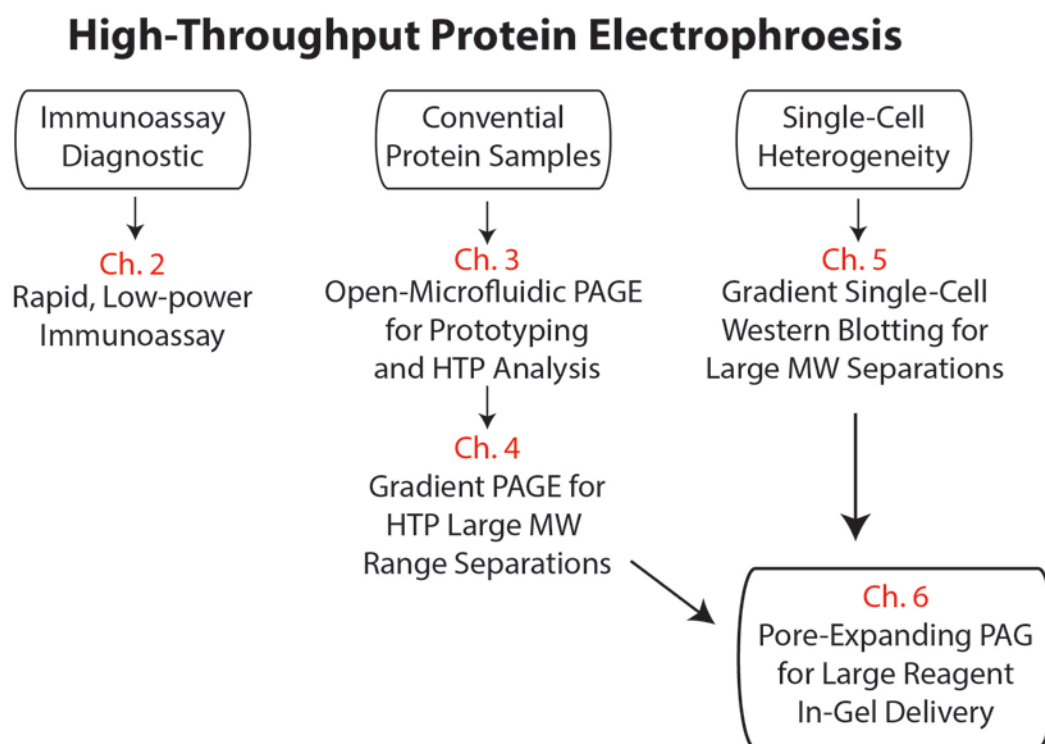


Figure 1.4: Thesis Overview. Microfluidic Bioanalytical Device and Assay Development for High-Throughput Electrophoretic Protein Analysis.

In chapter 2 we describe a new type of assay, PAG moving boundary electrophoresis, and demonstrated it by rapidly performing a protein separation in less than a minute and under a millimeter in separation length. We extended the technique to perform an electrophoretic immunoassay in a 1 mm channel using just a 9 V battery to run the separation – the lowest ever reported voltage for performing a protein separation.

In chapter 3 we describe the development of an open-microfluidic hydrogel platform, free-standing PAGE (*fsPAGE*). The directly photo-patterned gels can be easily modified to quickly realize new geometries for PAGE prototyping. We up-scaled *fsPAGE* into a large array - to achieve 96-plex protein separations in parallel. In chapter 4, we utilized grayscale masks to directly pattern complex spatial variation in gel density. Specifically, a periodic gradient gel array is created to perform high-throughput SDS PAGE.

In chapter 5, we applied the grayscale fabrication technologies to single cell western blotting. This enabled large molecular weight range protein separations from the contents of single-cells in less than a 1 mm separation length. In chapter 6, we create a composite cross-linked PAG that can be stimulated to partially decrosslink and expand in pore-size. This eliminates antibody probing bias throughout the gradient gel and allows access to dense PAGs that were not previously accessible to the single cell western blotting platform.

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Polyacrylamide Gel Electrophoresis Moving Boundary Electrophoresis

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2.1. Introduction

Owing to parallel advances in protein electrophoresis assay design and separation platform design, the past 80 years have seen a striking 10^3 reduction in needed separation distances (i.e., mm to μm) and a similarly striking 10^9 reduction in sample volume consumption.¹⁻³ These advances build from the first electrophoretic protein separation, as reported in Arne Tiselius’ 1930 doctoral thesis.^{4, 5} In this seminal work, Tiselius employed a tube network with an integrated mechanical switch. Along the tubes axis, midway between the anode and cathode, he introduced a central U-shaped section that could be quickly inserted or removed from the network. Protein separations were performed by filling the U-tube with the sample of interest and the rest of the network with buffer. Shifting the U-tube into place completed the electrical circuit to initiate electrophoresis and introduced a sharp initial boundary between the buffer and sample. Using this U-tube apparatus, Tiselius electrophoresed human serum and detected distinct concentration boundaries as species entered and migrated along a buffer-filled tube.⁵ These moving boundaries or fronts corresponded to analytes with differing mobilities. The technique is termed moving boundary electrophoresis (MBE). While copious sample consumption and inclusion of a mechanical switch to define sharp injected concentration boundaries were hallmarks of Tiselius’ remarkable separation science advance, so, too, was design simplicity arising from use of a simple tube to comprise the separation axis.

Building on the conceptual framework of MBE, adaptation of protein electrophoresis to capillary and microfluidic platforms – along with the inclusion of molecular sieving materials – has driven recent performance gains.^{6, 7} Chiefly, gains arise from efficient heat dissipation owing to a high surface area-to-volume ratio on the microscale.⁸ Consequently, 10x higher electric fields can be applied without the need for cooling or appreciable dispersion losses. These higher operating electric fields enable electrophoretic separations in short separation channels. Accordingly, these conditions yield reduced run times as compared to MBE and other more modern competing formats (i.e., slab-gel electrophoresis).^{9, 10} Gains also arose from the advent of zone electrophoresis (ZE), where a zone or ‘plug’ of sample is defined at an injection “port” and injected into a separation capillary or channel for subsequent analysis.^{6, 11} The majority of capillary and on-chip electrokinetic separations use ZE. ZE’s popularity stems from advantages over MBE in sample consumption, readout and extraction simplicity (discrete bands), and the elimination of ‘boundary anomalies’ observed by Tiselius and early separations scientists.¹² Increased sample consumption in MBE is, for most applications, inconsequential when working in the microfluidic regime. Notably, the latter boundary anomaly observations were later termed isotachopheresis and utilized advantageously in discontinuous electrophoresis.^{6, 9, 10} For MBE, isotachopheresis effects are readily eliminated by matching sample and buffer conductivity.⁴

Microfluidic ZE involves use of an injection junction and an adjacent (often orthogonal) separation channel.¹³⁻¹⁵ A cross or double T-channel format are typical architectures comprised of 2 intersecting channels (mm-to-cm in length) and 4 fluid ports. Dramatic improvements in reducing injection dispersion have been realized by incorporating concentrating, or stacking, mechanisms in conjunction with the physical injection. Examples include stacking approaches inspired by macroscale slab gel electrophoresis,^{9, 10} including the use of a size based filters and transient-isotachophoresis.¹⁶⁻¹⁸ Also inspired by slab-gel approaches, our group reported on a photo-patterned PAG pore-size discontinuity in a separation channel as means to create a migration shift for sample stacking with a homogenous buffer system (stacking gel).¹⁹

While powerful in terms of separation performance, the 4-port microchip ZE architecture has a relatively large device footprint,²⁰ making it difficult to scale down for high density multiplexing. Consequently, exciting recent microfluidic design efforts have sought to simplify on-chip electrophoretic systems to a minimal set of channels, sample/buffer ports, and interfacing requirements. For example, Mathies and colleagues used a 3 port direct injection technique to realize a 384-lane capillary array for genetic analysis on a single 20 cm glass wafer.²¹ Sample is directly electrophoretically loaded from a single port and then separated along an orthogonal 8 cm long channel. The approach presents a tradeoff between a larger initial zone size, compared to conventional ZE, and smaller overall device footprint.

To further reduce device footprint, Ross and colleagues introduced gradient elution moving boundary electrophoresis (GEMBE) for high resolution separations in a short (3 mm) single microchannel or capillary.^{20, 22-26} GEMBE utilizes a constant electro-osmotic counter-flow in conjunction with a modulated hydrodynamic counter-flow to controllably introduce analytes into a single inlet, single outlet channel based on electrophoretic mobility. Much like MBE, each species is detected as a moving boundary. Despite the short channel lengths, kiloVolts are required for the establishment of fast electroosmotic flow, resulting in relatively high power consumption – acceptable for many applications yet not desired for others such as those considered in the present study.

In this chapter, we describe a protein electrophoresis platform that relies on a single-inlet, single-outlet design powered simply by voltage control (no pumps, valves, or injectors). In designing the assay and platform, we realize the potential of MBE for minimal interfacing and sample handling now in a microfluidic format and detail the design, fabrication, and demonstration of polyacrylamide gel MBE (PAG MBE). By photopatterning a free-solution-PAG injection interface (stacking gel) within a microchannel, MBE fronts are sharpened by migration shift stacking from the PAG sieve. We optimize the PAG MBE separation for low-power, rapid native protein electrophoresis in a single short microfluidic channel operated by one applied electric potential gradient. Further, in a low-power homogeneous electrophoretic immunoassay (EIA) format,²⁷⁻²⁹ we scale down the channel length (1.3 mm) to power the separation with an off-the-shelf 9 V battery. The EIA completes in 30 s and consumes just 3 μ W, a 3×10^2 reduction in power demand from conventional microfluidic electrophoretic separations.¹⁹

2.2. Materials and Methods

Reagents and Protein Samples

Solutions of 30% (w/v) (29:1) acrylamide/bis-acrylamide, 3-(trimethoxysilyl)-propyl methacrylate (98%), glacial acetic acid and methanol were purchased from Sigma Aldrich (St. Louis, MO). Photoinitiator 2,2-azobis[2-methyl-N-(2-hydroxyethyl) propionamide] (VA-086) was purchased from Wako Chemical (Richmond, VA). AlexaFluor 488 conjugated Trypsin Inhibitor (TI*), Ovalbumin (OVA*), and Bovine Serum Albumin (BSA*) were purchased from Sigma. Polyclonal OVA antibody was purchased from Abcam (ab1221). Tris-glycine (10x) native electrophoresis buffer was purchased from Bio-Rad Laboratories (Hercules, CA). The 'protein ladder' used throughout this chapter consisted of 100 nM TI, OVA, and BSA fluorescently labeled with AlexaFluor 488 in Tris-glycine buffer (pH 8.3).

Fabrication of Glass Microfluidic Chips

Straight 1 cm, 10 μm x 70 μm glass microfluidic channels were fabricated by a foundry service (Caliper Life Sciences, Hopkinton, MA) using standard HF wet etch processes.³⁰⁻³² Access holes were drilled in-house using a Cameron Micro Drill Press (Sonora, CA) with a 2.1 mm diameter Triple Ripple drill bit rotating at 30,000 RPM. For the low-power demonstration, access holes were located 1.3 mm apart, in all other devices holes were located 1 cm apart. The chip was thermally bonded to a blank glass chip in a programmable oven (Vulcan 3-550, Neytech, York, PA) using the temperature program: 30 min at 440°C, 30 min at 473°C, 6 hours at 592°C and finally 30 minutes at 473°C.³³

Methacrylate Surface Functionalization in Glass Microchannels

To prepare the channels for PAG photo-polymerization, they were washed with a 0.1 M NaOH for 10 minutes and then flushed with DI water and purged with vacuum. To create covalent linkages between the PAG and the channel walls during free-radical polymerization, we used an oxide self-assembling monolayer to functionalize the channel walls with a propyl methacrylate group. A 2:3:5 (v/v/v) mixture of 3-(trimethoxysilyl)-propyl methacrylate, glacial acetic acid and DI water was mixed with an analog vortex mixer and then degassed in a sonicator bath under house vacuum for 5 minutes.³⁴ The solution was introduced into the channels using capillary action and allowed to incubate for 30 minutes. After the incubation, channels were rinsed with methanol and DI water, and then vacuum-purged.

PAG Stacking Interface Fabrication

After channel surface preparation, fabrication of the discontinuous pore-size PAG was conducted. Gel precursors consisted of 3% - 14% (w/v) acrylamide concentration with a bis-acrylamide crosslinker ratio of 3% (w/w), and 0.2% (w/v) VA-086 dissolved in Tris-glycine buffer (pH 8.3). After degassing, 3 μl of gel precursor was added to a well and allowed to fill the channel through capillary action. Once filled, an additional 3 μl of gel precursor was added to the opposite well. Photo-polymerization was carried out above a Blak-Ray® UV lamp for 10 minutes. Lamp intensity was measured at 10 mW/cm² with a UV light meter (Lutron Electronic Enterprise Co, UV-340A, Taipei, Taiwan). At 1 minute into the exposure and without removing the chip, 10 μl of Tris-glycine buffer was pumped in and out of the sample well four times. The addition of the buffer prevents polymerization in the well, creating a free solution – PAG stacking interface within the micro-channel. After polymerization, the device was stored

Chapter 2

submerged in Tris-glycine buffer at 4°C until experimentation. Care was taken to avoid trapping of air bubbles in the reservoirs during storage.

PAG MBE Operation

To conduct an electrophoretic separation, the sample wash reservoirs were loaded with Tris-glycine buffer and a platinum electrode was inserted into both wells. A ~2 μ l sample was aliquoted directly into the sample loading reservoir and an electric field was immediately applied. In the low-power demonstration, electrophoretic protein migration was controlled by a standard off-the-shelf 9 V battery (Energizer[®], St. Louis, MO). Current monitoring was performed by placing a 50 k Ω resistor in series with the microchannel and monitoring the voltage drop with a digital multimeter (Agilent 34401A, Santa Clara, CA). In all other experiments, continuous control and monitoring of voltage and current levels at each electrode was accomplished using a custom built high-voltage power supply with current/voltage feedback control. All experiments on the 1 cm channel devices used applied electric fields of 300 V/cm unless otherwise noted.

Fluorescence Imaging

Inverted epi-fluorescence imaging of AlexaFluor 488 labeled protein fronts was performed with a Peltier cooled charge-coupled device (CCD) camera (CoolSNAP HQ2, Roper Scientific, Trenton, NJ) and a 10x objective (UPlanFL, N.A. = 0.3, Olympus, Center Valley, PA) on an Olympus IX-70 microscope. Camera exposure times ranged between 50 and 150 ms with 4 x 4 pixel binning resulting in an acquisition resolution of 3.3 μ m per data point. Light from a 100 W mercury arc lamp was filtered through a XF100-3 filter (Omega Optical, Battleboro, VT) for illumination. To enable comparison between different exposure times as well as to quantify the stacking enrichment factors, the imaging setup was calibrated prior to each experiment. The difference in fluorescence before and after loading labeled sample into the PAG filled channels was divided by the difference in fluorescence before and after the sample was added by capillary action into an empty microchannel. Image analysis was performed with ImageJ software³⁵ (NIH, Bethesda, Maryland). Post processing was performed using an in-house algorithm implemented with MATLAB[®]. Briefly, the cross section of the channel for each position along the separation axis (i.e., x axis) is averaged to yield a single value. A five pixel or frame moving average filter was used in plot profiles or electropherograms, respectively. Front position and variance in either location or migration time was determined by taking the derivative of the plot profile or electropherogram, respectively, and applying a Gaussian peak least-square fitting algorithm. The curve fitting program determined the Gaussian curve conditions (mean and standard deviation) in the last frame of a video sequence (when species are well resolved) and then the videos were regressively analyzed frame by frame passing along curve fitting results from one frame to the initial conditions of the next to automate the process and improve each fit.

Peak Capacity Calculations

Peak capacity for the separations demonstrated in figure 2.3 were calculated for each gel density by taking the protein elution time for 1 mm of PAG migration divided by the temporal bandwidth of the protein after all species in the ladder had been resolved. This was performed reported for both TI and BSA, the smallest and largest proteins of the ladder, respectively.

2.3.PAG MBE Rational Design

We sought to design protein separations realizable in minimal microchannel architectures with compact device footprints and simple interfacing. More specifically, imposed design constraints included optimizing protein electrophoresis for short (< 1 mm) separation lengths in microchannels having just one inlet and one outlet, thus eliminating the use of T-junction, double T-junction, or cross junction geometry. To minimize sample and buffer loading steps, we employed a homogeneous, not discontinuous, buffer system under purely electrokinetic control (i.e., no pressure-driven flow). Owing to these imposed constraints, MBE was pursued here as a promising separation modality for this on-chip protein analysis.

Obtaining high separation performance in short separation lengths requires minimizing injection dispersion.³⁶ For macro-scale separations, where low applied electric fields (< 30 V/cm) separate proteins in centimeters of migration distance, band broadening is dominated by molecular diffusion ($\sigma_{inj}^2/\sigma^2 \rightarrow 0$). Here, σ represents the total spatial standard deviations – proportional to the widths (4σ) – of each Gaussian distribution and σ_{inj}^2 is the injection dispersion. Injection dispersion is largely determined by the sample injection strategy and, thus, the initial zone width ($4\sigma_{inj}$) prior to the separation. As required separation distances are reduced, the injection dispersion becomes a dominant source of dispersion ($\sigma_{inj}^2/\sigma^2 \rightarrow 1$).³⁷ This latter regime has grown in relevance as analytical chemists have begun to realize separations in shorter distances (e.g., owing to the higher electric field strengths attainable in microfluidic devices).

When assessing injection quality and subsequent separation resolution (RS) for resolving species specifically in MBE, a simple transformation allows for separation performance to be analyzed in a manner similar to that of ZE. In MBE, if we consider the spatial derivative of the concentration profile ($\frac{dC(x)}{dx}$), we thus transform the concentration front description into a Gaussian distribution:^{25, 38}

$$\frac{dC(x)}{dx} = \frac{A}{\sqrt{2\pi}\sigma} \exp\left(-\frac{(x-x_0)^2}{2\sigma^2}\right) \quad (1)$$

where A is a constant, x is the axial position along the channel, and x_0 is the mean axial location of the resultant Gaussian distribution. Consequently, RS is represented for MBE in for a manner analogous to ZE and our conclusions regarding minimization of injection dispersion for ZE extend to MBE. Minimization of MBE injection dispersion has been accomplished using a mechanical switch, in Tiselius' U-tube apparatus, or by using a counter-flow to both gate the introduction of species with different mobilities and sharpen the injected concentration fronts, in capillary and microfluidic GEMBE.²⁰ Here, the assay design constraints under study include minimizing interfacing including flow control. Consequently, we explored inclusion of a PA stacking gel at the head of the MBE separation channel (Figure 2.1A, B) to both reduce injection dispersion and impart molecular sieving for electrophoretic mobility based protein separations.

As is relevant to the PAG stacking gel, recall that in PAGE analyte mobility (μ), which is directly proportional to migration rate (U), can be related to PAG pore-size via the Ferguson relationship,³⁹ where $\mu = \mu_0 10^{-KT}$. Where μ_0 is the free solution mobility, K is the retardation

coefficient and T is the total acrylamide concentration in the precursor solution (gel density). As the gel density is increased, PAG pore-size is reduced.⁴⁰ The retardation coefficient is closely tied to protein size and shape, resulting in protein mobility decreasing exponentially with an increase in molecular mass or gel density. Thus, MBE front sharpening would arise from a physically induced shift in migration rate as an analyte moves from free solution (U_0) into the sieving matrix (U). Assuming a short separation length and fast separation time (t), where $\sigma_{inj}^2/\sigma^2 \rightarrow 1$, RS for PAG MBE can be described by:

$$RS \cong \frac{\Delta U t}{4\sigma_{inj}} \cdot \frac{U_0}{U} \quad (2)$$

Thus, the PA stacking gel would act to both enrich the sample and steepen the concentration front by a factor of U_0 / U ,⁴¹ thereby enhancing RS by the same ratio. Increased gel density would result in lower protein in-gel mobility, thus the width of the analyte front should be further reduced until the limiting case, where species are excluded from the PAG region. Further, the PAG size selective sieving matrix acts to enhance the differential mobility between proteins (ΔU). On the basis of these considerations and metrics, design of a PAG MBE separation has the potential to satisfy the single-inlet single-outlet, homogeneous buffer constraints while offering protein analysis under minimal transport control in a compact microchannel network.

2.3.1. Minimizing MBE Injection Dispersion with a PAG Stacking Interface

To characterize the impact of the injected concentration front on PAG MBE separation performance in short microchannels, we studied the injection dispersion of analyte fronts electromigrating through various free solution - PAG stacking interfaces (Figure 2.1C, D). The entry region condition of free solution was selected to maximize the migration shift (U_0/U) to improve separation performance, as described in Eqn. (2). Additionally, the presence of the PAG region downstream of the free solution region prevents bulk flows in the injector region thus eliminating Poiseuille flow induced dispersion. For all conditions considered, the free solution-PAG stacking interface was located roughly 600 μm into the MBE separation channel, thus nominally at the head of separation channels of total length 10 mm. Figure 2.1C reports PAG MBE stacking of a single model analyte front (TI*, 500 nM) as the species electromigrates through each of three different stacking conditions (free solution to PAG regions of 3%T, 10%T and 12%T). These three PAG pore-size conditions were chosen owing to broad applicability in sieving a broad range of protein molecular masses.

Inspection of each concentration front in Figure 2.1C shows that a transition from free solution to the smaller pore-size PAG regions (10%T, 12%T) notably increases the amplitude of the front and reduces axial penetration of the concentration front into the microchannel, as compared to larger pore-size PAG regions (3%T). To quantitatively evaluate injection dispersion in PAG To determine the location and width of each MBE front, standard deviations for each Gaussian distribution were computed from the spatial derivative of the MBE concentration profiles (Eqn. 1) (Figure 2.1D). The resulting standard deviation for each analyte front (i.e., in each of the three PA stacking gel configurations) yielded values of: $\sigma = 196 \mu\text{m} \pm 34 \mu\text{m}$ for injection into a 3%T PAG, $\sigma = 66 \mu\text{m} \pm 1 \mu\text{m}$ for 10%T PAG, and $\sigma = 28 \mu\text{m} \pm 1 \mu\text{m}$ for 12%T PAG ($n = 3$ for each). By using the 3%T as free-solution approximation ($\sigma_0 \cong \sigma_{3\%T}$), we see reduction in front width

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($\sigma_0/\sigma_{\%T}$) as 3.0 ± 0.5 for the 10%T PAG configuration and up to 7.0 ± 1.3 for the 12%T PAG configuration. Thus, with a small pore-size, the PAG stacking interface reduced the equivalent injection peak width by over 85% for the three cases considered here. Consequently, even with the homogeneous buffer system employed, PAG stacking concentrates and sharpens protein fronts while the presence of sieving matrix eliminates detrimental bulk flow effects and reduces the rate of molecular diffusion.

We next consider the stacking gel configuration in the context of realizing PAG MBE as a separation. As shown in Figure 2.1C, D, the mobility of the front slows with an increase in PAG density. For example, at an elapsed separation time of 4 s, the concentration front locations were measured at: $x_0 = 400 \mu\text{m} \pm 6 \mu\text{m}$ for a 3%T PAG, $x_0 = 139 \mu\text{m} \pm 5 \mu\text{m}$ for a 10%T PAG, and $x_0 = 55 \mu\text{m} \pm 8 \mu\text{m}$ for a 12%T PAG ($n = 3$ for each). Using the 3%T PAG measurements as a free-solution approximation of the TI* mobility, we evaluate TI* migration shifts ($U_0/U_{\%T}$) in PAG of higher densities, yielding: 2.9 ± 0.1 for $x_{0, 3\%T}/x_{0, 10\%T}$ and 7.3 ± 1.1 for $x_{0, 3\%T}/x_{0, 12\%T}$. Based on Eqn. (2), the reduction of front width from the free-solution case should be directly related to the measured migration shift. The close match between migration shift ($U_0/U_{\%T}$) and MBE front sharpening ($\sigma_0/\sigma_{\%T}$) for both the 10%T and 12%T PAGs investigated here indicates that PAG MBE front sharpening can be accurately described by the PAG sieve induced migration shift.

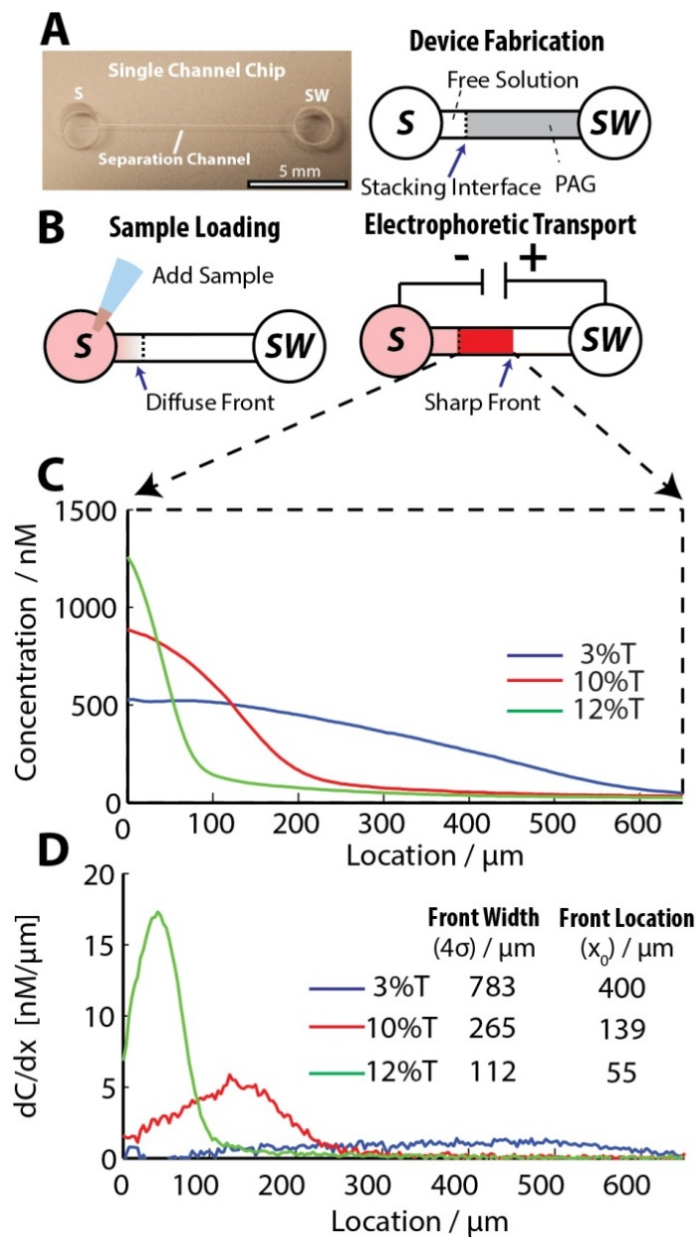


Figure 2.1 PAG MBE utilizes a photo-patterned stacking gel to define a sharp injection front necessary for adaption of MBE to a microchannel format. (A) Photograph of the single glass channel PAG MBE microdevice (*left*). Schematic illustrating the two media housed in the straight microchannel: a free solution region abutting a PAG sieving matrix region yielding a sharp stacking interface, indicated with an arrow (*right*). (B) Sample was pipetted into a fluid reservoir (*left*), an electric field was then applied and analytes electromigrate into the microchannel according to mobility (*right*). (C) The free solution-to-PAG transition at the head of the PAG MBE separation axis was tuned by decreasing the pore-size of the PAG region (3%T, 10%T, 12%T). Fluorescence traces indicate the concentration distribution of TI* as the front electromigrates into PAG MBE channels. PAG density varied, all other conditions held constant ($t_{\text{separation}} = 4 \text{ s}$, $E = 100 \text{ V/cm}$, $[\text{TI}^*] = 500 \text{ nM}$). (D) Corresponding concentration spatial derivatives, dC/dx , for the TI* front injections shown in (C).

2.4. Native Protein PAGMBE

To establish PAG MBE for native proteins over short separation lengths, we characterized the influence of MBE separation conditions on RS . Figure 2.2 shows full field imaging results from a PAG MBE separation of a ladder of three native proteins. A free solution – 12%T PA stacking gel interface located 600 μm from the sample well was again employed (see *SI* for time evolution video microscopy data). The false color micrographs in Figure 2.2A report the electromigration of concentration fronts into the PAG MBE separation channel. Within a 3 s elapsed separation duration, all three fronts have transitioned through the free solution PA stacking gel and are visually detectable. Identification of each ladder species was verified a priori through MBE analysis of each species alone thus yielding an apparent electrophoretic mobility for each.

For the three ladder pairs studied (BSA*-OVA*, OVA*-TI*, BSA*-TI*), RS was monitored in time as the critical metric for determining the elapsed separation time and total required separation length needed to baseline resolve the native species. Figure 2.2B shows that within 5 s all protein pairs are resolved, with a RS of 1.0, 1.1 and 2.3 for the BSA*-OVA*, OVA*-TI* and BSA*-TI* pairs, respectively. Figure 2.2C shows the PAG MBE separation at 7 s of elapsed separation time. Each of the three species present has been enriched upon passing through the 12%T PAG interface, with BSA* being enriched most effectively (5.1 factor) and TI* being least enriched by the 12%T interface (2.5 factor), with this sized-based bias in enrichment expected owing to the size-based migration shift mechanism operating at the PAG stacking interface. Further, the TI* front (peak) was notably more disperse than those of either of the larger protein species assayed. The observed differences in enrichment and dispersion between BSA* and TI* are an expected consequence of the known differences of their retardation coefficients ($K_{BSA} > K_{TI}$). The resolved PAG MBE separation of the model protein ladder required ~ 500 μm of migration in the PAG sieving matrix with the full field imaging readout used here for PAG MBE characterization. The native assay results presented in Figure 2.2 support the design assertion that PAG MBE is capable of providing useful functionality in a single straight 2 port channel, thus complementing similar assays that use a 4 port double T-channel injector¹⁹ or 3 port direct injection scheme.²¹

To evaluate minimization of the required separation lengths needed for native protein analysis, electropherograms were collected at sequential locations along the channel for PAG MBE separations in five PAG densities (5%T, 8%T, 10%T, 12%T, and 14%T). Figure 2.3A reports the electropherogram derivative at 90 μm intervals along the PAG sieving matrix. Evolution of the separation in time was monitored by computing RS as a function of migration distance for each PAG density (Figure 2.3B). No separation was observed in the 5%T gel. Over the same distance the BSA*-TI* separations were resolved ($RS = 1$) at 283 μm , 242 μm , 128 μm and 61 μm for the 8%T, 10%T, 12%T, and 14%T PAGs, respectively. As expected, for each experiment the RS grew with the square root of the migration distance.⁴² These separation distances are 10x shorter than the best reported on-chip PAGE separation lengths to date.⁴³ The improved resolution in dense gels was also apparent in projected peak capacity for a 1 mm channel. When TI is used as the reference analyte (see *SI* for more details), which has the highest mobility and diffusivity of the protein ladder, the peak capacity was 3.5 in a 10%T gel, 5.2 in a 12%T gel, and 6.5 in a 14%T gel. When BSA is used, the peak capacity was 4.0 in a 10%T gel, 8.9 in a 12%T gel, and

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10.1 in a 14%T gel. Sufficient for migration shift assays, but for more complex separations, channels greater than 1 mm would be required. While performance does improve with PAG density, practical limitations on the maximum usable gel density exist. In the optimally performing 14%T PAG configuration, all separations are completed in the first 200 μm of sieving, with a *RS* of 1.0, 2.2 and 3.7 observed for the OVA*-TI*, BSA*-OVA* and BSA*-TI* separations, respectively in Figure 2.3C.

In the 14%T separation a “system peak” (negative signal) was observed behind the BSA peak at 360 μm in Figure 2.3A. We attribute the decreased signal to physical exclusion of a BSA dimer (commonly resolved in native PAGE⁴⁴) at the stacking gel interface. The system peak can be further enhanced by gel non-uniformities near the solution–PAG interface. Characterized previously for photo-patterned discontinuities,¹⁹ the interface gel can exhibit significantly smaller pore-size than bulk PAG. This reduced pore-size at a free solution–PAG interface has a two-part impact. Firstly, the interface can reject large analytes which normally would be able to migrate in a given PAG density. We address this consideration through use of slightly lower gel densities than employed in bulk uniform gels. Secondly, the non-uniformity can make spatial detection of fronts close to the interface difficult ($< 100 \mu\text{m}$), as indicated in Figure 2.2C. Care must be taken in analysis of the intensity spatial derivative (dI/dx), as change in a signal as a result of gel non-uniformity could be confused with the presence of a protein front. For this reason, we choose to utilize temporal detection when performing protein PAG MBE analysis within short separation lengths.

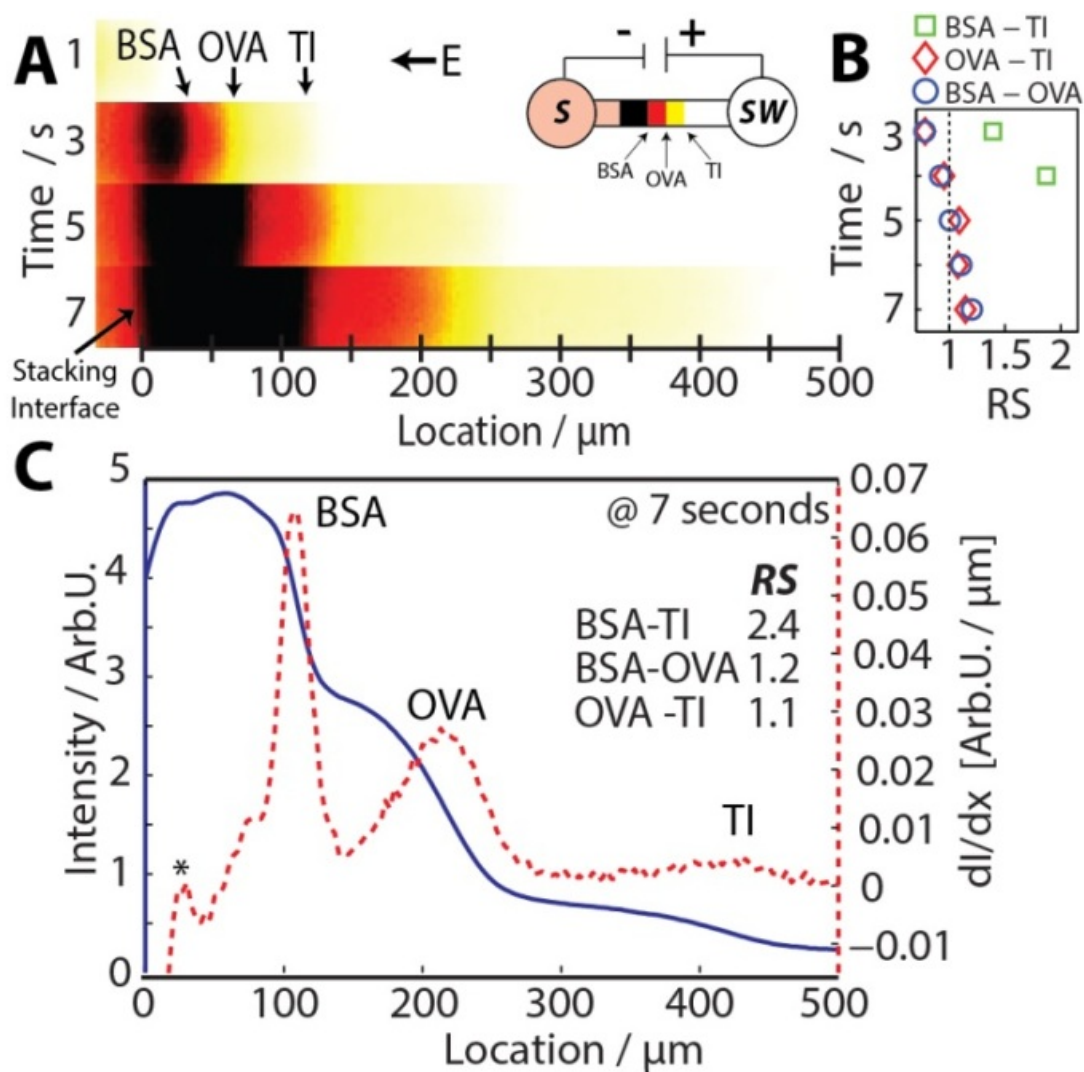


Figure 2.2. PAG MBE for rapid analysis of native proteins. (A) Epi-fluorescence micrographs show time evolution of a PAG MBE separation of a native protein ladder (BSA*, OVA* and TI* at 100 nM each). False color CCD images are labeled to indicate protein front locations at 3 s elapsed separation time. (B) Time evolution of RS shows critical separation duration needed for each analyte pair. (C) The spatial concentration profile (solid trace) and the spatial derivative of the intensity signal (dI/dx , dashed trace) at 7 s of elapsed separation time. RS for each protein pair is indicated in the inset. A system peak (*) at 20 μm is attributed to gel non-uniformities at the interface and subsequent protein retention. $E = 300 \text{ V/cm}$, 12%T PAG.

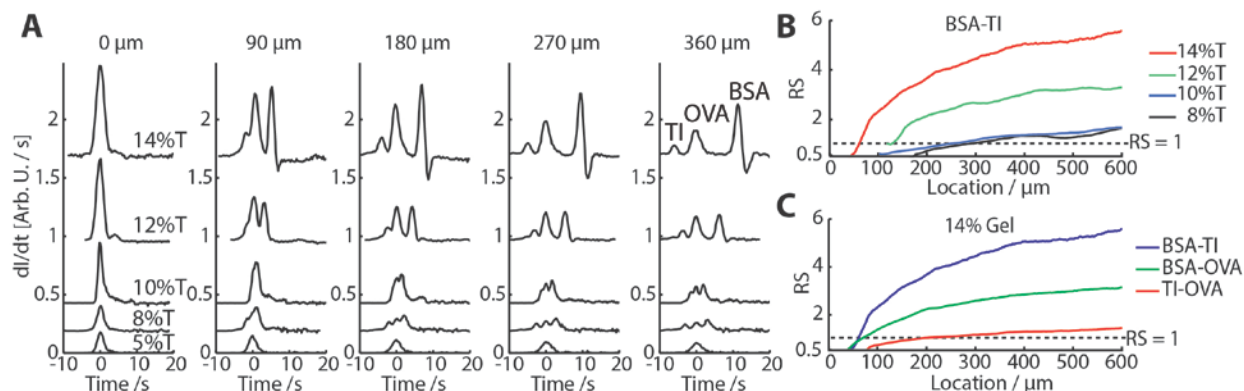


Figure 2.3 Tuning PAG density optimizes subsequent PAG MBE of native proteins. (A) The electropherogram derivative for each PAG density collected at 90 μm intervals along the PAG region. To enable visual comparisons, the x-axis of each plot was shifted such that the OVA* peak is centered at 0 seconds. (B) RS for the BSA*-TI* versus migration distance over a range of gel densities. (C) RS for each protein in the 14% gel density case. At 200 μm the separation was complete for all proteins: a RS of 1.0, 2.2 and 3.7 for the OVA*-TI*, BSA*-OVA* and BSA*-TI* separations, respectively. $E = 300 \text{ V/cm}$.

2.5. Low-Power Homogenous Electrophoretic Immunoassays

In light of the separation performance in PAG MBE (small separation distance and peak capacity) and the scalability of the single channel 2-port architecture, we sought to further develop an EIA²⁷⁻²⁹ using this format (Figure 2.4), now powered by an off-the-shelf 9 V battery with no voltage amplification (Figure 2.5). Extending electrophoretic analysis to a portable, battery powered platform for applications such as remote environmental sensing and point-of-care medical diagnostics has long been a goal of diagnostics developers. Previous work has implemented conventional capillary electrophoresis assays directly into the field through the development of the electrical infrastructure for amplifying a low-voltage consumer battery into a portable high voltage power supply (1000's of volts).⁴⁵⁻⁴⁷ Here our design goals include leveraging the short separation distances required to eliminate the need for applying high voltage at all. With short fabricated channels, the desired electric field strengths and rapid time-to-result¹ could be achieved under low applied voltage conditions.

First, we extended the separation performance of PAG MBE to homogeneous EIAs in the 1 cm long separation channels described in Figure 2.1A, still with conventional high-voltage control. For direct comparison to the native protein PAG MBE assays detailed above, we applied the PAG MBE separation developed earlier to the three protein ladder. Then an EIA for OVA was added by including an unlabeled polyclonal OVA antibody (75 nM) in the three protein native ladder. The combined OVA EIA and protein separation were conducted in a 10%T PAG device, the highest density gel employed without noticeable size-based immobilization of immune-complexes in the gel structure. As reported in Figure 2.4A, the OVA immunoassay was completed at 300 μm beyond the stacking gel (Figure 2.4B). The migrating front of the protein ladder sample passes at 5 s followed by the slow migrating OVA*/anti-OVA immune-complex front at 15 seconds. At lengths farther downstream, the protein ladder separations are completed

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as detailed in the previous section. The EIA was verified with a negative control in Figure 2.4C, with the same protein ladder and an antibody non-specific to any protein in the ladder.

Next, we implemented the EIA in a short channel developed for low-power electrophoresis. We fabricated a 10x shorter (1.3 mm long) glass microchannel housing the PAG gel configuration shown in Figure 2.1A (6%T PAG). Here, though, the OVA proof-of-principle EIA was powered by a 9 V off-the-shelf battery (Figure 2.5A). The time-evolution of the homogeneous EIA is displayed in Figure 2.5B. The PAG MBE allowed resolution of the migrating OVA* and immune-complex peaks in 25 s with a required separation length of just 140 μm beyond the stacking gel (Figure 2.5C, D). Although just 11% of the total fabricated separation length was utilized for the PAG MBE immunoassay, fabrication of the channel length was limited to 1.3 mm owing to interference between the 2 mm diameter S and SW ports employed. Use of smaller drill bit diameters or alternate methods of fabricating the fluid reservoirs will allow for fabrication of even shorter separation channels, which should prove sufficient for completing the EIA. Nevertheless, the shorter 1.3 mm long separation channels were appropriate for use with 9 V battery operation.

In this low-power assay, the 9 V battery drove a 300 nA current resulting in demonstration of a 3 μW immunoassay. Thus, the use of the PAG MBE format and the ultra-short channel reduced power demands by 4×10^5 from standard lab medicine assays and 3×10^2 from conventional microfluidic-based homogeneous EIAs.^{19, 48, 49} To highlight the impact of reduced power consumption in this present study, other portable battery powered electrophoretic technologies reported 15 hour continuous operation times,⁴⁶ whereas the 3 μW assay demonstrated here could theoretically be powered by an off-the-shelf 9 V battery for over 200 years (ignoring natural degradation), an important design consideration for deployment to low-resource settings.

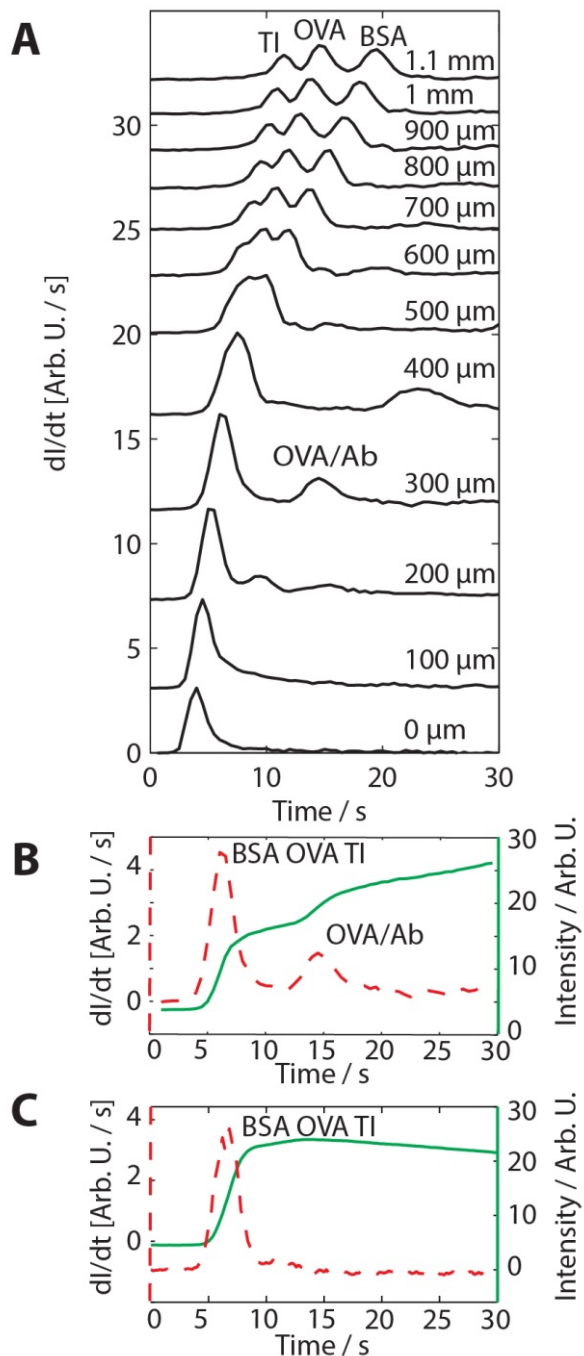


Figure 2.4. PAG MBE enables efficient homogeneous EIAs. (A) Time evolution of a PAG MBE protein separation and OVA immunoassay. The temporal derivative of the analyte concentration distribution is reported at progressive locations along the 10%T PAG. Native protein multimers were detected at 600 μm and 200 μm (BSA dimer and trimer,⁵⁰ respectively), and at 900 μm (72 kDa OVA isoform).⁵¹ Electropherogram of the concentration fronts (solid trace) overlaid with the corresponding time derivative (dashed trace) for the (B) homogeneous electrophoretic OVA immunoassay and a (C) negative control using an antibody non-specific to any proteins in the system. Detection point was 300 μm downstream of the stacking gel, 900 μm from the injection port. $E = 300$ V/cm.

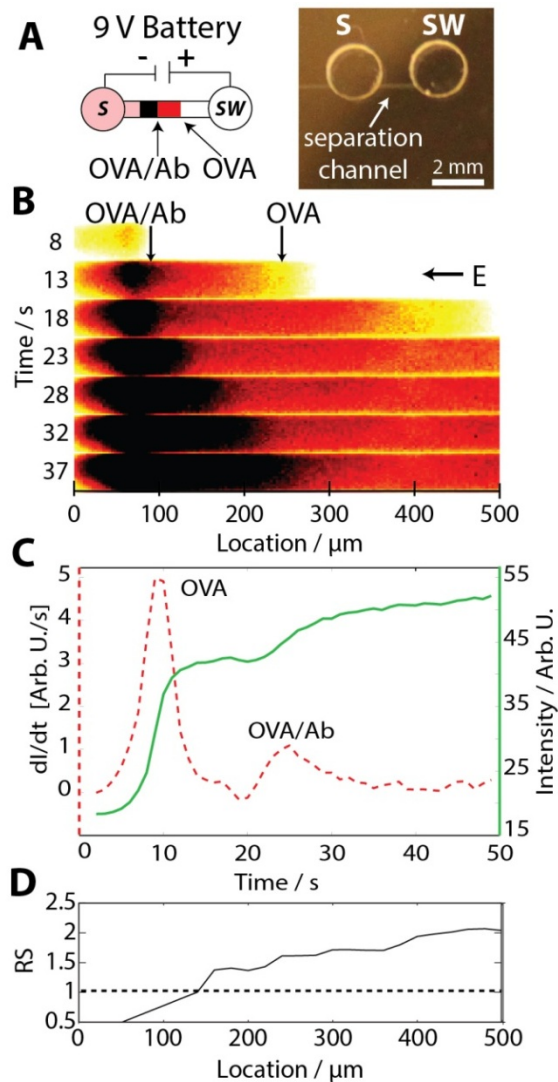


Figure 2.5. Low-power 3 μW EIA is completed using PAG MBE driven by a 9 V battery in a 1.3 mm channel (A). (B) A false color CCD time evolution montage of the EIA (200 nM OVA*, 250 nM OVA-antibody) displays the low-power separation which completes in less than 25 seconds. (C) The electropherogram (solid trace) and the temporal derivative (dI/dt , dashed trace) are overlaid at the (D) 140 μm separation length.

Conclusions

By introducing a PAG stacking interface in the classic MBE protein separation, we introduce a variant of the MBE assay that is appropriately designed for implementation in a microchannel. Careful control of frontal dispersion through a stacking gel yields high resolution MBE protein separations driven exclusively with electrophoresis, as opposed to a physical switch or bulk flow elution. Outcomes of this microfluidic adaption of MBE are two-fold. Firstly, PAG MBE enables rapid protein analysis in a compact microdevice footprint. The single channel, 2-port architecture is well suited for scale up into, for example, a dense array of separation channels. We see this approach as broadly relevant to high-throughput targeted proteomics and migration shift

screening.⁵²⁻⁵⁵ Secondly, PAG MBE enables “down-scaling” of protein electrophoresis to short separation and channel lengths. Importantly, this aspect of the PAG MBE performance uniquely enables low-power EIAs, as demonstrated here using a 9 V battery. We illustrate the concept by reporting an EIA that consumes just 3 μ W of power. We see the low-power EIA formats as amenable to powering by a myriad of inexpensive, readily available power sources including, for example, salvaged photovoltaic cells, cell phones, or other consumer biomedical or communication electronic devices.

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Free-Standing Polyacrylamide Gel Electrophoresis

T.A. Duncombe & A.E. Herr, “Photopatterned free-standing polyacrylamide gels for microfluidic protein electrophoresis”, *Lab on a Chip*, **2013**, 13, 2115-23. Reproduced In part by permission of The Royal Society of Chemistry

3.1. Introduction

Prior to 1955, electrophoretic separations were conducted in either buffer-filled glass tubes or porous filter paper.^{1, 2} That year was a turning point for electrophoresis when Oliver Smithies introduced starch as a molecular sieving matrix and dramatically altered electrophoresis assays.³ The gel matrix is important to separation performance, as the material minimizes molecular diffusion and retards analyte electromigration yielding a broad range of electrophoretic mobilities.⁴ Attesting to the power of the separation matrix, contemporary slab-gels (e.g., polyacrylamide, agarose) remain remarkably similar to Smithies’ original mid-twentieth century starch gel separation system.

Recently, substantial efforts have been undertaken to advance slab-gel electrophoresis performance. Commercial products supporting forty eight and ninety six concurrent protein separations are feasible (Agilent TapeStation®, Invitrogen E-PAGE™). Research-grade slab-gel electrophoresis systems supporting hundreds of concurrent separations have been introduced as well, with Ciaccio *et al.* utilizing a nanoplotter to array nanoliter sample volumes directly atop a large format slab-gel.⁵ While a striking demonstration of multiplexing capacity, both assay speed and separation performance would still benefit from advances. As the nanoplotting of samples required hours and diffusion during sample injection reduced separation performance.

In contrast to slab-gel based formats, microfluidic electrophoresis systems enable low sample injection dispersion and high efficiency separations.⁶ Channel networks (cross-t and similar injector geometries) are designed to minimize injection dispersion.⁷ Confining the electrical current path to a microchannel provides efficient heat dissipation enabling the application of 10x higher electric fields with no additional cooling.⁸ Consequently, microfluidic formats facilitate unprecedented efficiency in protein sizing (e.g., < 1 min separations in < 1 mm separation length).⁹⁻¹¹ Multiplexing and enhanced throughput have been achieved, including arrays of tens to hundreds of microchannels supporting concurrent electrophoretic separations.^{12, 13} Such approaches have, for example, advanced on-chip nucleic acid sequencing.¹⁴

While microfluidic protein separations can perform markedly better than slab-gel polyacrylamide gel electrophoresis (PAGE) systems in terms of assay speed and multiplexing, separations conducted in enclosed microfluidic channels suffer from significant shortcomings. Filling and addressing dense microchannel arrays generates new challenges in fluidic and electrical interfacing, requiring complex manifolds for operation.^{12, 15, 16} Importantly, enclosed channels also complicate common and often essential post-separation protein manipulation steps including protein staining, western blotting, mass spectrometry, and sample collection.¹⁷⁻²⁰ Further, microfluidic PAGE systems are primarily housed in wet etched glass microchannels. As such, PAGE assay has not yet benefitted from the rapid prototyping approaches used to drive

innovation in other bioanalytical techniques, including whole cell manipulation and analysis, lateral flow assays, and droplet microfluidics.

To address these lingering shortcomings in microscale electrophoretic separations, we introduce rapid prototyping of free-standing polyacrylamide gel (*fsPAG*) structures – with customizable injection reservoir and separation gel properties – on planar substrates amenable to scanner-based imaging. We find the < 1 hr *fsPAG* design-fabricate-test cycle beneficial for ready customization of assay parameters. Using the *fsPAG* structures, we characterize the impact of key design choices on protein *fsPAGE* performance in moving boundary and zone electrophoresis formats. We characterize the injection mode and resultant injection dispersion and introduce a device geometry for concurrent operation of 96 *fsPAG* assays through a single electrode pair and the variation of migration across the array. We observe that the *fsPAGE* system offers a hybrid of advantages between macroscale PAGE and microfluidic electrophoresis.

3.2. Materials and Methods

Reagents

Solutions of 30% (w/v) (29:1) acrylamide/bis-acrylamide, glacial acetic acid, glycerol, ethanol, methanol, SYPRO Ruby protein gel stain, Brij L23, and Triton X-100 were purchased from Sigma Aldrich (St. Louis, MO). Photoinitiator 2,2-azobis[2-methyl-N-(2-hydroxyethyl) propionamide] (VA-086) was purchased from Wako Chemical (Richmond, VA). Molecular biology grade (DNase-, RNase-, and Protease-free) water was purchased from Mediatech, Inc. (Manassas, VA). GelBond® PAG Films and Gel Slick® glass plate coating were purchased from Lonza (Base, Switzerland). Photo-masks were designed using CleWin (PhoeniX Software, Enschede, Netherlands) and printed on mylar transparencies at CAD/Art Services (Brandon, OR). Green fluorescent 15 µm FluoSpheres® were used for EOF visualization as purchased from Invitrogen Life Technologies Corporation (Carlsbad, CA). FluoSpheres® are sulfate based microspheres and have a net negative charge at neutral pH. AlexaFluor 488 (AF488) conjugated Trypsin Inhibitor (TI*, 21 kDa), Ovalbumin (OVA*, 45 kDa), and Bovine Serum Albumin (BSA*, 67 kDa) were purchased from Life Technologies Corporation. Unlabeled BSA was purchased from Sigma and unlabeled OVA from Thermo Scientific (Rockford, IL). Tris-glycine (10x, pH 8.3) native electrophoresis buffer was purchased from Bio-Rad Laboratories (Hercules, CA) and 1 M Tris-HCl (pH 8.6) was purchased from bioWORLD (Dublin, OH). The protein ladder used in this study consisted of 500 nM each of TI*, OVA*, and BSA* fluorescently labeled with AF488 in the Tris-glycine buffer. Fluorescently labeled proteins are denoted with a “*”. Unless otherwise noted, all sample buffers contained 1x Tris-glycine (25 mM Tris, 192 mM glycine, pH 8.3), 10% glycerol and 0.5% Triton X-100 and all gel buffers contained 1x Tris-glycine and 20% glycerol. While SDS PAGE is anticipated to be fully compatible with *fsPAGE*, all demonstrated separations are in native conditions. As is necessary for measuring protein complexes.

fsPAGE Operation

Operation of *fsPAGE* was performed in the environmental chamber detailed in figure 3.1 and is similar to slab-gel semi-dry electrophoresis. The *fsPAG* devices were soaked in run buffer for 5 min. When removed, the back side of the GelBond® was quickly dried with a Kimwipe (Kimberly-Clark Corporation, Neenah, WI) and placed atop a piece of borosilicate glass (CBS

Scientific, San Diego, CA). Residual buffer was wicked from the reservoirs and from the top of the PAG surfaces using another Kimwipe[®]. For *fs*PAG MBE devices, the sample of interest is pipetted into the sample well and a run buffer is pipetted in to the opposite, sample wash well. Platinum electrodes are aligned and inserted from above into the two wells and electrophoresis is immediately initiated by a Caliper high voltage power supply. For zone electrophoresis *fs*PAGE devices, electrode wicks (Serva, Heidelberg, Germany) were wetted in run buffer, excess buffer was removed with a Kimwipe, and the damp electrode wicks were then placed atop the gel contact pad. Protein samples were pipetted into the reservoirs and the device was placed in the environmental chamber. Graphite electrodes (Bio-Rad Laboratories) were placed in contact with the electrode wicks. The environmental chamber was sealed with a borosilicate glass plate and a voltage was applied with a PowerPac[®] HV power supply (Bio-Rad Laboratories) to initiate electrophoresis.

Imaging

Fluorescence imaging in figures 3.4 through 3.8 was conducted on an inverted epi-fluorescence microscope (Olympus IX-70) equipped with a Peltier cooled charge-coupled device (CCD) camera (CoolSNAP HQ2, Roper Scientific, Trenton, NJ) and a 2x objective (PlanApo, N.A. = 0.08, Olympus, Center Valley, PA). Camera exposure times were 300 ms, unless otherwise indicated. Illumination was sourced from an X-Cite[®] exacte mercury lamp (Lumen Dynamics, Mississauga, Canada) filtered through a XF100-3 filter (Omega Optical, Battleboro, VT). Large area imaging in figures 3.9 and 3.10 was performed using a ChemiDoc XRS+ trans-illuminator with an XciteBlue conversion screen and standard ChemiDox XRS+ 548-630 nm emission filter (Bio-Rad Laboratories). Image analysis was performed with ImageJ software²¹ (NIH, Bethesda, MD). Intensity plots were extracted across the transverse axis of the separation gel, thereby including any added dispersion from protein band bowing. Post processing was performed using an in-house algorithm implemented with MATLAB[®] made by MathWorks (Natick, MA).

To create particle streak lines in the electroosmotic flow (EOF) studies, exposure times of 6 s were employed with an image acquisition rate one frame per 7 s. During image collection, the applied electric potential was removed midway through exposure and the beads slowed to a stop during the acquisition, yielding an apparent ‘head and tail’ for each migrating bead. The circular head represents the final location of a bead while the tail indicates the beads path and relative velocity during motion, approximating transport in a manner analogous to a velocity vector field.

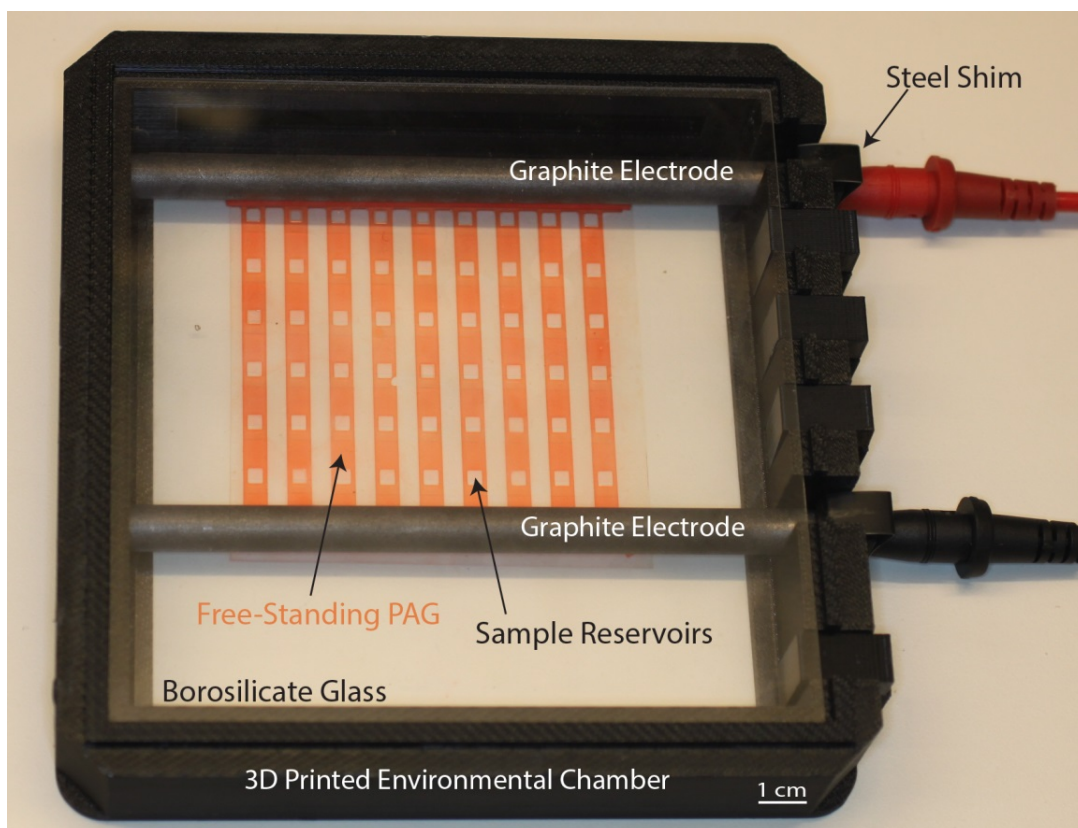


Figure 3.1 We constructed an environmental chamber to minimize evaporation during *fsPAGE*. The 3D printed holder was designed in Solidworks (Waltham, MA) and 3D printed using a uPrint[®] sold by Stratasys (Eden Prairie, MN). The .STL and .SLDPRT files are available on request. Graphite bar electrodes (#1702980) and M4 to banana plug connectors (#9007004) were purchased from Bio-Rad Laboratories. Steel shim stock with a 0.1 mm thickness was purchased from OnlineMetals.com (Seattle, WA). Borosilicate glass plates with 1 mm thickness were purchased from CBS Scientific (San Diego, CA). Electrode wicks (300 mm x 6 mm x 1 mm) manufactured by Serva (Heidelberg, Germany) were purchased from Crescent Chemical Company (Islandia, NY) and were placed between the *fsPAG* and the graphite electrodes.

3.3. Rapid Prototyping *fsPAGE* for Accelerated Innovation in PAGE Design

For protein PAGE analysis, we fabricate *fsPAG* microstructures that comprise both the sample injection reservoir and separation lane. The *fsPAG* fabrication process (figure 3.2A) was adapted from fabrication protocols for 3D hydrogels used to study cellular interactions.^{22, 23} After mask design and printing, the first fabrication step is sandwiching a PAG precursor solution (containing photo-initiator) between a support surface and a lid; here, the lid is coated with Gel Slick[®] to minimize gel attachment. The support surface should present exposed unsaturated hydrocarbons for covalent bonding with the PAG during the free-radical polymerization process. We use a GelBond[®] PAG support but note that the desired chemistry can be coated on glass²⁴ and some polymer surfaces.²⁵ Second, the precursor solution is exposed to UV light through a

mask to photo-polymerize the desired *fsPAG* structure geometries. Exposure proceeds for 35 s using a 13 mW/cm² UV intensity and for up to 240 s for a lower UV intensity of 8 mW/cm² (measured by a UV light meter). Third, the lid is carefully removed and unpolymerized precursor solution is gently washed away leaving behind the three-dimensional structures. Once UV intensity and time has been optimized for a given gel density the fabrication method has excellent yield (> 95%, $n \sim 100$). For the purpose of realizing zone electrophoresis devices, we fabricated structures with μ m-scale heights (120 μ m) and mm-scale in-plane features. Without optimizing for hydrogel photopatterning resolution, as our application does not require it, we have realized a minimum features size of 75 μ m in width. This is on-par with previously reported photopatterned hydrogels structures which range from 50 μ m to 5 μ m.^{23, 26} The PAG microstructure height (z-axis) is adjusted by modifying gasket height with spacers. The z-axis resolution is determined by the precision of spacer height.

If a buffer exchange step is desired prior to PAGE, a 5 min soak in the run buffer of interest is required.²⁷ All devices used in this study were stored in run buffer solution prior to use. From start to finish, the *fsPAG* fabrication process requires < 10 min. If mask printing capability is available on site, researchers can complete the entire ‘design-fabricate’ cycle in well under 1 hour, notably faster than the common and powerful rapid prototyping processes using polydimethylsiloxane (PDMS) for micro-device prototyping. Fabrication of *fsPAG* microstructures does not require mold fabrication, as the features are directly photopatterned on the support substrate.

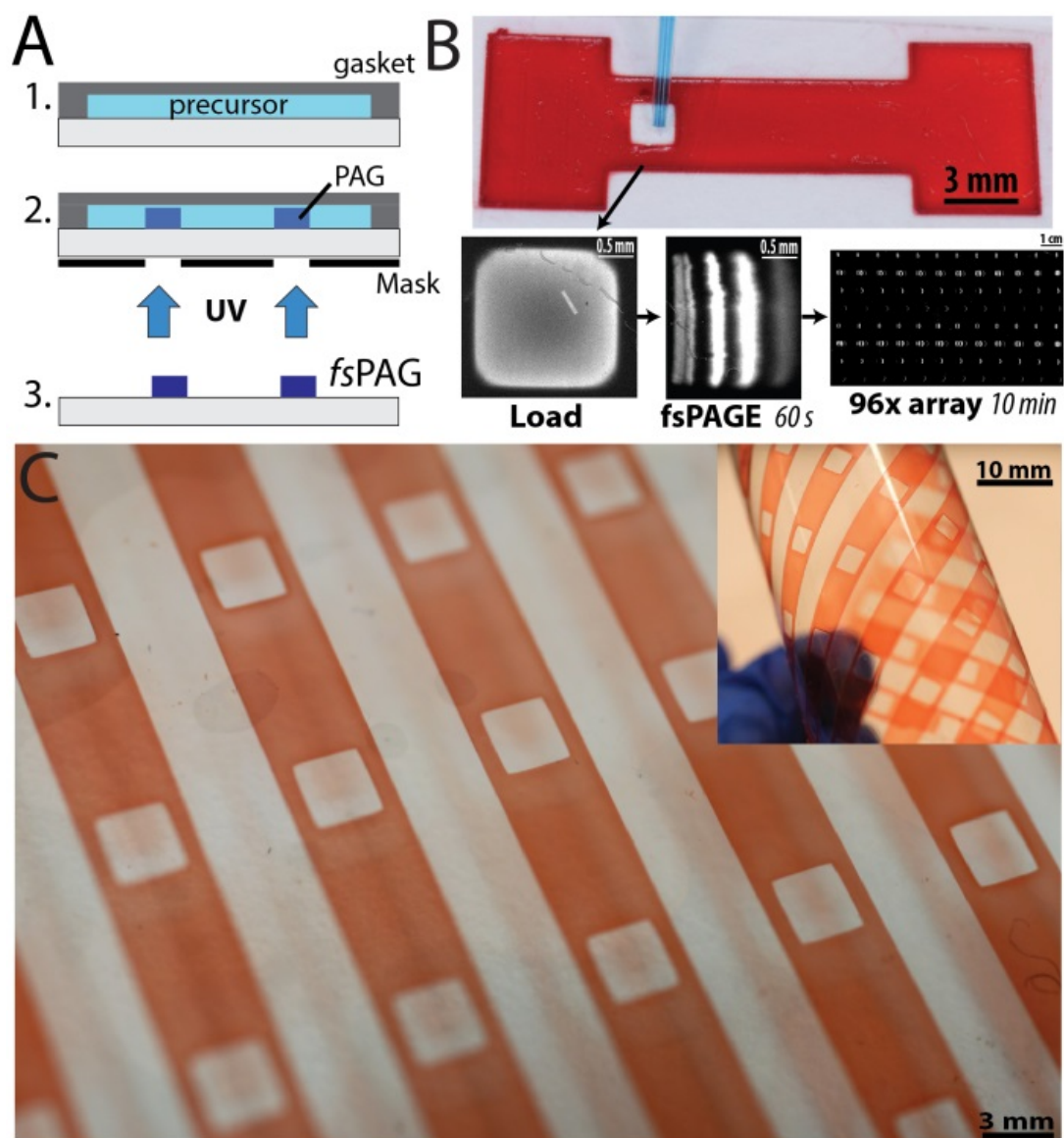


Figure 3.2 Free-standing polyacrylamide gels (*fsPAGs*) couple precise transport control at the micro-scale with the simplicity of a macro-scale slab-gel. (A) *fsPAG* fabrication requires 10 min to complete and consists of three steps: 1. Sandwich PA precursor solution between a gasket and a GelBond[®] substrate. 2. Expose the solution to UV light through a photo-mask. 3. Wash away excess precursor solution. (B) To perform *fsPAGE*, a protein sample is pipetted directly into a reservoir and then electrophoretically injected into and separated by the PAG structure with an applied voltage to the *fsPAGs* contact pads. We demonstrate the utility of *fsPAGE* for both a single separations and 96-plex separations. (C) The resulting 120 μm tall microstructures can be patterned over a large area enabling massively multiplexed protein electrophoresis on large *fsPAG* arrays operated with a single anode-cathode pair. [²⁸ Reproduced by permission of The Royal Society of Chemistry]

3.4. *fs*PAG MBE

Here, we use the *fs*PAG platform to fabricate free-standing microchannels and to perform PAG MBE (figure 3.3). In MBE the moving boundary of analytes are analyzed, as opposed to discrete zones, thus eliminating the need for an injection channel. Polyacrylamide gel MBE enables rapid protein separations in short single channels.²⁹ The MBE format allows separations to be realized on a small device footprint and with low power consumption, both important when implementing a multiplexed array.

A protein separation was performed in the free-standing polyacrylamide gel format and is displayed in Figure 3.4. A fluorescently labeled sample of 250 nm BSA, 250 nm OVA, and 250 nm TI in 1x tris/glycine is baseline resolved in 2 minutes and in the first 250 μm of migration. In Figure 3.4A the protein moving boundaries are clearly visible several millimeters along the separation channel. Figure 3.4B displays the first 250 μm of migration at the 130 seconds into the separation. The image is false colored such that the TI, OVA, and BSA fronts can be clearly distinguished. The corresponding fluorescence intensity plot is shown in Figure 3C and is overlaid with the derivative of the intensity over location (dI/dx). In the dI/dx plot the unique protein species can be clearly identified.

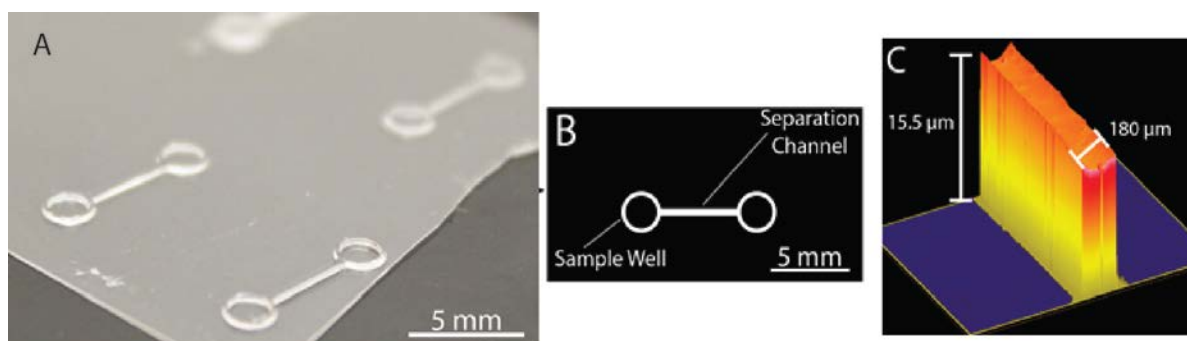


Figure 3.3 *fs*PAG MBE devices are covalently attached to a planar surface (A) and consist of two circular sample reservoirs connected by a microchannel (B). (C) Optical profilometry reveals the microchannel crosssection as 15.5 μm high and 180 μm wide.

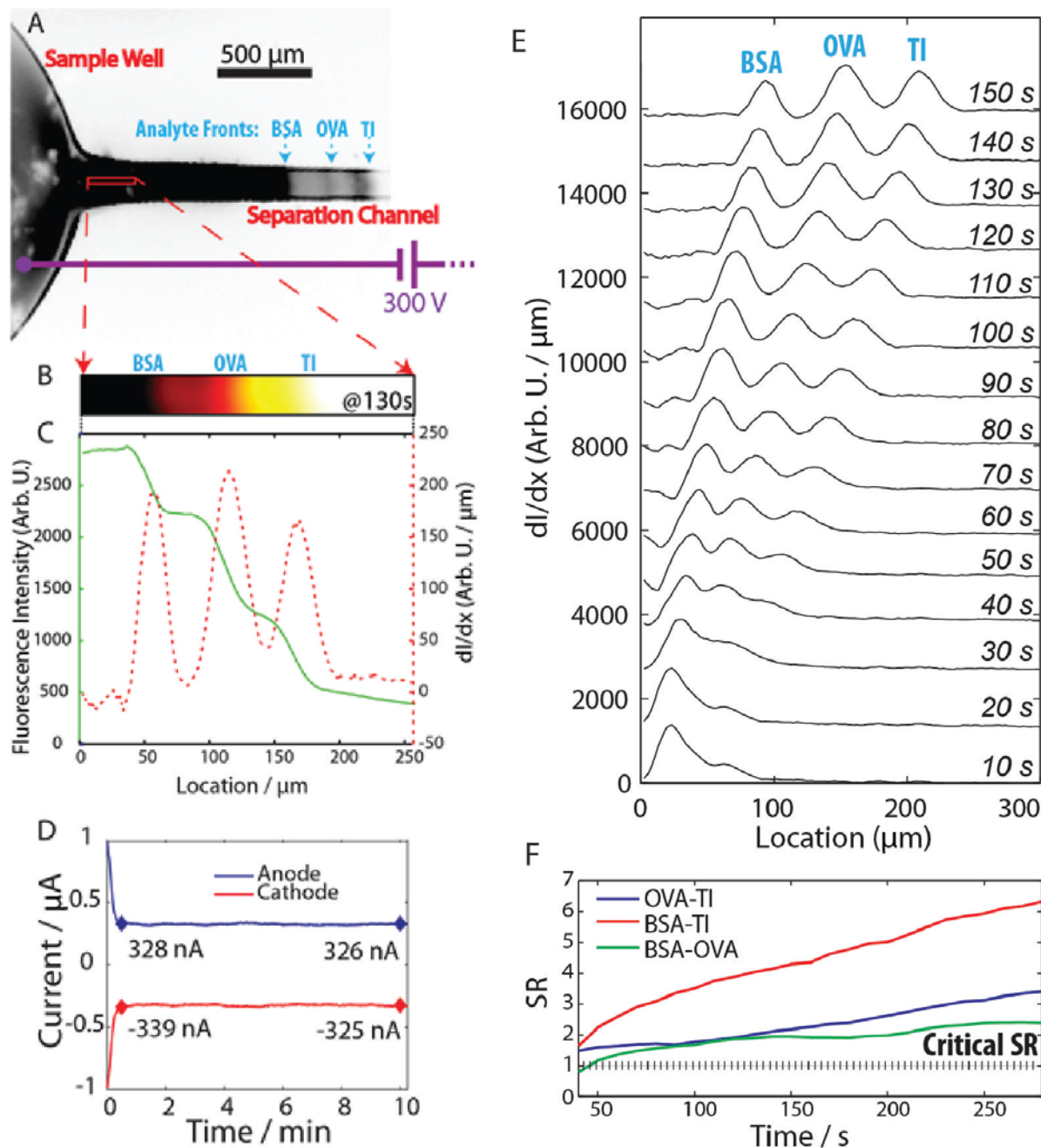


Figure 3.4 A free-standing 10% (w/v) acrylamide gel acts as both the microchannel and sieving matrix for a native MBE separation (A). 250 nM fluorescently labeled protein ladder consisting of Trypsin Inhibitor (TI), Ovalbumin (OVA) and Bovine Serum Albumin (BSA) is captured in an epi-fluorescence image. (B) A false color image of the first 250 μm of the separation channel at 130s clearly shows well separated proteins, a 2.7x reduction in separation length as compared to on-chip.²⁹ The corresponding (C) intensity profile and the derivative of the front dI/dx are overlaid. (D) The environmental chamber prevents appreciable current reduction. (E) dI/dx is staggered in time to visualize the protein migration with the (F) separation resolution plotted for each protein separation from the Figure 3 experiment.

3.5. *fs*PAG Zone Electrophoresis Sample Injection

While PAG MBE has several distinct advantages, including minimal injection dispersion, short separation distances and times – its utility is inherently limited as separations are only accomplished at analyte fronts. To accomplish a zone electrophoresis separations in *fs*PAGE we introduced an alternative design geometry that required additional optimization described below.

3.5.1. Reservoir Geometry

Using the *fs*PAG fabrication process, we investigated a sample injector geometry analogous to slab-gel PAGE formats: a free solution sample reservoir (i.e., an area with no polymerized gel) fabricated in-line with the PAG separation axis (Figure 3.2B). We explore use of the in-line injection approach in *fs*PAG microstructures for several reasons, including: to allow one-step injection to *fs*PAGE, to take advantage of sample stacking anticipated to occur at the reservoir-separation gel interface, and to simplify the microstructure geometry and footprint in anticipation of multiplexing requirements. The in-line injector design contrasts with canonical microfluidic sample injector geometries defined by two intersecting microchannels and requiring four sample ports.⁷ Consequently, common microfluidic electrophoresis designs require > 1 inlet port per sample and are able to inject only a fraction (~0.01%) of the total sample volume loaded into the channel terminus reservoir.^{30, 31} In contrast, to load the *fs*PAG microstructure, an aliquot of sample (1 μ L) is pipetted directly into the free-solution reservoir. To inject sample, voltage is applied at the terminal extents of the *fs*PAG structure, causing the entirety of the loaded sample volume to be injected into the subsequent *fs*PAGE assay. Reservoir array layouts with a standard pitch (well-to-well spacing) facilitate integration of the *fs*PAGE platform with common fluidic handling systems. The *fs*PAGE loading volume is customized by adjusting reservoir dimensions, which offers a tradeoff between sensitivity and sample consumption.

3.5.2. Injection Dispersion

Given the geometry and materials characteristics of the injection reservoir, we hypothesized that a surface charge would exist on the floor of the injection reservoir from the GelBond[®] film. This charged material could, in turn, support EOF during application of an electric field. Given the closed geometry of the injection reservoir, we further hypothesized that recirculation arising from EOF during injection – if not corrected during sample stacking – could induce dispersion in the subsequent *fs*PAGE assay as sample front migrates into the *fs*PAG.

To investigate the presence of EOF during electrokinetic sample injection from the reservoir into the *fs*PAG, we performed particle tracking in the reservoir (figure 3.5A). A solution of 15 μ m diameter fluorescent microbeads was pipetted into the 2 mm x 2 mm reservoir and an injection potential was applied ($V = 200$ V). Epi-fluorescence imaging of bead streak lines revealed two axially symmetric vortices in the reservoir. At the reservoir centerline, both vortices flow towards the cathode, and recirculate towards the anode at the edges of the reservoir. These observed flows are not unexpected given similar circulating EOF vortices reported in microsystems with non-uniform zeta potentials.^{32, 33} Though the exact chemistry of the GelBond[®] support surface is proprietary, the polyester support is coated with a layer of adherent resin with

ethylenically unsaturated groups.³⁴ The GelBond[®] surface is hydrophilic, with a water contact angle of less than 10°, thus, suggesting surface polarization.³⁵

We next sought to understand the impact of reservoir EOF on sample injection into a 15%T PAG at 100 V/cm from the 2 mm x 2 mm reservoir. We evaluated injection for both discontinuous and homogeneous electrophoresis in figure 3.4B and 3.4C, respectively. In discontinuous electrophoresis, transient isotachopheresis is used to pre-concentrate a sample within the reservoir region between a trailing ion (glycine) and a leading ion (Cl) prior to a PAGE separation.^{36, 37} The increased local electric field in an isotachophoretic stack results in enhanced EOF.³⁸ Images of the transient isotachopheresis injection of OVA* show a non-ideal injection, with a pronounced streak observed along the reservoir centerline in agreement with the qualitative bead tracking in figure 3.5A. Both results indicate that the GelBond[®] has a surface charge that induces EOF. In the homogeneous electrophoretic injection, the desired axially orthogonal OVA* band was observed, but significant streaking was seen along the edges of reservoir. The reduced band distortion in the homogeneous system is due to the combination of a slower EOF and a shorter reservoir migration time. As is especially relevant to the isotachophoretic injection mode, suppression of EOF should reduce and perhaps eliminate the highly distorted and dispersive sample zone injected into fsPAGE.

Given the evidence of surface charge in the reservoir, we conjectured that protein adsorption to the reservoir may result in either dispersive sample injection or sample mass loss. Adsorption was apparent for both the discontinuous and homogeneous injections with residual proteins seen in the reservoir 30 s after the electric field was applied. We evaluated the protein adsorption in figure 3.5C by monitoring fluorescence signal in the sample reservoir during electrophoresis. Upon application of the sample injection voltage, a 75% reduction of the initial fluorescence signal was observed in the first 20 s. Continued monitoring of the reservoir fluorescence showed an exponential decay in signal. The steady decrease in signal suggests gradual leaching of retained sample off of the surface over time. As sample adsorption to the reservoir material results in sample loss and non-ideal sample injection (i.e., sample streaking from the reservoir), we investigated the addition of commonly accepted capillary EOF suppressors to the reservoir in figure 3.4D and 3.4E for discontinuous and homogeneous electrophoresis, respectively.^{39, 40} For homogeneous electrophoresis with EOF suppression additives, 0.35% Brij or 0.5% Triton X-100, we observed >95% of sample fluorescence signal exiting the reservoir in the first 20 s of field application. The resulting injection of a discrete, well defined sample zone was observed when the suppressor was only in the run buffer, only in the sample buffer, or in both. In figure 3.5 we demonstrate a 20-30% improvement in separation performance and a 16-42% reduction of sample mass loss with an EOF suppressor additive in homogeneous electrophoresis. For discontinuous electrophoresis, protein adsorption was also minimized with the addition of an EOF suppressor, but the OVA* protein band was still distorted by EOF. We observed that, in combination with EOF suppressors, the band distortion can be minimized in discontinuous injection by reducing the electric field (figure 3.7), but a trade-off is made with the duration of the injection. In cases where EOF is not desired, coating the GelBond[®] substrate with a neutral polymer to mitigate or perhaps eliminate EOF in discontinuous electrophoresis is feasible. In cases where EOF-induces stirring in the reservoir is desired (e.g., sample preparation, timed reactions), EOF can alternately be enhanced through selection of charged coatings and/or spatial patterning.

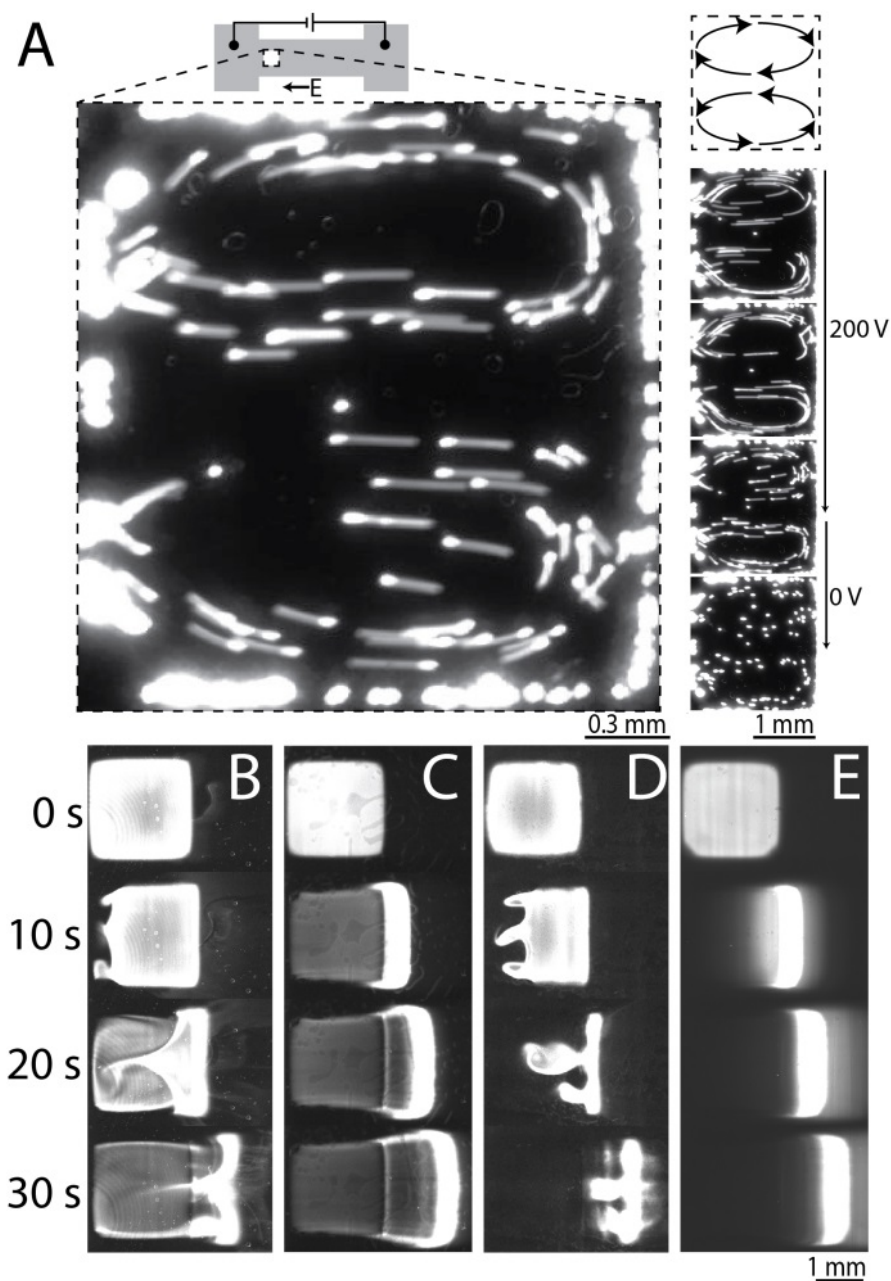


Figure 3.5 EOF suppressors minimize injection dispersion from the free-solution reservoir.

(A) The motion of fluorescent beads in a 2 mm x 2 mm solution-filled reservoir (left) shows the formation of two axially symmetric vortices driven by EOF. A time-lapse montage (right, 7 s between frames) of bead transport demonstrates the dependence of flow on the applied voltage. (B) Discontinuous electrophoresis injection of protein (500 nM OVA* in 150 mM Tris-HCl) is severely distorted by EOF. (C) While homogeneous electrophoretic injections resulted in the desired axially orthogonal protein bands, pronounced streaking from the reservoir resulted in injection dispersion and sample mass loss. By adding an EOF suppressor, 0.5% Triton X-100, to the sample solution, protein adsorption was effectively eliminated for both (D) discontinuous and

(E) homogeneous electrophoretic injections. $E = 100 \text{ V/cm}$, 15%T PAG. .^[28] Reproduced by permission of The Royal Society of Chemistry]

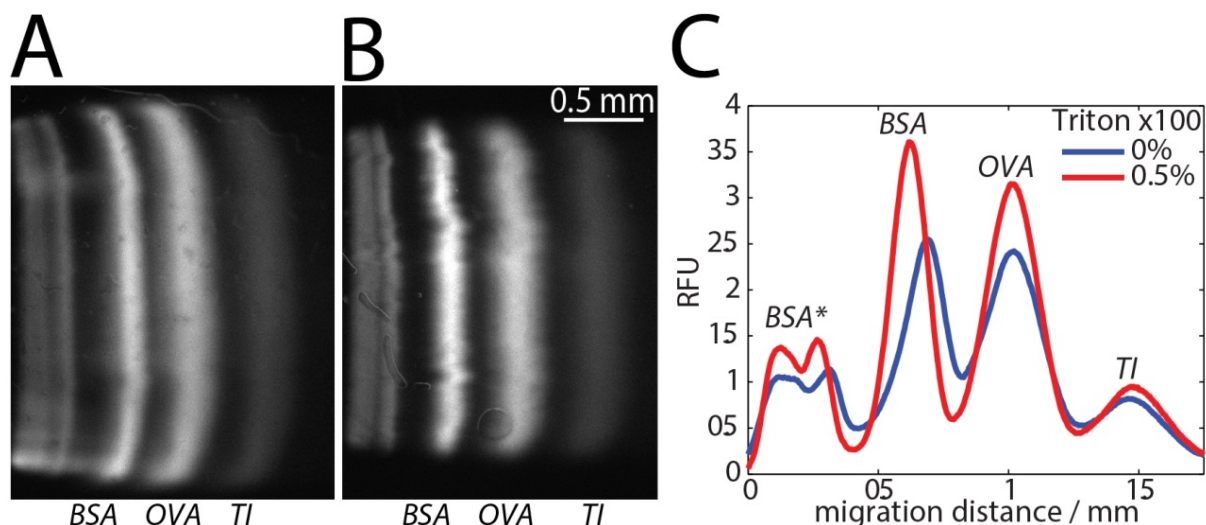


Figure 3.6 The addition of an EOF suppressor enhances separation performance and protein band linearity. The separation of a protein ladder in a 20%T *fs*PAG at 100 V/cm was performed (A) without an EOF suppressor and (B) with 0.5% Triton X-100. (C) The intensity plot profiles of the corresponding images, and intensity plots are aligned at the point where OVA* has migrated 1 mm, 40 seconds for the suppressor-less separation and 51 seconds for the 0.5% Triton x100 separation. The 0.5% Triton x100 increases the solution viscosity and reduces migration velocities by ~25%. In the separation with the EOF suppressor the protein peaks were both better resolved and larger than the separation without an EOF suppressor. As detailed in Fig. 2, the improvement is from a reduction of both EOF and protein adsorption in the reservoir. .^[28] Reproduced by permission of The Royal Society of Chemistry]

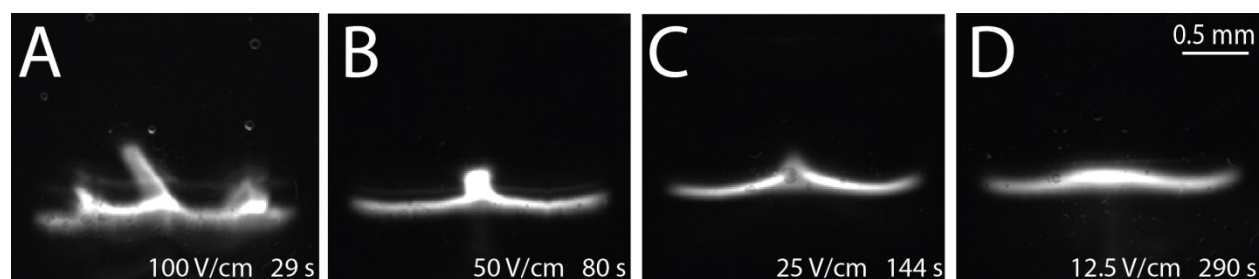


Fig. 3.7 High electric field negatively impacts protein injection in discontinuous buffers. Discontinuous electrophoresis injection of a 300 nM OVA* from a 1 mm (axial) by 2 mm (transverse) sample reservoir was performed at (A) 100 V/cm, (B) 50 V/cm, (C) 25 V/cm, and (D) 12.5 V/cm into a 20%T PAG. As expected with EOF induced dispersion, the band distortion was improved with a lower injection electric field. Consequently, the reduced injection potential also resulted in longer injection times: (A) 29 s, (B) 80 s, (C) 144 s, and (D) 290 s. .^[28] Reproduced by permission of The Royal Society of Chemistry]

3.5.3. Sample Stacking

In this work, the free-solution reservoirs act in an analogous manner to a stacking gel. The sample migrates at its free-solution mobility (μ_0) in the reservoir but slows down after it enters the PAG molecular sieve to an in-gel mobility (μ). The degree of sample stacking is equivalent to the ratio of these mobilities (μ_0/μ), effectively enriching the sample and reducing the peak width by the same ratio. In PAGE, in-gel mobility is determined using the Ferguson relationship,⁴ $\mu = \mu_0 10^{-KT}$, where K is the retardation coefficient of an analyte and T is the total acrylamide concentration in the precursor solution (gel density). Therefore, an increase in gel density will improve stacking and separation performance until the limiting case where proteins are excluded from the molecular sieving matrix. To test the relationship between *fs*PAG pore-size and stacking, we electrophoretically loaded a sample of 500 nM OVA* at 100 V/cm from a 2 mm x 2 mm reservoir into *fs*PAG structures of 10%T, 15%T and 20%T PAG. Figure 3.8A displays intensity plots of the first 1.5 mm of migration in each gel after 14 s. RFUs were normalized to the initial intensity within the reservoir to correct for any variation in loaded sample volume. As expected from the Ferguson relationship, stacking was most significant in the 20%T experiments with sample enrichment of 3.83 ± 0.69 and a half-peak width of 308.7 ± 19.4 μ m. The 15%T and 10%T PAGs showed enrichments of 2.63 ± 0.14 and 1.89 ± 0.24 with a half-peak width of 627.0 ± 40.4 μ m and 1021.9 ± 106.7 μ m, respectively.

To elucidate the importance of stacking in *fs*PAGE separations, we electrophoresed the protein ladder into each gel density at 100 V/cm and monitored the separation resolution, RS . Here $RS = X/4\sigma$, where X is the distance between the neighboring peak maxima and 4σ is the average peak width of neighboring peaks.⁴¹ In the 10%T PAG, no protein species were resolved (resolution is defined as $RS \geq 1$)⁴¹ over the total 9.5 mm length. In Fig. 3B, montages for the first 60 s of the 15%T and 20%T separations are displayed. In the 20%T case, both the BSA*-OVA* and OVA*-TI* separations were completed in just 60 s with RS values of 1.3 and 1.0 and in separation lengths of 1 mm and 1.7 mm, respectively. In contrast, the separations were still unresolved in the 15%T PAG at 60 s. The BSA*-OVA* species eventually resolved in 100 s at a separation length of 3.4 mm, and the OVA*-TI* was nearly resolved ($RS = 0.92$) at 380 s in 9.1 mm. For context, this geometry and performance translates into 25 unique separations within the length of a typical 10 cm slab-gel lane, assuming a 2 mm reservoir length and a separation length of < 2 mm (e.g., 20%T case). These results demonstrate both the importance of sample stacking in realizing high resolution protein separations and the design amenability to multiplexing. We apply a maximum electric field of 100 V/cm in this study for fast electrophoretic separations with minimal gel shrinkage over the course of a 20 minute separation. By increasing the electric field to 250 V/cm we can resolve TI*, OVA*, and BSA* in less than 15 seconds, but observe a dramatic increase in dispersion for separations longer than 15 s - a result of gel drying (observed physically and through electric current).

While the TI*, OVA*, and BSA* separation is completed in just 1 minute, if we continue for 5 minutes we can resolve the commonly observed BSA dimer and trimer (BSA² and BSA³, 138 and 207 kDa, respectively).⁴² The demonstrated 5 minute separation over a 21-207 kDa size range in 1 cm is comparable to the dynamic range seen in many 10 cm uniform slab-gel separations. As anticipated from first principles, the trade-off in using a short separation length versus a 10 cm slab-gel arises in the peak capacity of the system (the maximum number of

resolvable peaks).⁴¹ While the molecular weight ranges are comparable, a 1 cm *fs*PAGE could resolve approximately 1/10th of the species resolvable in a 10 cm uniform slab-gel. In such cases where a large peak capacity is needed in a short separation distance an time, design and development of a pore-size gradient *fs*PAGE gel optimized for the molecular mass range of interest is relevant – which is presented in Chapter 4.

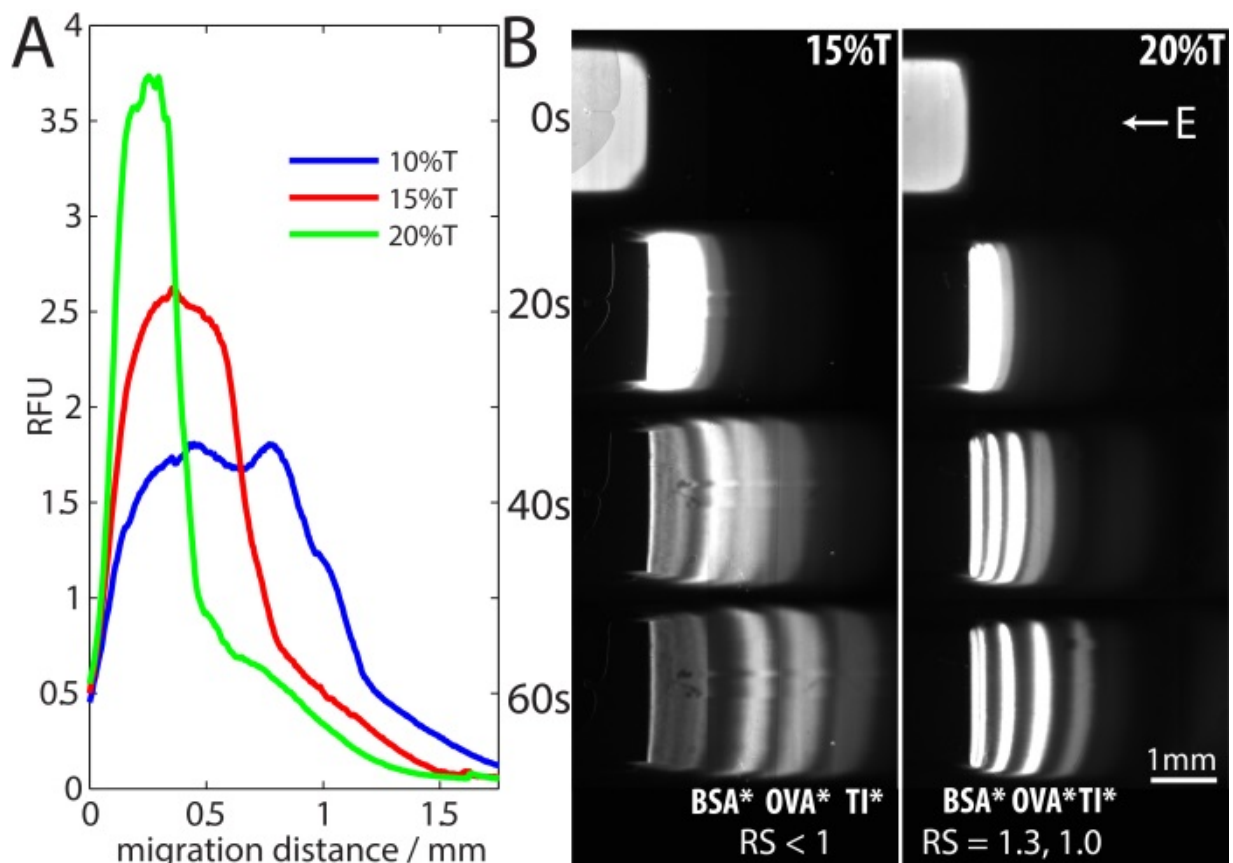


Figure 3.8 Sample stacking in a 20%T *fs*PAG facilitates the separation of a protein ladder over 2 mm in 60 s. (A) Sample stacking was evaluated in 10%T, 15%T, and 20%T *fs*PAGs by electrophoretically loading a sample of 500 nM OVA* at 100 V/cm. The intensity plot profile for each gel density is displayed at 14 s in the first 1.5 mm of the PAG. (B) Separation montages in 15%T and 20%T *fs*PAG devices were compared in the first 60 s of migration for a protein ladder. In the 20%T case (right), the BSA* -OVA* and OVA*-TI* species were fully resolved in 1 minute, with RS values of 1.3 and 1.0, respectively while for the 15%T (left), both separations require more than 1 minute of elapsed separation time. [²⁸ Reproduced by permission of The Royal Society of Chemistry]

3.6. Multiplexed *fs*PAGE Array

After establishing the sample injection conditions and composition of the separation gel, we next sought to scale-up the *fs*PAGE assay for multiplexing through concurrent assay operation. A key

design consideration was maintaining system simplicity by operating the full array with a single slab-gel power supply and two electrodes. As shown in figure 3.9A, electrical tracks connect the anode to cathode, with each electrical track comprised of more than one *fsPAGE*. A single *fsPAGE* module consists of a rectangular sample reservoir and contiguous free-standing separation gel; the module is termed a “ μ lane” for convenience. Several μ lanes in series comprise each electrical track. Electrical tracks connect to the same cathode and anode hardware, allowing concurrent separations in each μ lane, as well as in each electrical track. As is commonly done in electrophoresis platforms, the sample is diluted in a loading electrophoresis buffer prior to sample loading (discontinuous buffers are not compatible with the *fsPAGE* array format, Appendix B). Therefore, reservoir conductivity will largely be determined by the electrophoresis buffer as opposed to the protein sample – which mitigates potential electric field variations along an electrical track. Inclusion of internal migration standards assists with sample to sample protein migration comparisons. Sample reservoirs match the registration of a standard 96 well-plate, thus allowing integration with standard laboratory liquid handling technologies (e.g., handheld multichannel pipettors, automated robotic fluid delivery systems).

Here, we demonstrate a 96-plex PAGE separation of various samples in 9.6 min using the *fsPAGE* array. Samples in the 96 unique reservoirs were electrophoresed into 20% *fsPAGs* at 63 V/cm. Assays were performed on each individual ladder protein (TI*, OVA*, BSA*) and the ladder mixture. In figure 3.9C, we investigated the repeatability between μ lanes within a row by comparing the separations in column 8, rows 3-6. The ladder separation was compared to the sum of the μ lanes consisting of TI*, OVA*, and BSA* alone. The overlay shows a close migration match between the μ lanes with a migration percent variation of 1.2%, 4.2% and 3.8% for the TI*, OVA*, and BSA* species, respectively. In addition, the BSA*-OVA* and OVA-TI* separations were resolved with *RS* values of 1.3 and 1.2, respectively.

Protein mobility in μ lanes positioned in the middle of the *fsPAG* array was higher than mobilities observed in μ lanes along the boundary. For example, the BSA* on the top row migrated 25% slower than in row 5 and TI* in the bottom row migrated 15% slower than in row 4. The boundary dependent mobility likely arises from increased evaporation along the edges of the array resulting in a denser *PAG*. Our non-uniform sieve hypothesis is supported by the larger protein BSA* (67 kDa) experiencing a more significant mobility reduction than the smaller TI* (21 kDa), as the exponential Ferguson relationship would predict. Due to slower migration at the boundaries, variation across the 96-plex array was 17.1%, 11.5% and 15.1% for TI*, OVA*, and BSA*, respectively. If we just consider the middle of the array - μ lanes in rows 3-6 and columns 3-10 - absolute mobility variation is reduced to 8.4%, 4.6% and 6.0% for TI*, OVA*, and BSA*, respectively. The mobility variation results will inform future *fsPAG* array designs. Further, the results point to inclusion of an internal standard to account for μ lane to μ lane variation across the array. By considering the relative mobility to an internal standard such as OVA*, the percent variation across all ladder separations reduced to 6.2% for TI* and 3.2% for BSA*.

While we demonstrate the utility of a 96-plex *fsPAG* microtiter layout for a particular protein separation, this format is not necessarily suitable for all assays. For example, in cases where an assay requires separating proteins over a large molecular weight range or a higher level of multiplexing (e.g. 384-plex). Consequently, the *fsPAGE* fabrication process supports assay

optimization. For example, using the 1 hour design-prototype cycle, a modified design with longer separation channels or more reservoirs can be developed to address each specific need.

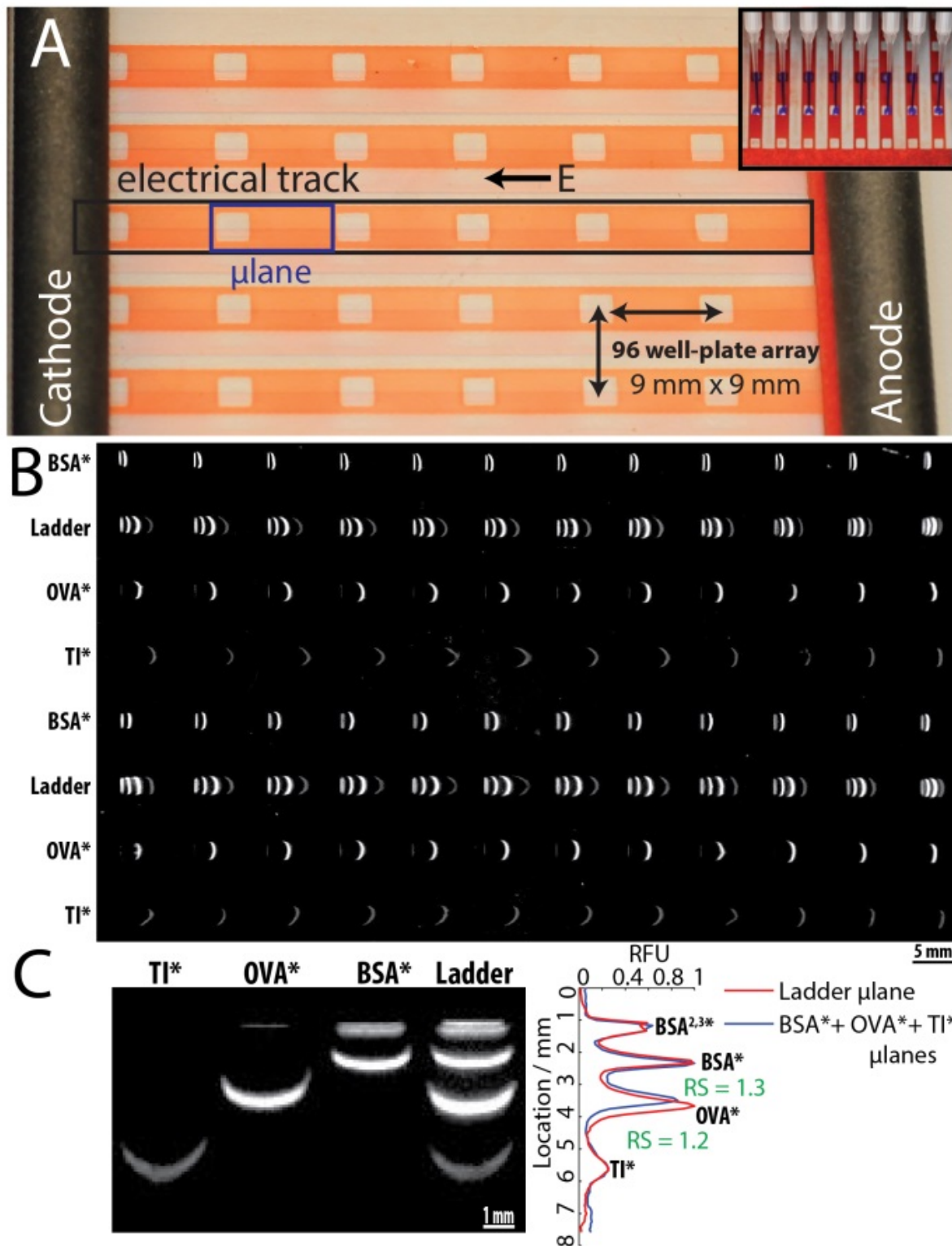


Figure 3.9 A collection of 96 discrete samples are separated in less than 10 min in a *fsPAG* array. (A) *fsPAG* arrays are fabricated to match a 96-well plate layout. Reservoirs are in an 8 x 12 array with 9 mm spacing to enable rapid sample delivery using a 12-channel pipette. The *fsPAG* planes are addressed with electrical tracks that span the two electrodes and operated with a standard slab-gel power supply. (B) A 96-plex 20%T *fsPAGE* separation is concluded in just 9.6 min. To investigate performance, the array was loaded with 4 different AF488 labelled samples: TI* (rows 4 and 8), OVA* (rows 3 and 7), and BSA* (rows 1 and 5), and a protein ladder (rows 2 and 6). (C) Separation uniformity is compared along one column in the array. An intensity profile of a ladder plane is overlaid with the summation of intensity profiles for the TI, OVA, and BSA* planes shows less than 5% variation in protein migration. [²⁸ Reproduced by permission of The Royal Society of Chemistry]

3.7. Downstream Protein Analysis

A principle rationale behind the *fsPAG* platform is microfluidic multiplexing in a format amenable to common slab-gel post processing reagents and tools. We demonstrate applicability of the most ubiquitous slab-gel downstream analysis reagent, a protein stain. In Fig. 3.10A, a 20 min separation was performed at 39 V/cm in a 20%T 96-plex *fsPAG* array and monitored with a pre-labeled ladder loaded into rows 1 and 8. Afterwards, the *fsPAGE* device was fixed in a solution of 50% ethanol and 3% acetic acid for 30 min, stained with SYPRO Ruby staining solution for 6 hours, and then de-stained in 10% methanol for 30 min.⁴³ Unlabeled proteins were detected (figure 3.10B) in rows 2 through 7 containing a dilution series of BSA (500 ng, 250 ng, 100 ng, 50 ng, 25 ng, and 10 ng of loaded mass, respectively). Protein quantification in figure 3.10C, was normalized to an internal standard to account for any plane to plane variation - 100 ng of unlabeled OVA. Figure 3.10D displays the resulting device image after the complete 7.5 hour staining procedure. The BSA and OVA peaks were clearly distinguished over the entire mass range with a BSA SNR of 359 ± 26 for the 500 ng samples to 75 ± 10 for the 10 ng sample ($n=12$). The calibration curve shows a linear relationship is across the 10 ng to 100 ng range, but does not hold for the 250 ng and 500 ng masses. The non-linearity at higher mass loads suggests shorter staining times are required for linear quantification over the entire mass range, 10 ng to 500 ng. In future work, we will exploit the open nature of *fsPAG* for more advanced downstream analyses including additional assay stages.

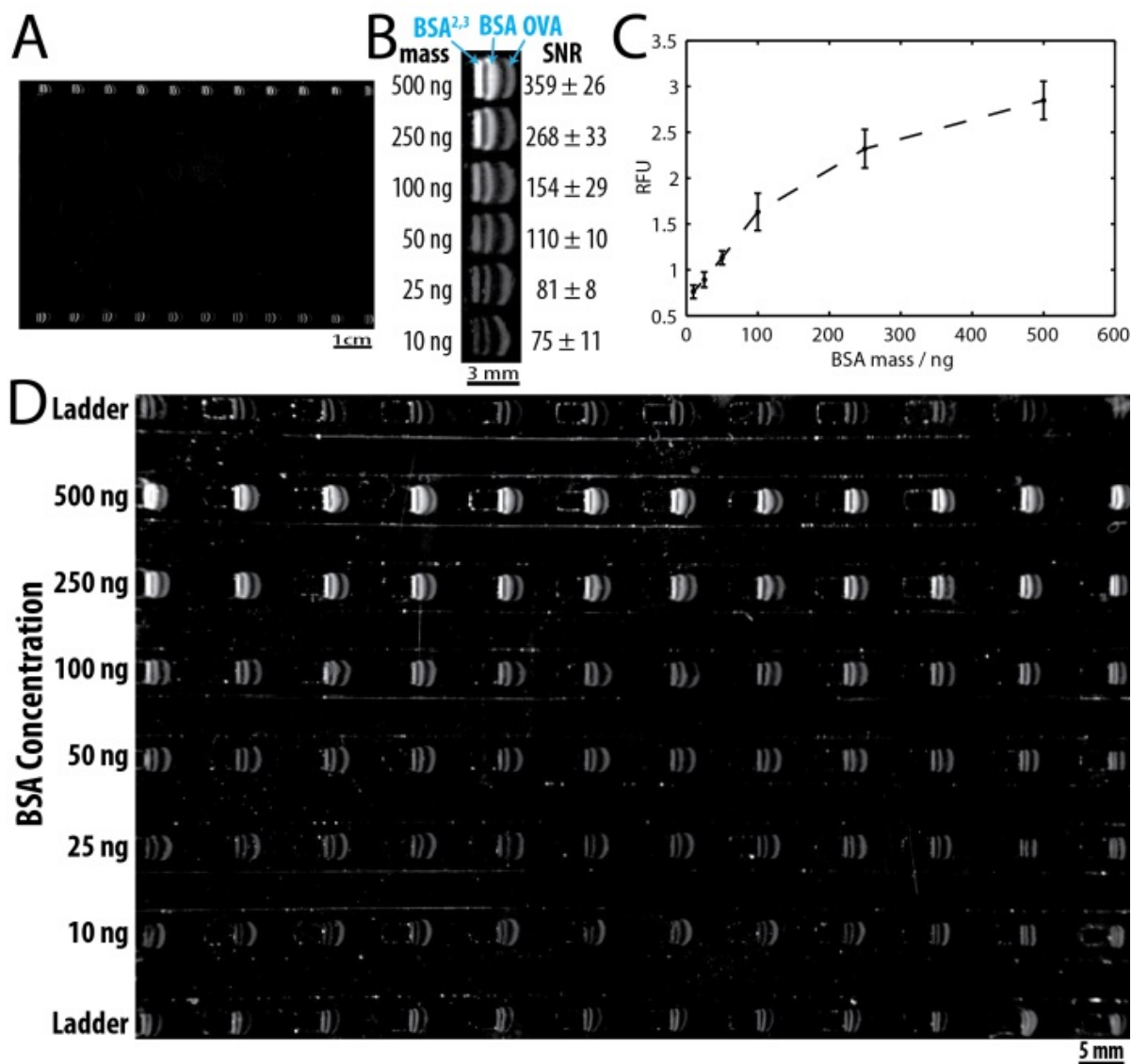


Figure 3.10 *fsPAG* array separates and detects 72 unlabeled native protein samples in a 7.5 hour assay. (A) The 20 min, 39 V/cm separation was monitored by pre-labeled ladder proteins loaded into rows 1 and 8, and unlabeled BSA and OVA in rows 2 through 7. (B) The unlabeled protein samples were fluorescently detected after a 6 hour stain with SYPRO Ruby. (C) The RFUs for the various BSA concentrations, ranging from 500 ng to 10 ng, were normalized to the OVA internal standard. (D) The *fsPAG* array enables massively multiplexed quantification of unlabeled protein samples. [²⁸ Reproduced by permission of The Royal Society of Chemistry]

Conclusions

We introduce a methodology for the rapid prototyping of PAG electrophoretic devices. The facile prototyping cycle extends the impact of a powerful movement towards fast iteration as a means to achieve optimization in microanalytical technologies. Rapid prototyping has been, most often, based on PDMS prototyping workflows with more exotic (yet accessible) materials recently gaining interest.^{44, 45} Polyacrylamide is a particularly appropriate prototyping material

for novel or optimized protein separations systems, owing to the important role this particular sieving matrix plays in standard slab-gel protein electrophoresis tools. A linear pore-size gradient for protein electrophoresis is described as one example.

Extending the *fsPAG* fabrication from one to multiple concurrent assays, we demonstrate 96 parallel separations in under 10 min anticipating that the *fsPAG* array could be scaled up to realize hundreds of separations in parallel. In contrast to other published or commercial electrophoresis arrays, which require researchers to purchase expensive fluidic delivery infrastructure (e.g. nanoplotters) or custom electrical interfacing and expensive cartridges for each use (Invitrogen's E-PAGE), our *fsPAG* array is operated with ubiquitous lab equipment (i.e., pipettes, a slab-gel power supply, slab-gel imaging reader). By lowering the infrastructure and prototyping barriers for developing and implementing PAGE systems, the *fsPAGE* platform enables standard laboratories to develop their own custom analytical tools to provide proteomic solutions to a research teams specific questions. In the future, photo-patterning of in-gel chemistries could similarly accelerate assay development – discussed in Appendix C.

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Gradient PAGE

4.1. Introduction

Pore-gradient polyacrylamide gel electrophoresis (PAGE) was first demonstrated by Gary Slater in 1965¹. In the technique, polyelectrolytes are electrophoresed into an increasing gradient in PAG density. As the density increases, the mobility of the polyelectrolytes decreases due to the progressively smaller pore-sizes. In the case of proteins - the gel density and mobility relationship can be described with the Ferguson relationship² (discussed in Section 1.3.1). This platform has several distinct advantages over uniform PAGE.

Gradient gels allow for the separation of highly heterogeneous mixtures of polyelectrolytes that span a large range of sizes but have a relatively small range of free-solution electrophoretic mobilities³. This is of particular relevance when performing protein sizing with sodium dodecyl sulfate (SDS) PAGE⁴. The negatively charged SDS detergent associates to proteins with a conserved SDS molecule to protein molecular weight basis, thereby leveling the effective free-solution mobility for all proteins. By driving electrophoretic transport through the nano-porous PAG sieving matrix, the SDS denatured proteins can be resolved by their size⁵. In a uniform gel, the density can be tuned to achieve the optimal separation for any given pair of analytes. In a highly heterogeneous mixture a gradient gel offers a compromise of separation performance for a given pair but enables many species to be resolved in unison.

During a polyelectrolytes migration into an increasingly dense gradient gel, its leading edge is at any moment in a denser region of the gel than its trailing edge. Therefore, there exists a differential mobility at the across the analyte due to the difference in density. This mobility difference sharpens the protein band continually during migration⁶ (discussed in Section 1.2.3). The progressive stacking helps mitigate dispersion that results from diffusion and correct for injection dispersion. It is of particular importance when separations are restricted to short separation lengths (e.g., less than 1 cm), as dispersion will rapidly reduce the number of resolvable species.

In Chapter 5, I introduce the free-standing PAGE (*fsPAGE*) platform for performing up-to 96-plex electrophoretic separations in uniform polyacrylamide gels a less than 1 cm separation length. This technique is highly useful, for example, when performing target separation assays, for example, for migration shift assays to measure binding or shifts molecular structure⁷. However, it is limited on performing separations on samples that contain a large molecular weight range. In this chapter, we introduce gradients into the *fsPAGE* platform to expand its utility for complex heterogeneous samples.

4.2. Materials and Methods

Reagents

Solutions of 30% (w/v) (29:1) acrylamide/bis-acrylamide, and glycerol were purchased from Sigma Aldrich (St. Louis, MO). Molecular biology grade (DNase-, RNase-, and Protease-free)

water was purchased from Mediatech, Inc. (Manassas, VA). GelBond® PAG Films and Gel Slick® glass plate coating were purchased from Lonza (Base, Switzerland). AlexaFluor 488 (AF488) conjugated Parvalbumin (ParV*, 12 kDa), Trypsin Inhibitor (TI*, 21 kDa), Ovalbumin (OVA*, 45 kDa), and Bovine Serum Albumin (BSA*, 67 kDa) were purchased from Life Technologies Corporation. Tris-glycine (10x, pH 8.3) native electrophoresis buffer was purchased from Bio-Rad Laboratories (Hercules, CA) and 1 M Tris-HCl (pH 8.6) was purchased from bioWORLD (Dublin, OH). Fluorescently labeled proteins are denoted with a “*”.

fsPAGE Electrical and Fluidic Interfacing, Operation and Imaging

The interfacing, operation of *fsPAGE* as well as protein imaging and analysis is described in Section 3.2. Unless otherwise noted, all *fsPAGE* sample buffers contained 1x Tris-glycine (25 mM Tris, 192 mM glycine, pH 8.3), 10% glycerol and 0.5% Triton X-100 and all gel buffers contained 1x Tris-glycine and 20% glycerol.

Laser Printing Grayscale- and Photo-masks

Grayscale and photomasks were designed using Adobe Illustrator (2400 dpi, CMYK) and printed directly with a Brother MFC-9320 laser printer on a transparencies (PP2500, 3M, St. Paul, MN). To overcome resolution limits presented by printed grayscale dot patterns on the mask itself (i.e., 30-80µm dots, figure 1B) we incorporate a light diffuser (rosco PN# 3010) in the UV light path, thus allowing for the fabrication of linear density gel-gradients.

Laminate PAGE fabrication

Laminate PAGE fabrication is described in Section 4.7.1.

4.3. Diffusion Gradient Fabrication for *fsPAGE*

While uniform pore-size PAGs allow electrophoretic separation of analytes with sufficiently large mobility differences, resolving analytes over a broad range of mobility differences can be problematic. During the course of prolonged PAGE, molecular diffusion and other sources of dispersion⁸ act to increase the peak width, effectively minimizing resolution gains achieved by an increased peak-to-peak distance. Consequently, gradient PAGs are used for high resolution separations.⁹ In a decreasing pore-size gradient PAG, migrating analytes constantly experience an increasing PAG density which acts to continually stack the zone as species migrate down the separation axis. This continual stacking is important for reducing peak width and, thus, enhancing *RS*.

Taking advantage of the quick design-fabricate-test cycle time of the approach introduced for *fsPAG*, we sought to extend the *fsPAG* fabrication protocol to allow creation of *fsPAG* structures with varying pore-size distributions. In figure 4.1A we demonstrate the fabrication of a decreasing 10%T to 20%T gradient pore-size *fsPAG* using a gasket with two inlets. Precursor solution for a low %T PAG is placed in one inlet and precursor for a high %T PAG is placed in the other. A 15 min diffusion step allows formation of gradient in %T along the separation axis. After diffusion establishes the gradient, UV cross-links the precursor solution (8 mW/cm² for 4 min) resulting in an *fsPAG* with non-uniform PA pore-size. For visualization, disodium 6-hydroxy-5-[(2-methoxy-4-sulphonato-m-tolyl)azo]naphthalene-2-sulphonate (Allura Red AC dye) was added to the high %T precursor to visualize an approximation of the diffusive gradient

formation process. Allura Red AC has a molecular weight of 496 Da, seven-times larger than acrylamide monomer, thus allowing a conservative estimate of the gradient formation process. The resulting *fsPAG* structure and the Allura Red AC concentration distribution is shown in figure 4.1A. The gel was then soaked in buffer for 10 hours to remove residual Allura Red AC.

After successful fabrication of a gradient *fsPAG*, we sought to compare separation performance of the gradient *fsPAGE* conditions and those for a uniform *fsPAGE*. We analyzed our protein ladder, (TI*, OVA*, BSA*) using a uniform 20%T *fsPAG* and a 10%T-to-20%T decreasing pore-size *fsPAG* ($E=100$ V/cm), as shown in figure 4.2B. As expected in the uniform gel, peak widths increased over the separation time. In contrast, we observed a slight reduction in peak widths over time, owing to the stacking nature of the decreasing pore-size *fsPAG*. Between 100 s and 500 s, protein velocities reduced from 17.2 $\mu\text{m/s}$ to 4.3 $\mu\text{m/s}$, 13.3 $\mu\text{m/s}$ to 3.4 $\mu\text{m/s}$, and 7.7 $\mu\text{m/s}$ to 2.0 $\mu\text{m/s}$ for TI*, OVA*, and BSA*, respectively. As a result, the gradient *fsPAGE* RS was notably enhanced compared to the uniform *fsPAGE* results. At 500 s of separation time, the uniform *fsPAGE* assay resolved the smaller molecular mass OVA*-TI* peak pair ($RS = 1.0$), whereas in the gradient gel, the same pair showed more than complete baseline resolution ($RS = 4.4$), see figure 4.2C. Baseline resolution for the smaller molecular mass OVA*-TI* pair was reached ($RS = 1.5$) after just 80 s of separation time in the gradient *fsPAGE* assay.

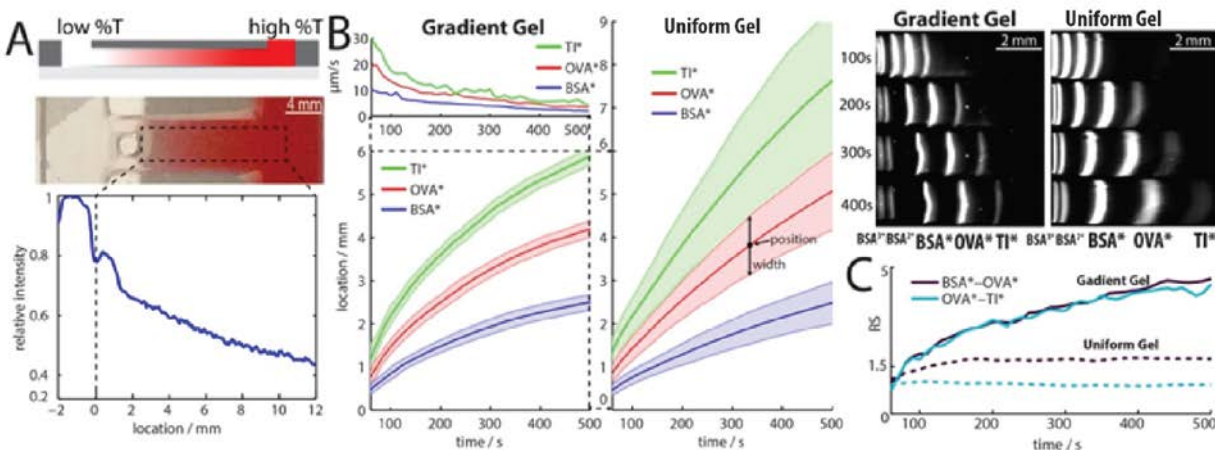


Figure 4.1 Gradient *fsPAG* suppresses protein dispersion over an 8 min separation. (A) Gradient *fsPAG*s were fabricated by establishing a PA density gradient beneath a gasket prior to photo-polymerization of the gel. Here, we use red food coloring to demonstrate the established gradient. (B) Gradient (10%T to 20%T) *fsPAGE* is compared side-by-side with the uniform *fsPAGE*. Peak position and width of the protein samples are plotted over time for each condition, demonstrating a stark contrast in dispersion over the course of a 500 s separation at 100 V/cm. (C) RS is plotted over the course of the experiment for the BSA*-OVA* and OVA*-TI* separations for both gel conditions. In the gradient gel, RS continues to improve with time due to the growing peak to peak separation between species and minimal bandwidth growth. All protein species are baseline resolved ($RS = 1.5$) in 80 s. In contrast, the uniform gel saw little improvement of RS beyond 100 s due to excessive bandwidth growth. The BSA*-OVA* species are baseline resolved in 110 s, while the OVA*-TI* are not baseline resolved. [¹⁰ Reproduced by permission of The Royal Society of Chemistry]

4.4. Grayscale Mask Photo-patterning of PAG Density

In Section 4.3 we demonstrated how diffusively fabricated gradient *fs*PAGE can enhance separation performance over large molecular range of proteins and reduce band broadening a unit device. Upscaling from this gradient PAGE unit device to a large *fs*PAGE array, introduced in Section 3.7, would be extremely difficult using diffusive fabrication. It would require complex gaskets, fluid handling, and a new *fs*PAGE array-design. Alternatively, we sought to adopt a different fabrication technique that facilitated rapid realization of spatially complex gel density patterns simply using a printed grayscale mask.

While grayscale based fabrication was first discussed in patent literature in the 1980s¹¹, its usage took off in the mid-1990s to realize three-dimensional structures with a single UV exposure step¹². The approach was first demonstrated for PAGs by Joyce Wong et. al. in 2003¹³ with radially varied PAG gradients (~1.5 cm in length) to modulate the stiffness experienced by seeded cells and study how they respond to various controlled stiffness asymmetries. We adopted this approach to create spatially complex (Figure 4.2) microfluidic PAGE devices for enhanced analytical performance. As shown in figure 4.2B, a dense polyacrylamide precursor containing a photo-initiator is confined under a gasket or in a microchannel. A laser printed grayscale mask which spatially attenuates UV light is aligned under the precursor solution. The uniform UV light is shone through the grayscale mask to create the desired UV intensity pattern. As PAG photo-polymerization kinetics is associated with UV intensity¹⁴, different sections of the precursor solution will polymerize with different kinetics. If the UV exposure is shut off prior to the completed polymerization a gel density pattern can be realized.

To maintain fabrication simplicity and the rapid-prototyping compatibility of *fs*PAGE, it was desirable to continue to use laser printed grayscale masks – as was done originally¹³. The challenge in our system was that we use a high-powered and collimated UV light to define the in-plane mesostructured *fs*PAG features. As opposed to the previous work, when non-collimated and relative low UV intensities were used. Due to the relatively low resolution of laser printers the grayscale pixels ranged between 50 to 100 μm in size. When grayscale gels were used directly to fabricate PAG structures, the proteins migrating through the PAG had a distinct pixelation pattern (figure 4.3A) due to micro-patterning from the laser printed masks dots directly patterned into PAG. To achieve the desired regional attenuation of UV intensity we added an off-the-shelf light diffuser (rosco PN# 3010), which is a tightly intertwined mesh of translucent fibers. Photographers typically use light diffusers to ‘soften’ light. Diffusers work by diffracting light to de-collimate it; for our application, diffuses smooth out the grayscale pixels, as seen in figure 4.3B. With the diffuser placed in the light-path (immediately after the grayscale mask), we can create a complete range of gray values while eliminating the pixelation artifact (figure 4.3C). This enables us to quickly create new grayscale designs in Adobe Illustrator, print them out on a laser printer and then directly use them. When the UV intensity is measured through different laser printed grayscale values (with the diffuser) we can realize a large range of UV intensities – with a linear grayscale value and UV transmission relationship from 10% to 90% ($R^2=0.98$, figure 4.3D).

To confirm the formation of gradient PAGs we added a fluorescent monomer into the gel precursor solution. Methacryloxyethyl thiocarbamoyl rhodamine B (Polysciences Inc., PN# 23591) is covalently incorporated into the PAG with its methacrylate group and can be directly imaged from the fluorescence of rhodamine (Ex. 548 nm, Em. 570 nm). Figure 4.4A shows a *fs*PAG created using a grayscale mask with variable grayscale gradients (90%-0% gray, 50%-0%, and 0% gray). The spatial variation in the resulting gel was directly observed with fluorescence. The spatial variation showed an agreement with the designed grayscale mask and demonstrated our ability to directly pattern PAG density and realize linear variations of density (figure 4.4B) for creating gradient PAGs.

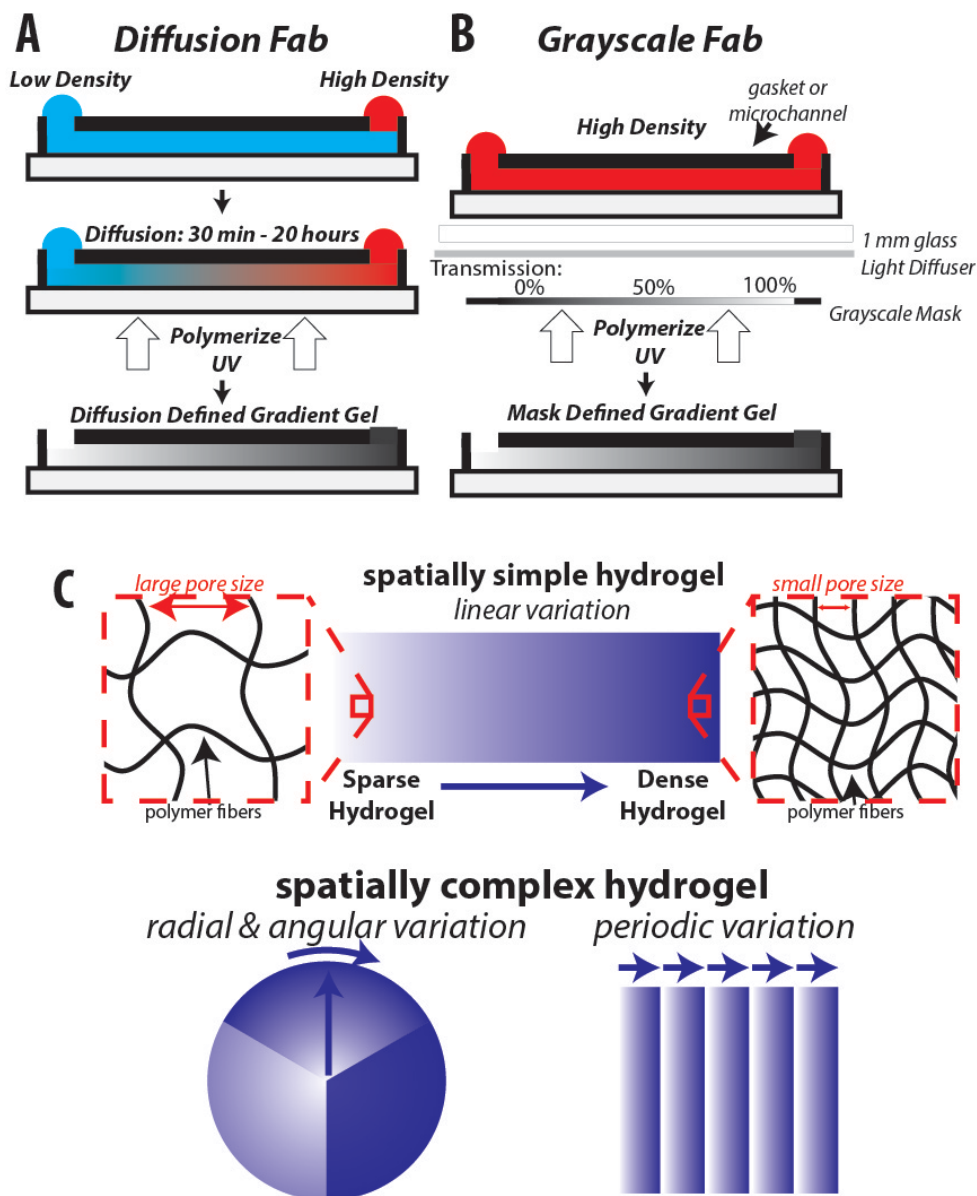


Figure 4.2 Gradient fabrication protocols. (A) Linear PAG gradients are realized with diffusion, where a high density and low density precursor solutions are allowed to diffuse and form a gradient of monomer density. Polymerization is then initiated using a photoinitiator and a

UV light. (B) Alternatively, grayscale masks can be used to create spatial patterns of monomer density. In this case, a high density precursor solution is used throughout and UV exposure is performed through a grayscale mask that spatially attenuates light in different regions of the precursor solution. This effectively modulates the polymerization kinetics, and if polymerization stopped (by turning the UV light off) prior to complete polymerization, a pattern of gel density can be achieved. (C) The key advantage of the grayscale fabrication approach is the design flexibility. Diffusion based gradient fabrication is limited to spatially simple linear gradient (Section 4.3). Grayscale patterned gels can be used to create spatially complex patterns for novel analytical applications – examples for periodic and radial / angular gradients are highlighted in Sections 4.4 and 4.5, respectively. © 2014 IEEE.

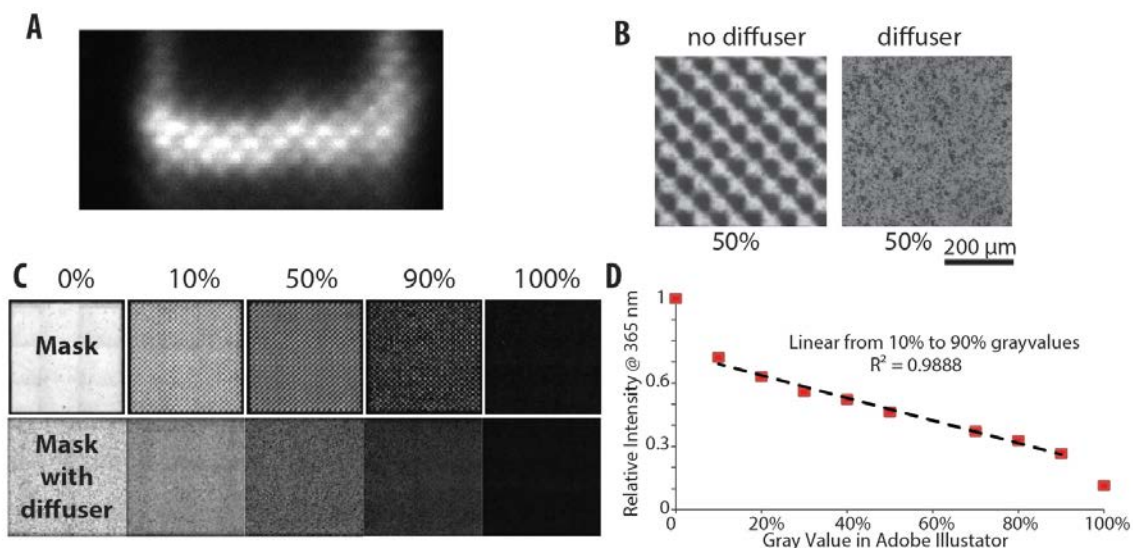


Figure 4.3 Laser printed grayscale masks enabled high-throughput prototyping of device architecture. (A) Direct PAG patterning through a grayscale mask without a light diffuser resulted in micro-patterning of PAG. As a result, an electrophoretically injected protein showed a pixilation pattern of the protein band due to the micro-heterogeneous gel density. (B) Incorporating an off-the-shelf 'light diffuser' (rosco PN# 3010) in the light path (shown in figure 4.1B) effectively diffracts the light such that the micro-patterns are avoided in the PAG structure. (C) The resulting UV intensity from different laser-printed grayscale patterns was linear from 10% to 90% gray. © 2014 IEEE.

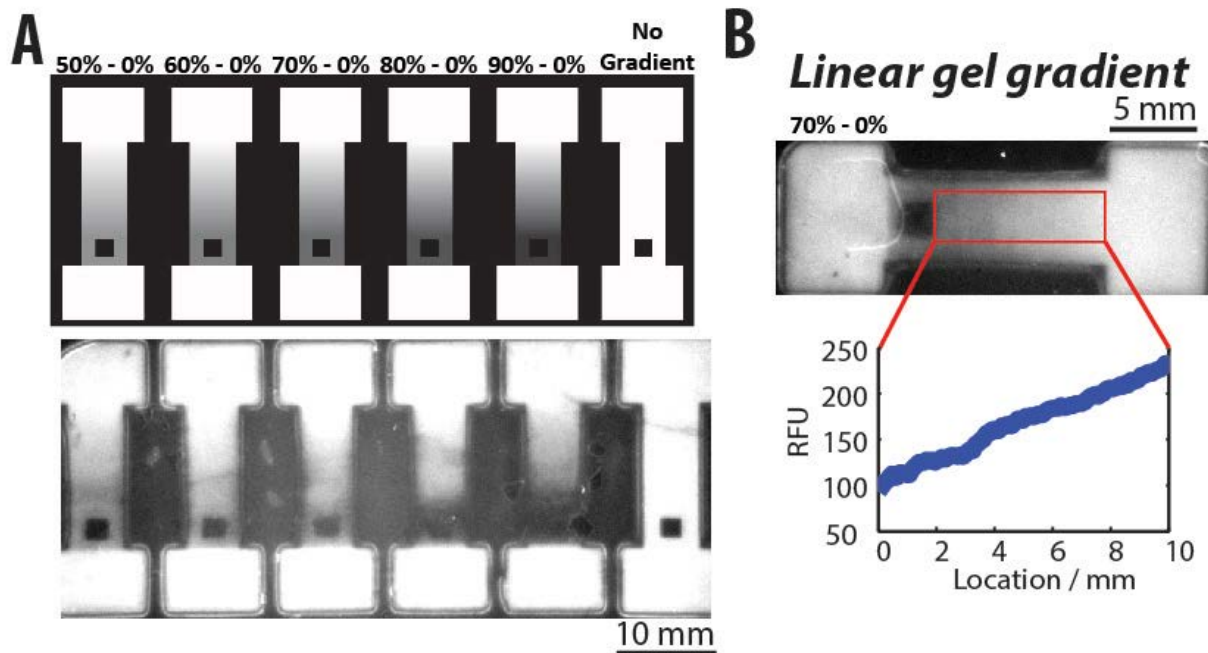


Figure 4.4 Incorporation of a fluorescent monomer enables the direct imaging of the spatial variation of PAG. (A) Gradient gel formation was tested over several gradient slopes by adding a fluorescent rhodamine methacrylate monomer to access relative monomer incorporation at different positions along the grayscale mask. This demonstrates the tune-ability of the grayscale photo-patterning platform – to realize a range of variable devices with one UV exposure. (B) Linear gel density gradients could be realized to create gradient gels. © 2014 IEEE.

4.5. Characterizing Grayscale Mask Fabricated PAG

The data shared in Section 4.5 was collected by the undergraduate researcher Darren Lim under Todd Duncombe's supervision.

Grayscale fabrication offers a powerful method for directly spatially patterning complex PAG density features in a single UV exposure step. One challenge with this technique is predicting the resultant gels sieving properties given the initial fabrication conditions – which includes the precursor concentration (%T), UV exposure time and the masks gray value (i.e. UV intensity). We sought to shed light on this relationship by creating a large data set of SDS PAGE protein velocity measurements over various precursor concentrations (20%T, 30%T and 40%T) and with proteins of different molecular weights (12 kDa, 21 kDa, and 67 kDa). For simplicity, pre-grayscale mask UV intensity (32.6 mW/cm^2 measured at 365 nm) and UV exposure times were held constant for each precursor concentration. The exposure time was chosen such that a robust fsPAG could be realized at a 90% gray value. The UV times were 90 second, 85 seconds, and 80 seconds for 20%T, 30%T and 40%T PAGs, respectively. Figure 4.5A shows the SDS PAGE protein velocity was extracted for the proteins Parv*, TI* and BSA* over 0% to 90% gray. To compare sieving between photo-patterned gels to that of chemically polymerized PAGs, chemically polymerized 20%T, 30%T and 40%T were fabricated and velocities were measured (dotted lines). The 0% gray photo-patterned gels had comparable velocities to chemically

polymerized gels. Over the 0% to 90% grayscale range we achieved up to a 19-times increase in velocity for the same protein at different gray values. For example, a velocity range of 0.25 mm/min to 4.92 mm/min is achieved for Parv for a 20%T precursor. Alternatively, a high velocity in one gray value and no transport in another could be achieved. TI travelled at a 3.6 mm/min velocity in 90% gray and had no velocity at 10% gray for the 30% precursor. The velocity data over a range grayscale conditions and protein sizes displayed in figure 4.5A was used to help inform assay design for grayscale PAG applications.

4.5.1. Relating SDS PAGE Protein Velocity and PAG Elastic Modulus

In the previous section we measured protein velocities in SDS PAGE over a range of molecular weights and grayscale fabrication conditions to develop prospective design rules describing how a given set PAG fabrication conditions will impact protein sieving performance. In this section, we develop a relationship between a measurable physical parameter of a fabricated PAG and the PAG protein sieving properties. By establishing this relationship, we enable a physical measurement on a resultant grayscale fabricated PAG to be used to predict the effective protein velocities. Specifically, we develop the relationship between SDS PAGE protein velocity and the elastic modulus of a PAG. In figure 4.5B we measured the elastic modulus for all grayscale conditions that were previously used to assess protein velocity (in figure 4.5A). Elastic modulus was extracted from measurements made using a MCR Rheometer (Anton Paar, Ashland, VA, USA) at 1 Hz. Using a mask and laser cut mold, 8 mm diameter 300 μ m thick were fabricated to match the diameter of the rotor. The storage and loss modulus was measured from the resulting PAG pieces, and the elastic modulus was calculated using the equation, $E = 2\sqrt{G'^2 + G''^2} * (1 + \nu)$. Where E is elastic modulus, G' is the shear storage modulus, G'' is the shear loss modulus, ν is PAGs Poisson ratio (0.48¹⁵). We observed a large range of E values in all precursor solution concentrations, from 0.2 kPa to 76 kPa, 0.8 kPa to 222 kPa, 15 kPa to 242 kPa for the 20%T, 30%T and 40%T precursor solutions, respectively. As expected, when the gray value of a grayscale mask is increased the elastic modulus of the resulting PAGs decrease and protein velocities increase (figure 4.6A).

To explore the elastic modulus and protein velocity relationship irrespective of the initial gray value – all protein velocities and corresponding E measurements (from figure 4.6A) are plotted for each protein in figure 4.6B. We chose to use an exponential model to describe the relationship between protein velocity and elastic modulus. This decision was based on the known Ferguson relationship², which describes protein velocity as an exponential function of gel density. Previous research has shown a power-law relationship between E and %T, specifically that %T is proportional to $E^{1/2.25}$ ^{16, 17}. We incorporated the power-law relationship in figure 4.6C, by plotting protein velocity versus $E^{1/2.25}$. The extracted constants of the exponential fits (for both E and $E^{1/2.25}$) are shown in Table 4.1. Qualitatively, the exponential models appear to describe the E and velocity relationship for high elastic modulus, $E > 1$ kPa, but diverge at $E < 1$ kPa.

The exponential velocity-E relationships appear to be dissimilar to the Ferguson relationship, which would predict $A_{\text{Parv}} = A_{\text{TI}} = A_{\text{BSA}}$ and, based on molecular weights $K_{\text{BSA}} > K_{\text{TI}} > K_{\text{PARV}}$. Instead we observe for E, $A_{\text{Parv}} > A_{\text{TI}} > A_{\text{BSA}}$ and for $E^{1/2.25}$, $A_{\text{TI}} > A_{\text{PARV}} > A_{\text{BSA}}$. For both relationships we observed that $K_{\text{TI}} > K_{\text{BSA}} > K_{\text{PARV}}$. These unexpected findings of the

exponential fit parameters for proteins of different molecular weights suggest a relationship different from the Ferguson relationship. While there is a clear exponential relationship between the elastic modulus of grayscale fabricated gels and protein velocity for a given protein (especially in stiff gels) – the nature of this relationship is still nebulous and requires a deeper investigation.

A possible hypothesis is that the incomplete polymerization of high gray value PAGs fundamentally alters the relationship between gel mesh size and gel density – thereby yielding non-Ferguson-like relationship. In a future investigation, we would extract the molecular weight between cross-links using rubber elasticity theory from the shear modulus measurements¹⁸ to determine the mesh size. Further, we would use dry mass measurements to determine the gel density. Then explore the relationship between mesh size, gel density to SDS PAGE protein mobility¹⁹.

Table 4.1 Preliminary results indicated that SDS PAGE protein velocity and elastic modulus of grayscale fabricated PAGs follow an exponential relationship that is dissimilar to the Ferguson relationship. The Ferguson relationship, $Velocity = Velocity_0 10^{-k \cdot T}$, describes the relative velocity of a polyelectrolyte traveling through PAG sieving matrix as function of free-solution mobility ($Velocity_0$), gel density (T) and the retardation coefficient (k) for a given polyelectrolyte. We tested whether the elastic modulus of a fabricated PAG could similarly be related to velocity in grayscale fabricated PAGs. All protein velocity and elastic modulus (E) from figure 4.5 were combined for each protein. A least square fit was performed with the exponential models to evaluate the relationship to E and apparent gel density, $Velocity = A 10^{-k \cdot EM}$ and $Velocity = A 10^{-k \cdot EM^{1/2.25}}$, respectively. The data and corresponding model is plotted in figure 4.6B and 4.6C. The apparent gel density was calculated using the previously reported and empirically measured power law relationship between E and PAG density ($T \sim E^{1/2.25}$)^{16, 17}. The exponential velocity- E relationship appears to be dissimilar to the Ferguson relationship, which would predict $A_{Parv} = A_{TI} = A_{BSA}$ and, based on molecular weights $K_{BSA} > K_{TI} > K_{PARV}$. Instead we observe for E , $A_{Parv} > A_{TI} > A_{BSA}$ and for $E^{1/2.25}$, $A_{TI} > A_{PARV} > A_{BSA}$. For both, $K_{TI} > K_{BSA} > K_{PARV}$

Figure 4.6B				Figure 4.6C			
$Velocity = Ae^{-k \cdot EM}$				$Velocity = Ae^{-k \cdot EM^{1/2.25}}$			
	<i>kDa</i>	<i>A</i>	<i>k</i>		<i>A</i>	<i>k</i>	
Parvalbumin (Parv)	12	3.057	1.77E-05		5.939	0.106	
Trypsin Inhibitor (TI)	21	1.794	2.53E-05		6.286	0.165	
Bovine Serum Albumin (BSA)	67	0.398	2.05E-05		1.174	0.14	

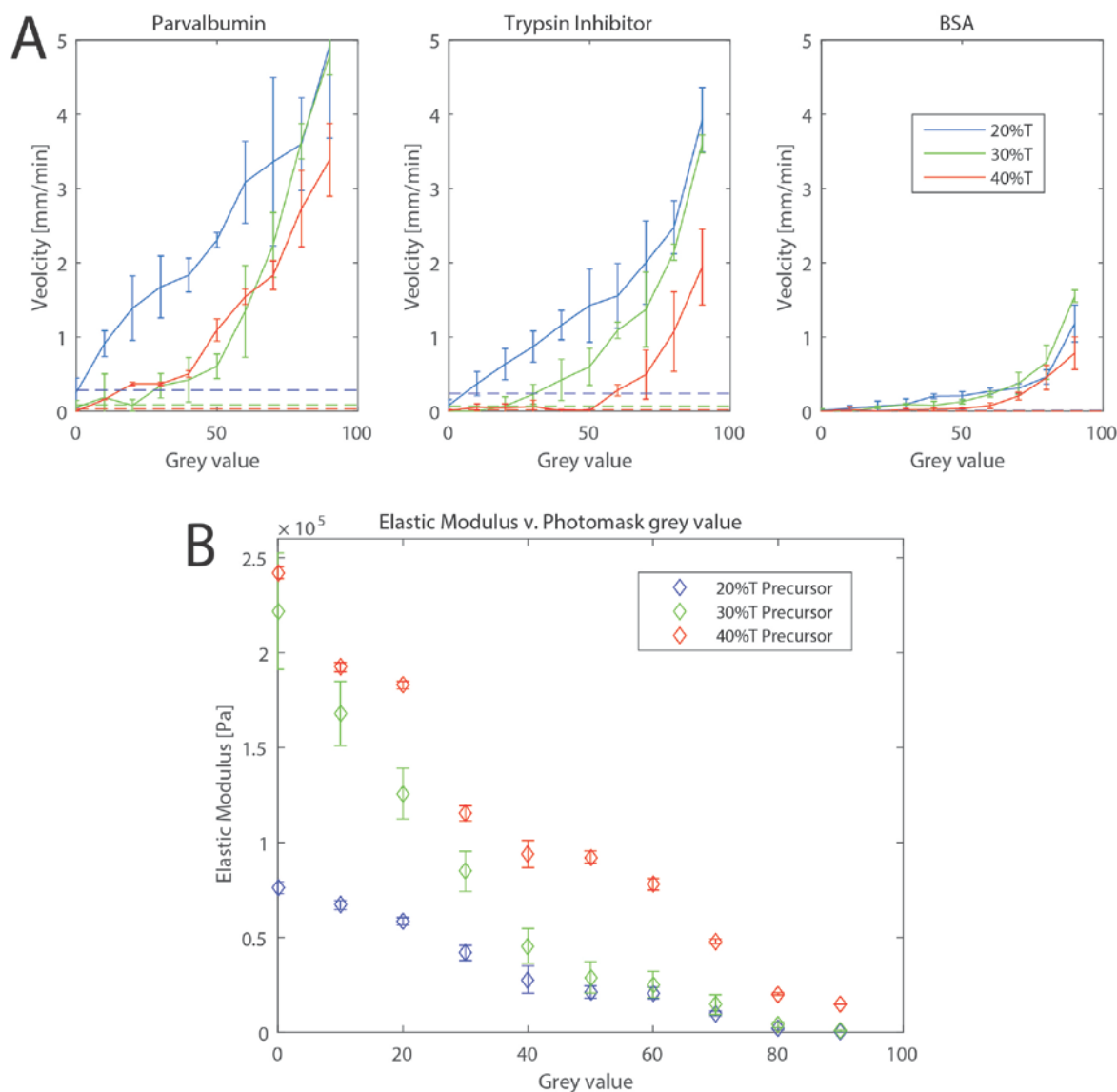


Figure 4.5 Uniform *fs*PAGs were fabricated at variable gray values (0% to 90%, at 10% intervals) in 20%T, 30%T and 40%T precursor solution concentrations (indicated in red, green, and blue lines, respectively) to assess PAG properties over a range of UV conditions. (A) To assess how grayscale fabrication impacts protein mobility, the velocity during SDS PAGE was measured in all *fs*PAGE conditions with Parvalbumin (Parv, 12 kDa), Trypsin Inhibitor (21 kDa) and Bovine Serum Albumin (BSA, 67 kDa). The dotted lines represent the velocity in chemically polymerized 20%T, 30%T and 40%T *fs*PAGs. The mean and STD is shown for three replicates in each condition. (B) The physical properties of the PAGs previously assessed for mobility, were tested using an MCR Rheometer (Anton Paar) at 1 Hz. Using a grayscale mask and laser cut mold, 8 mm diameter 300 μ m thick were fabricated to match the diameter of the rotor with over the range of grayscale conditions. The storage and loss modulus was measured, and the resulting elastic modulus is shown in (B) (using a Poisson ratio of 0.48^{15}). For each precursor concentration, the elastic modulus varied over an order a magnitude from 0% to 90% gray. *Data presented in this figure was collected by undergraduate researcher Darren Lim.*

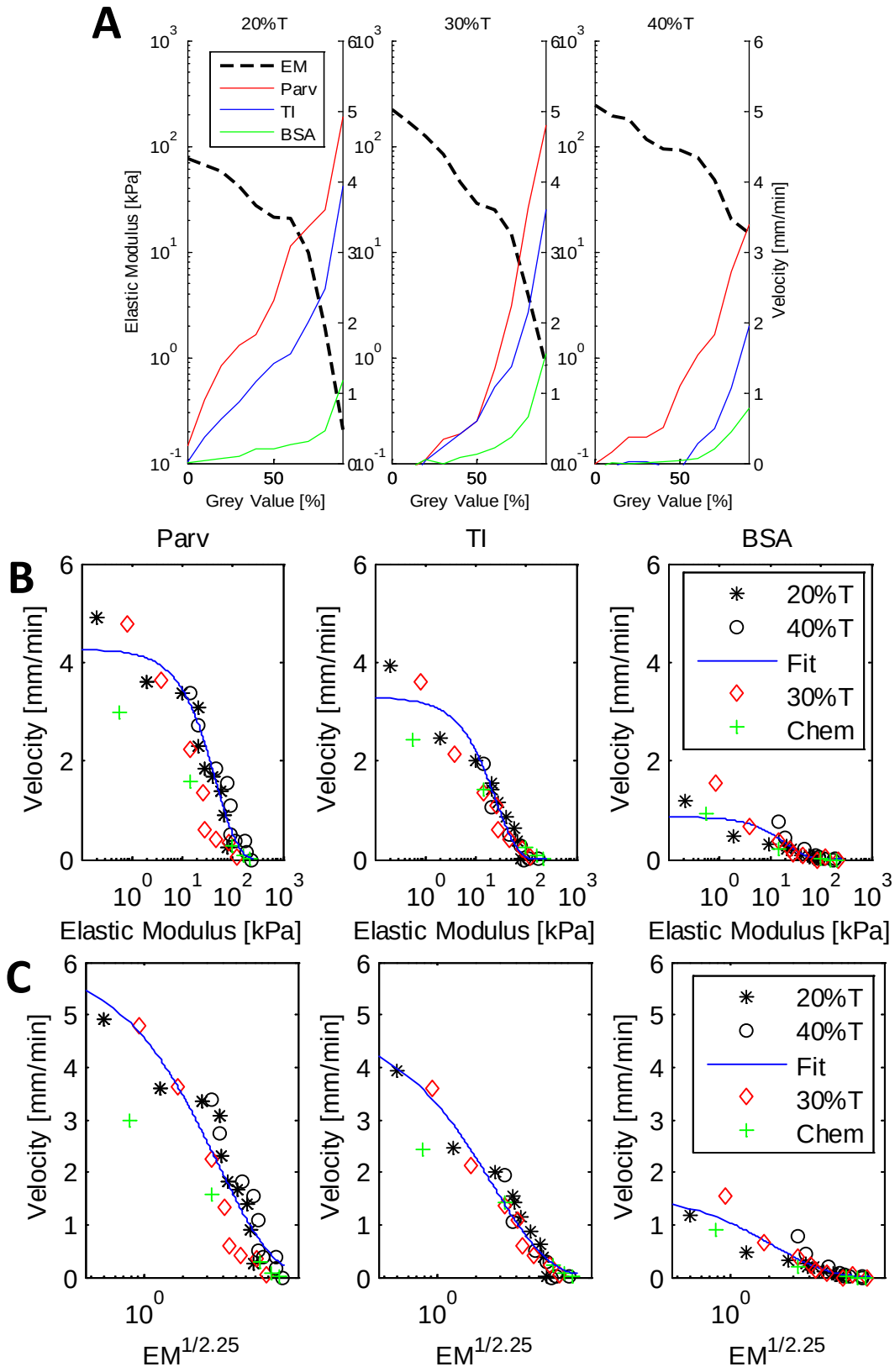


Figure 4.6 SDS PAGE protein velocity is qualitatively described with an exponential relationship to elastic modulus (E) or apparent gel density when $E > 1$ kPa. (A) SDS PAGE protein velocities for Parvalbumin (Parv, red), Trypsin Inhibitor (TI, blue), and Bovine Serum Albumin (BSA, Green) at different gray values are plotted for each gel precursor solution (20%T, 30%T and 40%T) and overlaid with the corresponding E (dotted black). As expected, as the gray value increases the elastic modulus of the PAG is reduced and the measured protein velocities increases. (B) The velocity for all measurements and their corresponding elastic modulus (in each precursor solution) is plotted for each protein. In addition, chemically polymerized PAGs for 10%T, 20%T, 30%T and 40%T are included (green). (C) The apparent gel density was calculated using the power law relationship between E and PAG density ($T \sim E^{1/2.25}$)^{16, 17}. The velocities were versus $E^{1/2.25}$. Exponential fits (blue, fit parameters reported in table 4.1) are included with the relationship $Velocity = A10^{-k*EM}$ and $Velocity = A10^{-k*EM^{1/2.25}}$, for B and C respectively. Qualitatively, the exponential models appear to describe the E and velocity relationship for high elastic modulus, $E > 1$ kPa, but diverge at $E < 1$ kPa. *Data presented in this figure was collected by undergraduate researcher Darren Lim.*

4.6. Periodic Gradient Gels for SDS fsPAGE Arrays

To adapt gradient PAG fabrication to the large 96-plex fsPAG array format previously introduced (figure 4.7A & Section 3.6), we used a periodically arrayed laser printed grayscale mask. Methacryloxyethyl thiocarbamoyl rhodamine B was added in the precursor solution so we could directly visualize with fluorescence the spatially varied density of the periodic gradient gel in figure 4.7B. We demonstrate a 96-plex gradient SDS PAGE for protein sizing over the molecular weight range from 12 kDa (Parv) to 134 kDa (BSA covalent dimer) in just 4 minutes of electrophoresis. Adaption of direct gel density photo-patterning enables numerous assay improvements including the high resolution protein sizing over a large molecular weight range, the reduction of injection dispersion, and the mitigation of contamination between samples.

As discussed in previously published work,²⁰ electroosmotic flow and sample adsorption in the injection reservoir may result in significant dispersion during electrophoretic injection that can reduce separation quality. The electroosmotic flow suppressor Triton-X 100, that was previously demonstrated for native fsPAGE (Section 3.5.2) is ineffective in the presence of SDS. Through the continuous stacking of a gradient gel, we largely eliminate injection dispersion to achieve tight protein bands despite not using an electroosmotic flow suppressor.

An additional advantage of a 96-plex gradient fsPAGE is the ability to place a high density, small pore size ‘halting’ region between consecutive lanes such that proteins cannot pass through the region and contaminate the subsequent lane. Enabling dense separation arrays with contiguous yet distinct separation lanes. In contrast, contamination can easily occur in a uniform gel array if the assay is run too long. The 96-plex protein sizing assay using free-standing gradient gel separations produces clearly resolved protein peaks in just 4 minutes of electrophoresis - as compared to hours for conventional tools. With the gradient gel we see no contamination from neighboring lanes, even after even a lengthy 15 minutes separation.

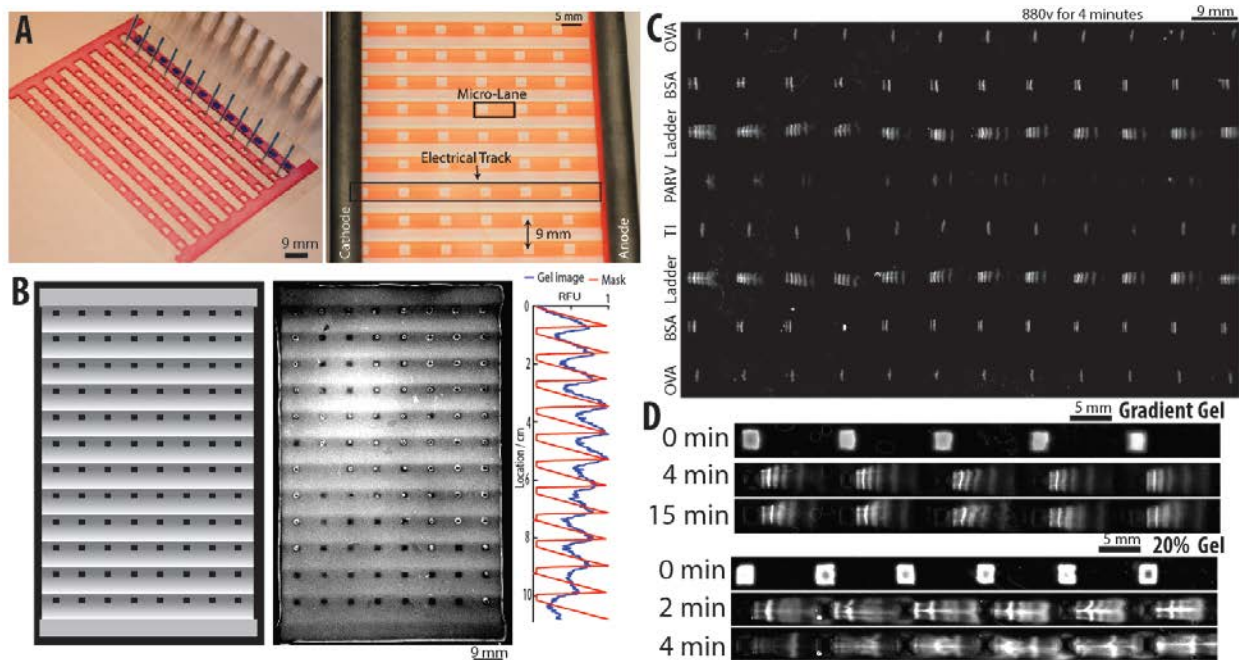


Figure 4.7 A 96-plex SDS PAGE gradient gel separation is performed for high resolution protein sizing within a large array. (A) We fabricate 96 separation lanes within the area of a 96 well plate. Each sample reservoir is spaced 9 mm enabling facile sample delivery using a multi-channel pipette. (B) A CAD image of the grayscale mask is compared to a fabricated gradient gel containing a rhodamine methacrylate monomer to visualize the spatial variation across the array. The apparent nonuniformity is caused by non-uniformity in the imaging system. (C) A 96-plex SDS PAGE gradient gel separation is performed in 4 minutes using four fluorescently labeled species: BSA (65 kDa, rows 2,7), OVA (45 kDa, rows 1,8), TI (21 kDa, row 5), PARV (12 kDa, row 4), and a ladder containing all four – in rows 3 and 6. (D) A key advantage of a gradient gel over a uniform gel array is the prevention of contamination from one lane to the next – we show five contiguous ladder separations in the gradient gel shown in (C) compared to in a uniform 20% gel. In the gradient gel, samples are uncontaminated for a 15 minute separation – while in the uniform gel lane contamination can be seen as early as 4 minutes. © 2014 IEEE.

4.7. Angularly & Radially-Patterned PAG Density for 24-plex PAGE Sieving Matrix Optimization Platform

In the previous section, we demonstrated the utility of using a periodic gradient gel for analyzing many different samples in parallel in duplicate gradient gels. In this section we demonstrate how angularly and radially varied grayscale PAG patterning can be used to screen multiple PAG conditions using the same sample for sieving matrix optimization.

4.7.1. Rapid Prototyping of Laminate PAGE Microfluidics

Under my mentorship and in-lab guidance, Kevin Maurer, a visiting masters student from ETH Zurich, developed PAGE compatible laminate microfluidic system. The fabrication approach is described in detail in Kevin Maurer's Thesis. In short, the laminate consisted of a top and bottom

layer of GelBond® (polyester material containing free-radical reactive alkene groups) and a double-sided adhesive layer (tesa® 68575). Designs drawn in Adobe Illustrator (figure 4.8A) were directly laser cut into the layers GelBond and double sided adhesive (figure 4.8B). Then a heated lamination is performed to assemble the final laminate device (figure 4.8C). Microfluidic channels were primed with 6% benzophenone in isopropanol for 30 seconds then removed. The purpose of this step is to pre-treat the adhesive material with free-radical generating molecules – such that polymerization can proceed uniformly throughout the channels. The polyacrylamide precursor solution is then added and photo-patterning is used.

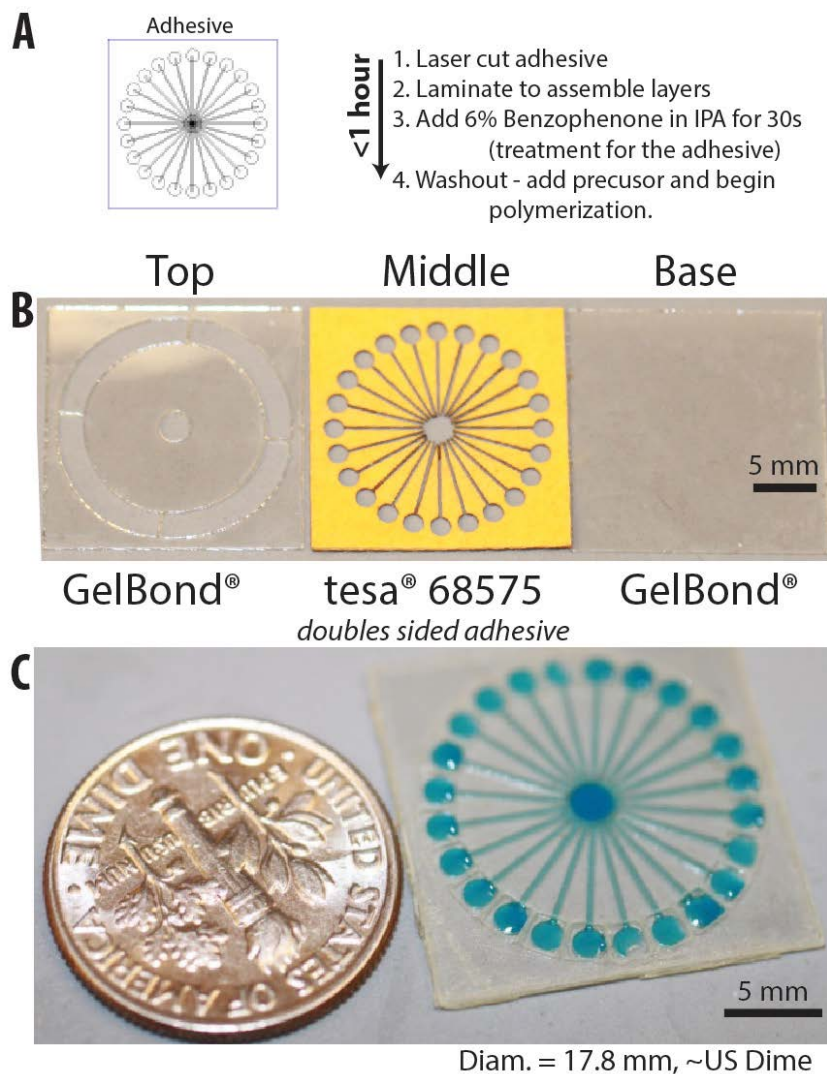


Figure 4.8 Laminated PAGE microfluidic platform is used to rapidly prototype a 24-plex circular adhesive microfluidic device in the area of a US dime. (A) CAD designs were directly laser cut in the (B) three layers of the laminate devices (GelBond, adhesive, GelBond). (C) The device is formed using a heated laminator. The circular 24-plex device has a shared central reservoir and discrete outer reservoir. After assembly – only a 30s soak in 6% Benzophenone is needed before PAG polymerization. Importantly, GelBond® - a commercial product that covalently attaches to PAG during polymerization – is used as the top and bottom

layer making the device PAG polymerization ready without channel surface modifications, supporting the rapid prototyping nature of the approach. © 2014 IEEE.

4.7.2. High-Throughput Sieving Matrix Optimization

Leveraging the rapid prototyping speed of adhesive microfluidics, we developed a 24-plex electrophoresis screening assay in a small circular chip (diameter of US dime). The device, shown in figure 4.8C, contains 24 radial channels with a shared central reservoir. A sample is injected from the central reservoir as a discrete zone into all 24 radial channels using transient isotachopheresis which had been previously demonstrated for single-channel injection²¹ but is here adapted to 24-parallel injections (figure 4.8A). Briefly, the device initially contains homogenous 500 mM Tris-HCl. Buffer in the central reservoir is removed using a kimwipe then replaced with protein sample containing 50 mM Tris-HCl, and an electric field is applied radially outward at 30 V/cm for one minute. Buffer in the central reservoir is then exchanged with a buffer 25 mM Tris 192 mM Glycine, which has a significantly lower conductivity, and an electric field is applied at 75 V/cm. The resulting isotachopheresis stack acts to concentrate the protein species at the gel solution interface prior to the zone electrophoresis separations radially in the 24 channels (figure 4.9B).

To screen for optimal gel conditions, we utilized a grayscale mask to vary gel density angularly in six regions shown in figure 4.9C (40%T precursor concentration). The six regions consisted of a 0% region, 100% to 0% grayscale gradient, a 100% to 20% grayscale gradient, a 100% to 40% grayscale gradient, a 100% to 60% grayscale gradient, and a 100% to 80% grayscale gradient. As a proof of concept, we benchmarked a wide-size range protein ladder (BenchMark™ Fluorescent Protein Standard). The separation results for the ladder are shown in figure 4.8E. For this case, the 60%-0% gradient gel was the optimal separation condition.

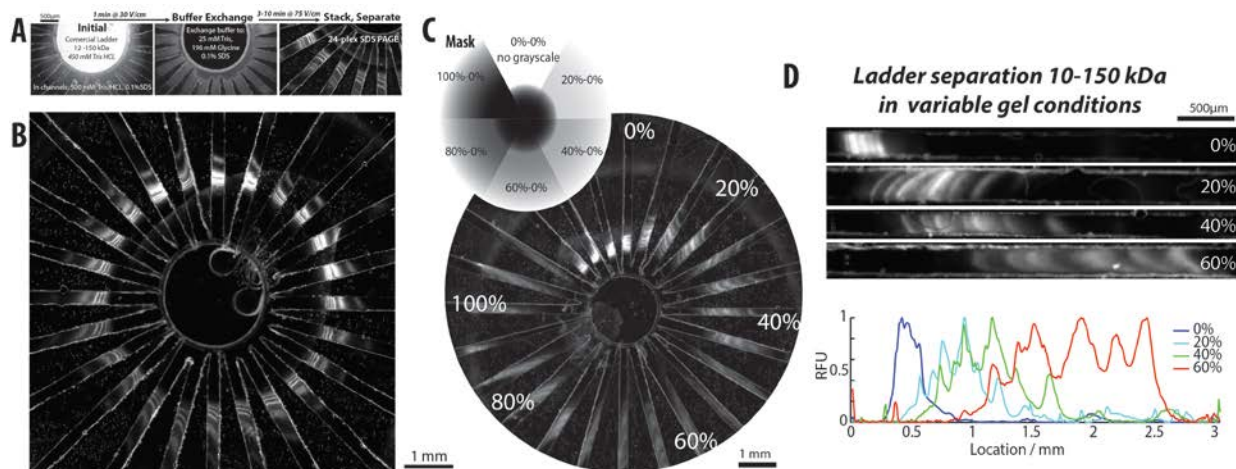


Figure 4.9 A 24-plex circular adhesive microfluidic device in the area of a US dime. (A) The 24 channel injection proceeds as follows: sample is added to central reservoir and an electric potential is applied uniformly towards the outer reservoirs causing sample to migrate into the channels. After loading, the remaining sample in the central reservoir is replaced with

Tris/Glycine. Due to the conductivity difference between Tris/Glycine and the Tris/HCl buffer in the sample transient isotachopheresis occurs resulting in sample stacking initially and then a separation in each channel²². Using this technique, (B) 24-separations can be performed from a single injected. (C) An angularly varied grayscale mask is used to differentially pattern channels within the circular chip. A commercial fluorescent SDS PAGE ladder is separated in all channels to determine the optimal gel conditions. (D) The 60% to 0% gradient demonstrated the optimal separation – with incomplete gel polymerization observed for the 80% and 100% gel gradients. © 2014 IEEE

Conclusions

Direct grayscale mask photo-patterning of spatially complex PAG density opens the door for new types of electrophoretic assays that are not realizable using other fabrication approaches. Further, due to the simplicity of laser printing new designs – new patterns can be rapidly prototyped. This technique can be extended to realizing sub-mm gradients by creating high-resolution grayscales – which is discussed in Chapter 5 for improving the performance of single cell western blotting. In the future, the technique could also be adopted for creating spatially complex immobilized chemistries.

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Chapter 4

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Single Cell Gradient Western Blotting

This work was conducted in collaboration with my colleague Dr. Chi-Chih Kang. Primary tissue analyzed in Section 5.6 was provided by collaborators Dr. Toby Ward and Dr. Mark Pegram from the Stanford Medical Center, Stanford, CA, USA.

5.1. Introduction

In 2012 the National Institute of Health (NIH) announced plans to invest \$90 million towards the development of new tools to analyze single cells (commonfund.nih.gov/SingleCell). The effort highlights the growing realization that grouping cells by “cell type” is an oversimplification that ignores significant cell to cell heterogeneity. Single cell interrogation has shown that clear differences exist within populations with important biological consequences stemming from these differences. The NIH initiative seeks to support new innovative techniques for quantitative single cell analysis, which historically was largely limited to flow cytometry and immunocytochemistry based analysis. Over the last decade, there have been numerous developments in single cell protein analysis, which have expanded upon the traditional immunoassays, to enhance multiplexing, throughput and quantitation^{1, 2}. While powerful and highly useful for some applications, these technologies are still limited by antibody specificity (discussed in Section 1.1). To improve the specificity of single protein analysis, our group introduced an integrated fractionation prior to antibody probing in the single cell western blotting (scWB) platform^{3, 4}.

The scWB platform utilizes a chemically polymerized polyacrylamide gel (PAG) containing a benzophenone photo-capture group⁵ and molded wells with a diameter comparable to the cells under investigation. In the assay workflow, single cells are settled into individual wells, lysed, fractionated with sodium dodecyl sulfate PAG electrophoresis (SDS PAGE), UV immobilized, and then probed with antibody to detect western blotting results for thousands of cells in parallel. For multiplexed protein detection, antibodies can be chemically stripped from the gel, which can then be re-probed for detecting multiple markers. Unfortunately, the uniform hydrogel in the seminal work is inherently limited in achieving high quality separations for the large range of proteins present in a cell. Injecting and separating the broad protein size range is not achievable with the previously published uniform gel scWB. There exists a specific uniform PAG density which is optimal for the separation for a pair of proteins with known sizes. When analyzing the entire proteome of a cell - any one gel density will be acceptable for a subset of proteins but inappropriate for the majority. Simply increasing the PAGE time to compensate for non-ideal separation conditions leads to significant diffusive losses.

In the ideal case, the scWB platform would function as a single cell proteomic catalog accessible to sequential immunoassays for multiplexed analysis. We sought to design a platform that could inject and subsequently separate proteins that span molecular weights between 20 kDa and 300 kDa. For the human proteome of NIH3T3 fibroblast cells (which has been extensively investigated) the 20 to 300 kDa range accounts for 86% of proteins⁶ (figure 5.1A). Further, to maintain a dense array for high-throughput single cell analysis, we limited separation lengths to 1 mm.

Pore-size gradients, as noted by D. Rodbard et.al. in a seminal analysis⁷, are the ideal separation medium for multi-component mixtures with a large range in size and a small range in free-resolution electrophoretic mobility, which is the exact case for a single-cells SDS denatured proteome. In this chapter, we demonstrate high-quality separation results for two protein pairs that span an order of magnitude in size with periodic 1 mm PAG pore-sized gradient arrays fabricated directly using a grayscale mask (figure 5.1B).

The U373-GFP human glioblastoma cell line and the SKBR3 HER2+ breast cancer cell line are used for gradient scWB characterization and validation. The new gradient scWB platform was applied to assay primary human HER2+ breast cancer tissues to investigate the single cell heterogeneity of the HER2 signaling pathway. The development of gradient scWB greatly advances our original platform by allowing proteins to be analyzed from a single cell over a large molecular weight range in a high-throughput dense-array.

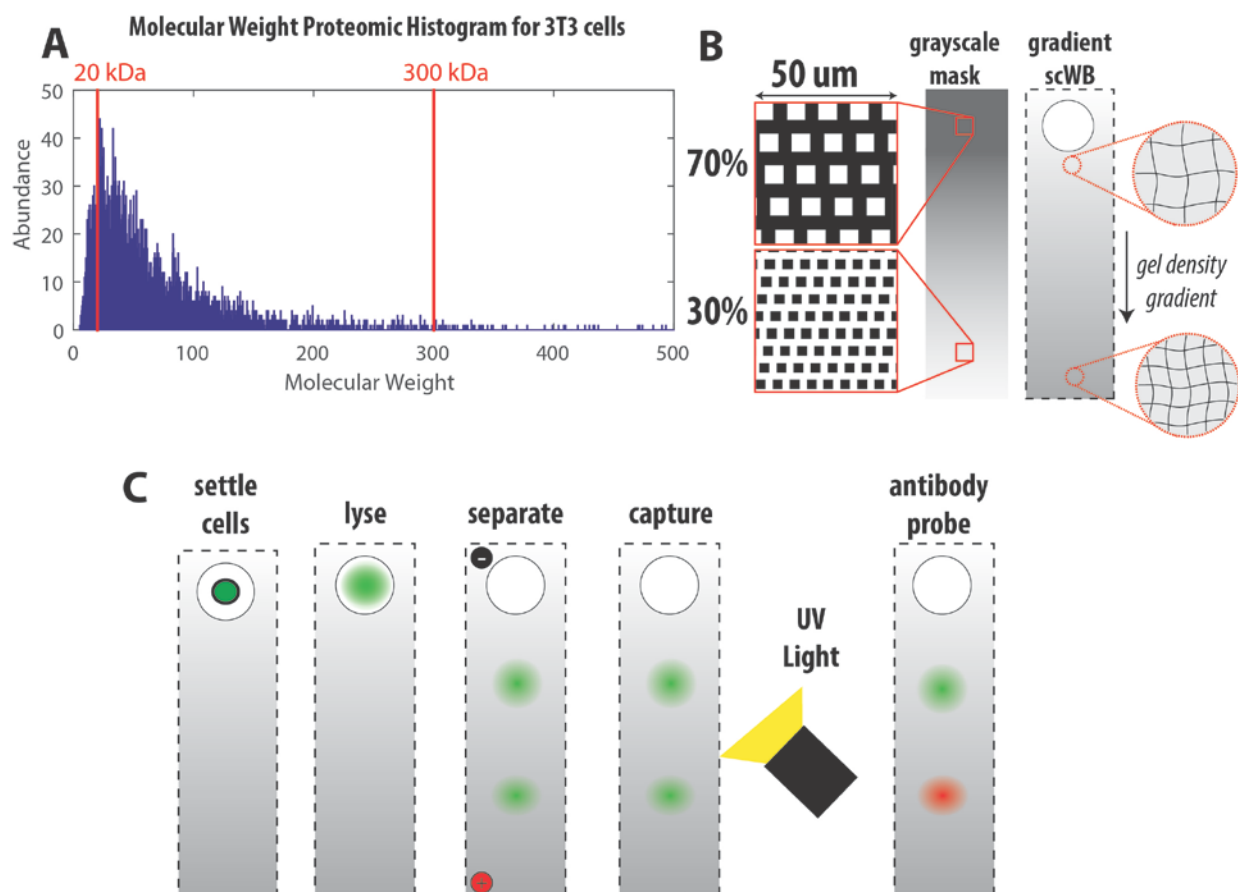


Figure 5.1 Gradient Single Cell Western Blotting (scWB) enables analysis over a large range of protein sizes. (A) A histogram of the proteome for 3T3 human fibroblast cells⁶ displays the molecular weight distribution of proteins. The assay objectives for the design of gradient scWB are shown in the red lines at the 20 kDa and 300 kDa positions. (B) We propose

the use of direct grayscale patterning to define PAG density gradient and an SU8 mold on a glass substrate (to enable photo-patterning) to define the microwells. (C) The assay workflow for scWB is as follows: 1) cell settling into microwells, 2) wash off unsettled cells, 3) lyse cells using a RIPA-like buffer for 15 to 25 seconds, 4) run electrophoresis to separate proteins at 40 V/cm for 10 to 60 seconds, 5) expose gel to UV light to photo-immobilize proteins, and 6) probe the gel with antibodies to detect the presence of specific proteins.

5.2. Materials and Methods

Reagents

Solutions of 40% (w/v) (29:1) acrylamide/bis-acrylamide, 3-(trimethoxysilyl)-propyl methacrylate (98%), glacial acetic acid, methanol, sodium deoxycholate, triton x-100, ammonium persulfate, tetramethylethylenediamine were purchased from Sigma Aldrich (St. Louis, MO). Photoinitiator 2,2-azobis[2-methyl-N-(2-hydroxyethyl) propionamide] (VA-086) was purchased from Wako Chemical (Richmond, VA). Tris-glycine (10x) native electrophoresis buffer and SDS were purchased from Bio-Rad Laboratories (Hercules, CA). PBS (10x) was purchased from Corning (Manassas, Virginia). TBST (20x) was purchased from Santa Cruz Biotechnology Inc. (Dallas, Texas). Glass microscope slides were purchased from VWR (Radnor, Pennsylvania). Glass half-slides (37 mm x 25 mm) were created using a diamond scribe (Ted Pella Inc., Redding, California) and glass pliers (Fletcher-Terry Company LLC, East Berlin, Connecticut). Water was UV sterilized and filtered.

SU8-Glass Mold Fabrication.

To achieve sub-100 μm microwells features in PAG along with a gel density pattern, we utilized a mold to define the wells and a grayscale mask to photo-pattern the density pattern. Precise alignment of the mold to the grayscale mask is required. Due to the reservoir of gel precursor solution, this fabrication setup is not compatible with conventional mask aligners. Therefore, to do alignment on a conventional epi-microscope, we created transparent SU8 molds using a glass wafer substrate. The adhesion of SU8 directly on glass is significantly worse than its adhesion on silicon and as a result, the majority of the micropillar array in our molds would detach after a single use. To address this, we adopted a protocol described by Seth Madren et. al.⁸, in which, an adhesion primer consisting of 2% titanium acetylacetonate in anhydrous isopropanol is used, in addition to an SU8 base layer. The use of an adhesion primer and an SU8 base layer enables the fabrication of robust transparent molds that can be used for ~20 to 40 slides with careful handling. The complete SU8 on glass mold fabrication protocol is described below (for a step by step protocol, see Protocol B).

Four inch silicon and glass wafers (borosilicate, 1000 μm thick) were purchased from University Wafer (Boston, Massachusetts) and SU8 2025 was purchased from MicroChem (Newton, Massachusetts). Photomasks were designed using CleWin (WieWeb software, Hengelo, Netherlands) and printed by CAD/Art Services, Inc. (Bandon, Oregon). SU8 glass fabrication method was adopted from Madren et. al.⁸ After a 10 min wafer dehydration at 110° C, the wafer was placed on the spin chuck. To improve the SU8/glass adhesion, a small volume (~1.5 mL) of 2% titanium acetylacetonate in anhydrous isopropanol (both purchased from Sigma Aldrich) was pipetted atop the wafer such that the full wafer was coated for 1 minute, then was spun dry. Immediately after, SU8 2025 was poured onto the wafer and spun at 500 RPM (with 100 RPM/s

for 30 s), followed by a spin at 2500 RPM (with 500 RPM/s for 30 s). The wafer was soft baked at 110° C for 3 min and then allowed to cool at room temperature. The wafer was next uniformly exposed (no photomask) to 250 mJ/cm² of light to polymerize the base layer. A post exposure bake at 110° C for 10 min was done to solidify the layer. The wafer was then allowed to cool to room temperature before being placed again on the spin wafer. SU8 2025 was poured on the wafer and spun at 500 RPM (with 100 RPM/s for 30 s) followed by a spin at 1900 RPM (with 500 RPM/s for 30 s) to define the top layer height (~50 μm). A soft bake was then performed (65° C for 6 min) – it was critical to keep temperatures low (< 80° C) at this step to avoid reflow of the upper SU8 layer. The wafer was then allowed to cool to room temperature, and then exposed to 250 mJ/cm² of UV through a photo mask defining the microwell array. A post exposure bake was performed at 65° C for 3 min, followed by 90° C for 7 min. After cooling to room temperature, the wafer was placed in SU8 developer (Microchem) for ~3 min. After development, the wafer was soaked in DI water for >2 min, and then inspected with an ADE MicroXAM-100 optical profilometer. The wafer was then hard baked for ~1 hour at 150° C. After the hard bake, chemical vapor deposition of dichlorodimethylsilane (ACROS Organics, Geel, Belgium) was performed in a desiccator (under house vacuum for 1 hr). The protocol used for silicon wafers (used for molding uniform gels) was similar. It excluded the titanium acetylacetonate adhesion priming, the first layer of SU8 and UV exposure was conducted at 150 mJ/cm².

scPAG Array Fabrication

Chemically polymerized uniform gels fabrication has been previously described.^{3, 4} Photopolymerization of gradient scPAG arrays was performed on a collimated and upward-facing 1000W near-UV light source with a 7 inch exposure array (OAI, San Jose, California). The UV intensity could be tuned between 18 mW/cm² and 30 mW/cm² (measured with the OAI 308 UV Meter at 365 nm). The fabrication setup was as follows (in the order of the light path): UV Filter Sheet, 3 mm borosilicate plate, grayscale chrome mask (sodalime glass), glass SU8 mold, gel precursor solution, and a methacrylate functionalized glass slide on which the PAG will attach after polymerization. To initiate free-radical polymerization from the VA-086 and avoid activating the benzophenone moiety on the BPMA groups, a UV Filter Sheet with a 400 nm cutoff (#39-426, Edmund Optics, Barrington, New Jersey) was used - with the UV filter in place, 30 mW/cm² would be reduced to less than 0.3 mW/cm² measured at 365 nm. This violet-light photo-patterning technique is compatible with the UV-active gels (Appendix D).

Polyacrylamide gel precursor solutions containing bis-acrylamide solution, with a concentration ranging between 11%T to 25%T, at 3.33%C, 3 mM BPMA, and 1% VA-086 was prepared and degassed under house vacuum and sonicated for 3 min. The SU8 mold was coated briefly with GelSlick[®] solution (Lonza, Basel, Switzerland) and then N₂ dried. The methacrylate functionalized glass slide was placed face down on the mold. The mold was then manually aligned to the gradient features on the chrome grayscale mask, such that the wells are ~20 μm below the top of the gradient period. Once aligned, the gel precursor solution was carefully added and allowed to wick between the mold and glass slide. Immediately after wicking, the setup was placed above the UV light and exposed. For the 11%T gradient gel used in this study, a UV intensity of 26.5 mW/cm² at 365 nm was used (measured without the UV filter sheet) and an exposure time of 121 s. After polymerization, the gels were carefully removed from the mold using a razor and placed in PBS for at least 10 minutes to remove excess acrylamide and photo-

initiator. The gels can then be used immediately or stored in the dark for extended periods of time (weeks or longer).

scWBing Operation

The gradient scWB operation is similar to the uniform scWBing previously described.^{3,4} For this work, cells were seeded with a 400 μ L solution of cells in PBS at a density of $\sim$ 500,000 cells/mL. The cell solution was passed through a 35 μ m nylon cell strainer (Corning #352235) prior to settling. Cells were allowed to settle into the PAG microwells for 20 min. Excess cells were washed off via PBS dispensed with a pipette while tilting the slide at an angle between 30-70°, angle chosen based on the abundance of un-settled cells. The slide was then placed in a 3D printed chamber with platinum wires at either side of the chamber (spaced by 5 cm). RIPA-like lysis buffer at pH 8.3 (0.5% SDS, 0.25% sodium deoxycholate, 192 mM glycine, 25 mM tris, and 0.1% triton x-100) heated to 50° C was poured to fill the chamber ($\sim$ 15 mL) and cell lysis was allowed to proceed for 10-25 s. A voltage of 200 V was then immediately applied using a BioRad PowerPac power supply to initiate electrophoresis at an electric field of 40 V/cm – electrophoresis times vary between 5 and 60 s depending on the experiment. Immediately after the power supply was turned off, photo-capture of proteins was initiated using a UV arc lamp (Lightningcure LC5, Hamamatsu, Bridgewater, NJ) for 45 s. Protective face shields were worn to prevent harmful UV exposure. After photo-capture, slides are placed in TBST for immunoprobng.

Antibody probing and stripping

Antibody solutions were diluted at either 1:10 or 1:20 fold in a TBST solution with 2% BSA (40 μ L volume of solution consumed for each slide). Immunoprobng was performed in a 5 hour total protocol. The probing protocol consisted of the following steps: primary probing (2 hr), TBST wash (1 hr), secondary probing (1 hr), and a final TBST wash (1 hr). After the final wash, the slides were soaked briefly in water ($\sim$ 2 min), dried under an N₂ stream, and then imaged on a microarray scanner. The primary antibodies used in this study and their respective fold dilutions were as follows: anti-Her2 (1:20 dilution, Mouse, Pierce, MA513105), anti-TruboGFP (1:10 dilution, Rabbit, Pierce, PA5-22688), anti-mTor (1:10 dilution, Rabbit, Pierce, 2983), anti-elf4E (1:10 dilution, Rabbit, Cell Signaling, 2067), anti- β -Tubulin (1:10 dilution, Mouse, Genetex, GTX11312), anti-ERK (1:10 dilution, Rabbit, Cell Signaling, s4695), and anti- α -Actinin (1:10 dilution, Rabbit, Cell Signaling, 6487p). The secondary antibodies used in this study and their respective fold dilutions were as follows: anti-Rabbit (1:20 dilution, Donkey, Invitrogen, A31573, Alexa Flour 647) and anti-Mouse (1:20 dilution, Donkey, Invitrogen, A21202, , Alexa Flour 488). After imaging, antibody stripping was performed overnight at 50°C in a 62.5 mM Tris buffer titrated with HCl to 6.8 pH, containing 2.5% SDS and 1% β -mercaptoethanol. The gels could afterwards be re-probed for new targets.

Microarray Imaging and Analysis

Slides were scanned with a GenePix 4300A microarray scanner at 5 μ m resolution and a 0 μ m focal depth with PMT gains of 550 and laser powers of 80-100%. Detection of turboGFP or Alexa Fluor 488 and Alexa Fluor 635 labeled secondary antibodies was performed using 488 nm and 635 nm lasers, respectively. Emission filters for the 488 nm spectral channel were from Omega Optical (XF3405) and a built-in far-red emission filter was used for the 635-nm channel. An outlier removal was performed on the generated image files to remove punctate spots of

bright signal resulting from either dust particles or antibody aggregates in FIJI imaging software.⁹ Further image processing and data extraction was performed using an in-house algorithm implemented with MATLAB version 2014b made by MathWorks (Natick, MA). Results attained in gel images were axially background subtracted to remove the non-uniform background resulting from the non-uniform gel density in gradient gels (images displayed throughout this chapter are background subtracted). Protein peaks were curve fitted to a Gaussian model and peaks with an $R^2 < 0.7$ or an $SNR < 10$ were discarded and not used in analysis. The total fluorescence was the integrated fluorescence from the background subtracted signal over a protein band peak width, i.e. over four standard deviations. If multiple peaks were present within four standard deviations, the extracted parameters from the Gaussian fit were used to estimate abundance for the peak of interest.

5.2.1. Grayscale mask design

The previously employed methods of forming gradient PAGs, via diffusion or active mixing of the precursor solution prior to polymerization¹⁰, are not capable of forming the spatially complex and high-resolution features our platform requires. Similarly, polymerization coupled with moving shutters to control light-dosage¹¹ would be extremely difficult to realize for a dense periodic array. The currently popular 2-photon patterning methods can achieve intricate high-resolution patterns, but are impractical for creating a large array of features¹² (e.g. microscope slide size).

In this work, direct spatial-patterning of PAG density is accomplished by photo-polymerizing the PAG gradient array with a near-UV light through a chrome grayscale mask adapted from the original demonstration of PAG grayscale patterning where laser printed grayscale (~50-100 μm dot size, figure 4.3B) created ~1 cm stiffness-gradients to manipulate the cellular microenvironment¹³. We sought to develop a chrome grayscale mask with pixel size less than 10 μm such that we could realize a smooth and controlled UV intensity profile within 1 mm or less in length. We developed a simple algorithm in a MATLAB script, described in figure 5.2 (code script shared in Appendix E), to directly define grayscale regions into the mask plotting program CleWin4 (PhoeniX Software, Enschede, Netherlands). The masks were fabricated on sodalime glass by aBeam Technologies (Hayward, CA, USA), which was able to create masks with tens of millions of features (required for the grayscale pattern) at no additional cost for the complexity.

Due to the computational memory limitations of drawing a mask with tens of millions of features, the script was used to create a feature pattern in 1 mm by 500 μm intervals and was then arrayed in CleWin4 to create the larger patterns. This results in a periodic 500 μm discontinuity which can be seen in figure 5.3. Due to the small feature size on the chrome grayscale masks (~3-10 μm dot size), diffraction happens quickly. At a 1 mm distance away from the mask we observe a smooth gradient, negating the need for a light diffuser used previously for the laser printed grayscale masks.

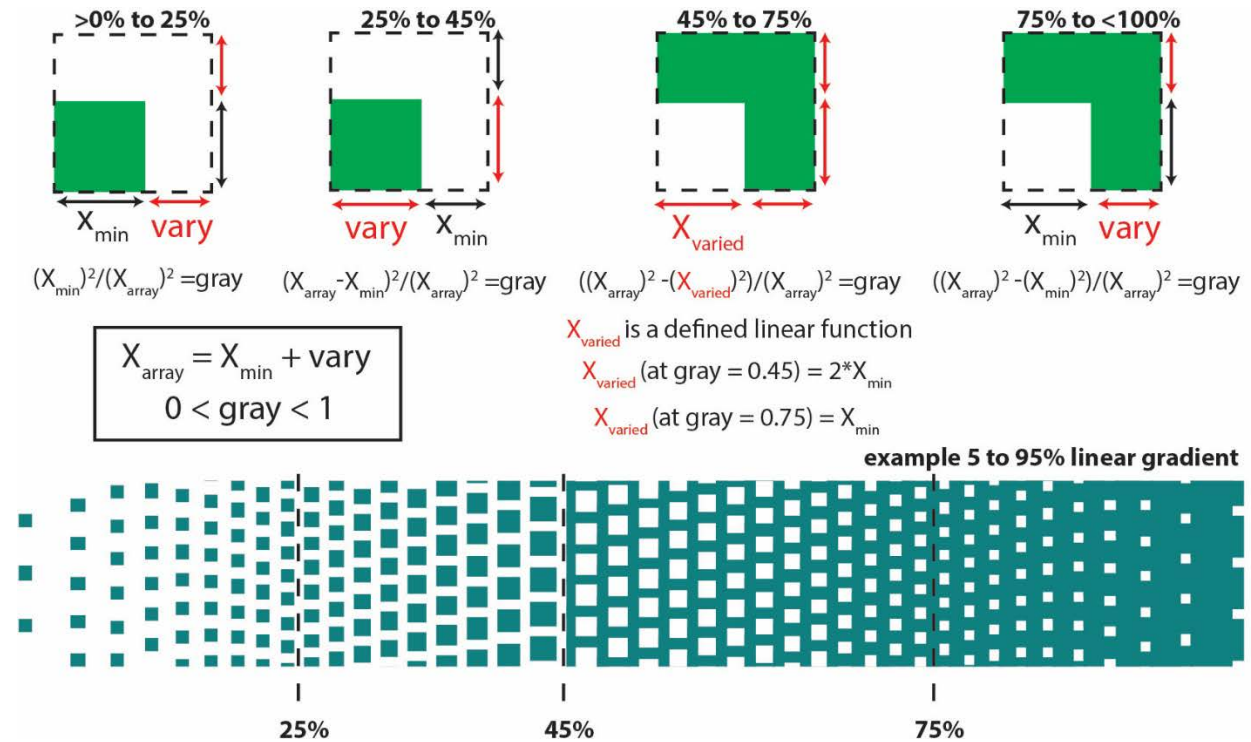


Figure 5.2 Simple algorithm to facilitate on-demand design of grayscale regions that can be fabricated into a chrome grayscale mask. The algorithm develops an array to represent a grayscale pattern solving different equations in four regions: 0 to 25%, 25% to 45%, 45% to 75% and 75% to 100%. These distinct regions were chosen to minimize the total size of pixels while remaining within the mask fabrication limitation from aBeam technologies (minimum square $3 \mu\text{m} \times 3 \mu\text{m}$, $X_{\min} = 3 \mu\text{m}$). With this approach, the maximum pixel size is $10 \mu\text{m}$ (at 45% gray). The mask would be fabricated as an inversion of the original design for simplicity, as it made the boundary around all grayscale features opaque. MATLAB script is shared in Appendix E.

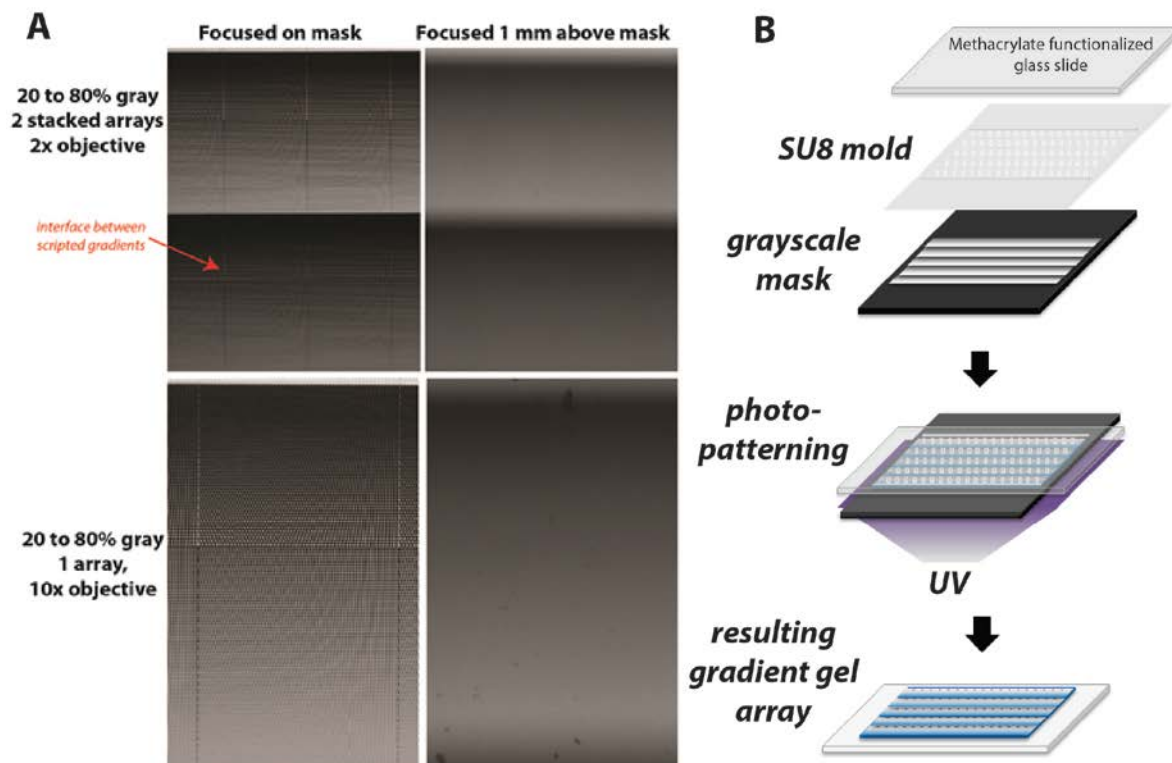


Figure 5.3 Fabricated grayscale masks and fabrication assembly. (A) The resulting masks were perfectly recreated by aBeam Tech, I highly recommend their services for complex mask designs. At a 1 mm distance from the mask the gradient is smooth, negating the need for a light diffuser. Interestingly, the darkest and brightest regions of the mask is slightly shifted indicating that the molded micro-wells should be oriented slightly below the interface when performing fabrication. In both images, the gradient is 1 mm in length. (B) Schematic of the fabrication setup highlights the alignment of the grayscale mask, transparent SU8 mold and the methacrylate functionalized glass slides.

5.3. Physical Characterization Grayscale Fabrication

To evaluate the presence of grayscale patterning, we directly imaged the spatial non-uniformity of the short PAG gradient array. Allylamine was added to the PAG precursor solution at a 1:100 molar ratio with acrylamide, and the gradient gel was fabricated as usual. We did find that allylamine inhibits polymerization, so we had to increase the polymerization time by ~30%. Allylamine directly incorporates into the PAG backbone with its allyl group. We soaked the resulting gel in 0.1 mg/ml FITC solutions for 1 hr to overnight and then washed it out for several hours in DI water. The primary amine presented by the allylamine group forms a surprisingly stable electrostatic interaction with FITC in deionized water solution¹⁴, stable even when the buffer is changed to Tris-Glycine pH 8.4 and a 40 V/cm electric field is applied. In figure 5.4A, we imaged the immobilized FITC with epi-fluorescence to directly observe the periodic spatial non-uniformity in the gradient PAG array. The micrograph reveals the periodic nature of the array and the smooth linear gradient region spanning 70% of the 1 mm period.

The graded topography of the short gradient gels can be clearly seen by eye after fabrication. Variable gel height was assessed with an ADE MicroXAM-100 optical profilometer (KLA Tencor, Milpitas, CA, USA) in figure 5.4B. Optical profilometer measurements on hydrated gels were unreliable, due to the need to remove excess solution atop the structures and a lack of an environmental chamber to prevent the rapid evaporation and drying process. Therefore, gels were dried prior to the measurements. The cross-section of the dried structure is shown in figure 5.4C. We observed a gradually increasing height along the gradient gel, ($3.5\ \mu\text{m}$ over the $1\ \text{mm}$ gradient). Interestingly, we normally observe a small bump adjacent to the microwell reservoir. The edge bump is $0.2\ \mu\text{m}$ above the lowest region observed along the gradient gel. This could be an artifact of the optical profilometer scan or of the gel drying process. Alternatively, it may indicate a polymerization edge effect stemming from free-radical inhibition by the adjacent SU8 micro-pillar. This has previously been shown to increase gel density at edge regions^{15, 16}. If a denser well interface is indeed being observed, it would be consistent with the well clogging commonly observed when injecting proteins in the PAG array.

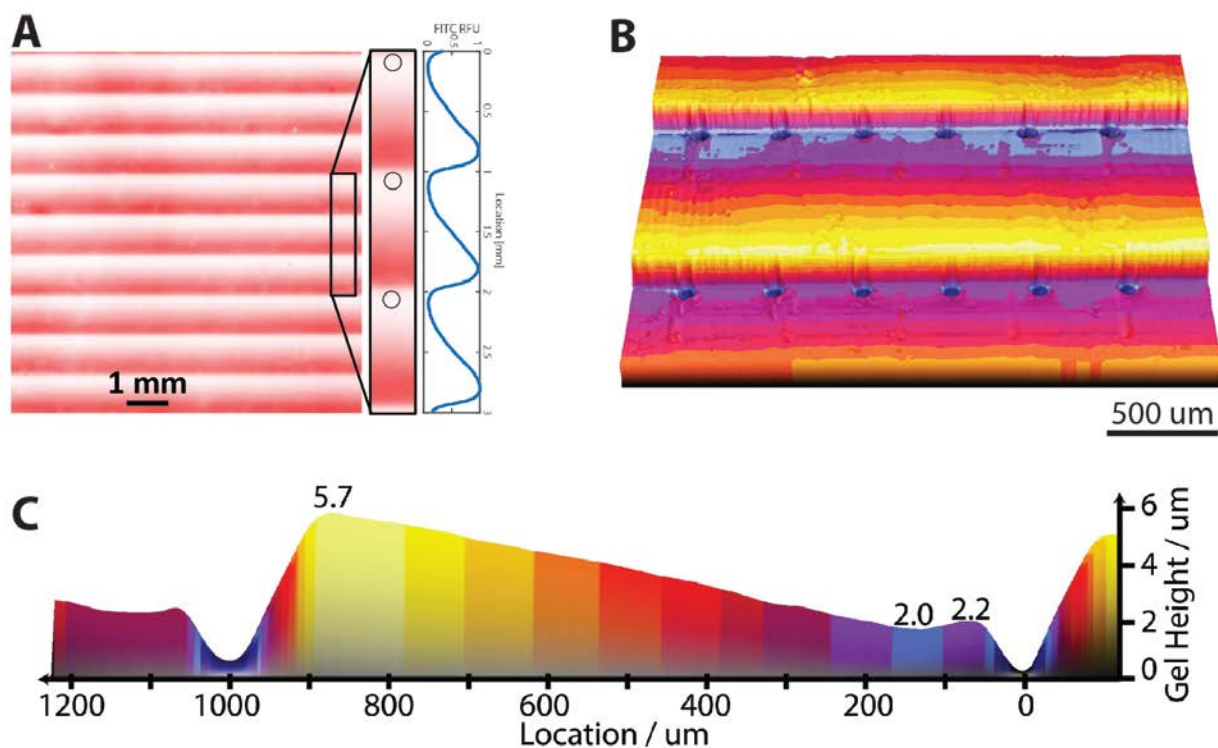


Figure 5.4 Direct Physical Characterization of 1 mm Gradient Gel Photo-patterning. (A) Spatial variation of gel density was measured by incorporating allylamine into the PAG backbone and staining the allylamine with FITC. The resulting epi-fluorescence image shows the periodic variation in gel density along the array and, of particular importance, the smooth linear region that will be used for the protein separation. (B) Optical profilometry revealed the topography of dried gradient gels for an array with a $400\ \mu\text{m}$ well to well spacing and a $1\ \text{mm}$ period. Small linear bumps that run axially along the gradient and are spaced transversely by $500\ \mu\text{m}$ are observed. This indicates that the method of arraying the drawn $500\ \mu\text{m}$ grayscale designs (described in the previous section) could be improved. Since these bumps are quite small, they

likely have a very small or no impact on the protein separations. (C) Cross-section of gel height over a period in the gradient gel. The gel height grows by 3.5 μm over the gradient gel period.

5.4. Non-Uniform Protein Sieving

In the evaluation of the non-uniform sieving properties of the gradient scWB platform, we utilized a U373 cell line transduced with cytoplasmic turboGFP. The U373 cells were seeded in the scWB platform and GFP migration was measured for uniform chemically polymerized gels (4%T, 6%T, 8%T, 10%T, 12%T, 16%T and 25%T) and gradient photo-patterned gels. For the GFP velocity measurements, an alternative 3D printed chamber with a glass bottom was used for real time imaging. To prevent excessive sample loss via diffusion from the well, the lysis buffer was cooled to 4°C prior to lysis. GFP migration was captured with an inverted epi-flourescence imager with a Peltier cooled charge-coupled device (CCD) camera (CoolSNAP HQ2, Roper Scientific, Trenton, NJ) and a 4x objective (UPlanFL, N.A. = 0.3, Olympus, Center Valley, PA) on an Olympus IX-70 microscope. Camera exposure times ranged between 100 and 500 ms with 1 x 1 pixel binning with an acquisition rate of 2 fps. Illumination was sourced from an X-Cite® exacte mercury lamp (Lumen Dynamics, Mississauga, Canada) and filtered through an XF100-3 filter (Omega Optical, Battleboro, VT). GFP mobility was extracted using a custom MATLAB algorithm that performed curve fitting for each frame to determine the mean location and peak width of the band.

Kymographs of the GFP migration are shown in figure 5.5A for a 25%T gradient gel, a 11%T gradient gel and an 8% uniform gel. In the gradient gels, the GFP band adopts an ellipsoid shape as it migrates further into the gel indicative of the axial stacking that is expected in a gradient gel. In contrast, the GFP band in uniform gels maintains its circular shape as expected. In figure 5.5B, we extracted the velocity as a function of time for all conditions. We did observe a significant reduction in velocity in the 25%T gradient gel (~50%) and a small reduction for the 11%T gel (~10%). We saw a severe exponential increase in the velocity for all uniform gels over time. This exponential relationship follows the expected changes in viscosity when the temperature is increased by joule heating. Due to these significant changes in viscosity over the course of electrophoresis, it was not possible to extract the effective gel density directly from the GFP gradient gel velocities.

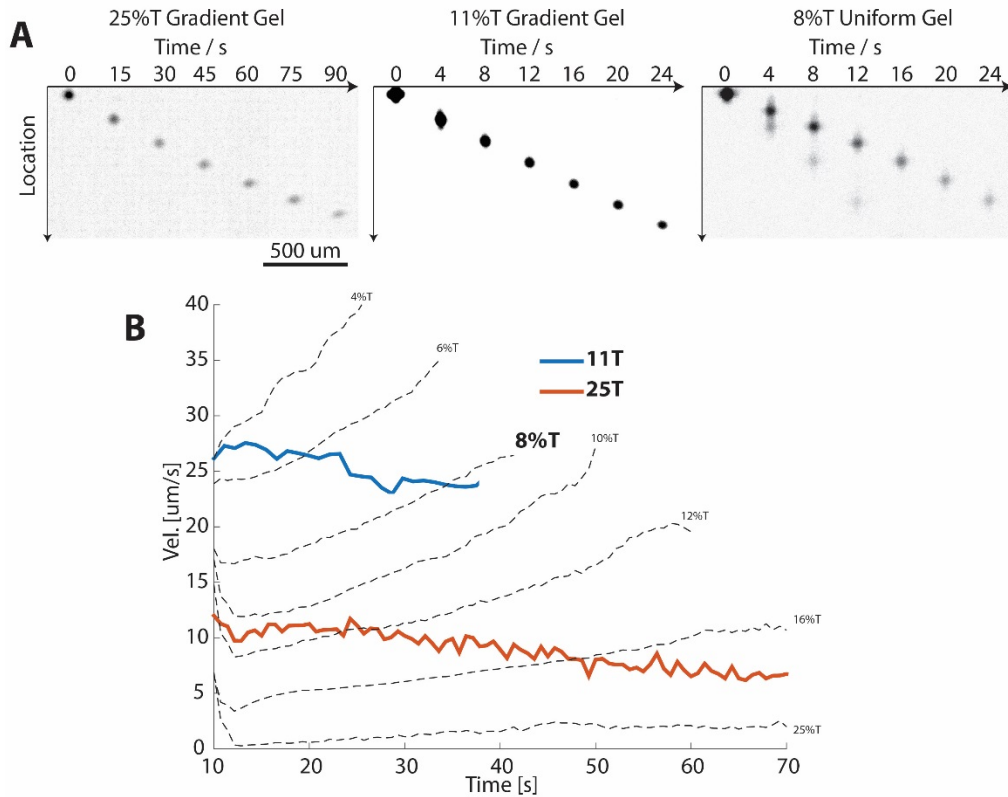


Figure 5.5 U373-GFP Velocity in Gradient and Uniform scWB Platforms. (A) Kymographs display GFP bands for 25%T and 11%T gradient gels and an 8%T uniform gel. From the kymographs, the non-linear velocity is noticeable for the 25%T but less apparent for the 11%T gel. In both gradient gels, axial stacking is apparent from the progressively ellipsoid shaped band as the GFP progresses into the gel density. (B) The velocity over time is plotted for two gradient gel conditions (11%T and 25%T, blue and red, respectively) and a range of chemically polymerized uniform gels (4%T, 6%T, 8%T, 10%T, 12%T, 16%T, and 25%T, dotted black lines). The exponentially increasing velocities for the uniform gels indicate significant joule heating during the separation.

5.4.1. Joule Heating Driven Dispersion

To map out the effective gel density as a function of location for the gradient gels, we need to correct for the impact of joule heating and the resulting temperature increase on velocity. As discussed in Section 1.2.1, the free-solution electrophoretic velocity (U) is inversely proportional to the dynamic viscosity (η) of the solution, $U \propto 1/\eta$. The viscosity of water is known to have an inverse exponential relationship with temperature (T)¹⁷, $\eta \propto e^{-T}$, over the temperature range considered here. We assume a linear relationship between the change of temperature and electrophoresis time (t) with the slope b , $\Delta T = b \cdot t$. This assumption was supported by experimental measurements which are not shown here. From these relationships, we can describe how velocity changes in time using the formula $U = U_0 e^{bt}$, where U is the instantaneous velocity, U_0 is the initial velocity, t is the electrophoresis time. To determine b for our system, we applied an exponential fit to the observed GFP velocities for the uniform gels (4%T, 6%T,

8%T, 10%T, 12%T and 16%T, $n=3$ for each) and found the average b value between the gel densities, $b = 0.021 \pm .003$. Using this extracted parameter, we modified the velocity versus time and velocity versus location plots (figure 5.6A and 5.6B, respectively) by multiplying the observed velocity by e^{-bt} in figures 5.6C and 5.6D. As expected, the heat correction produces largely constant GFP velocities for the uniform gels. With this modification, we can now view the non-uniform velocities in gradient gels and extract the relative sieving matrix impact as a function of location along the gel.

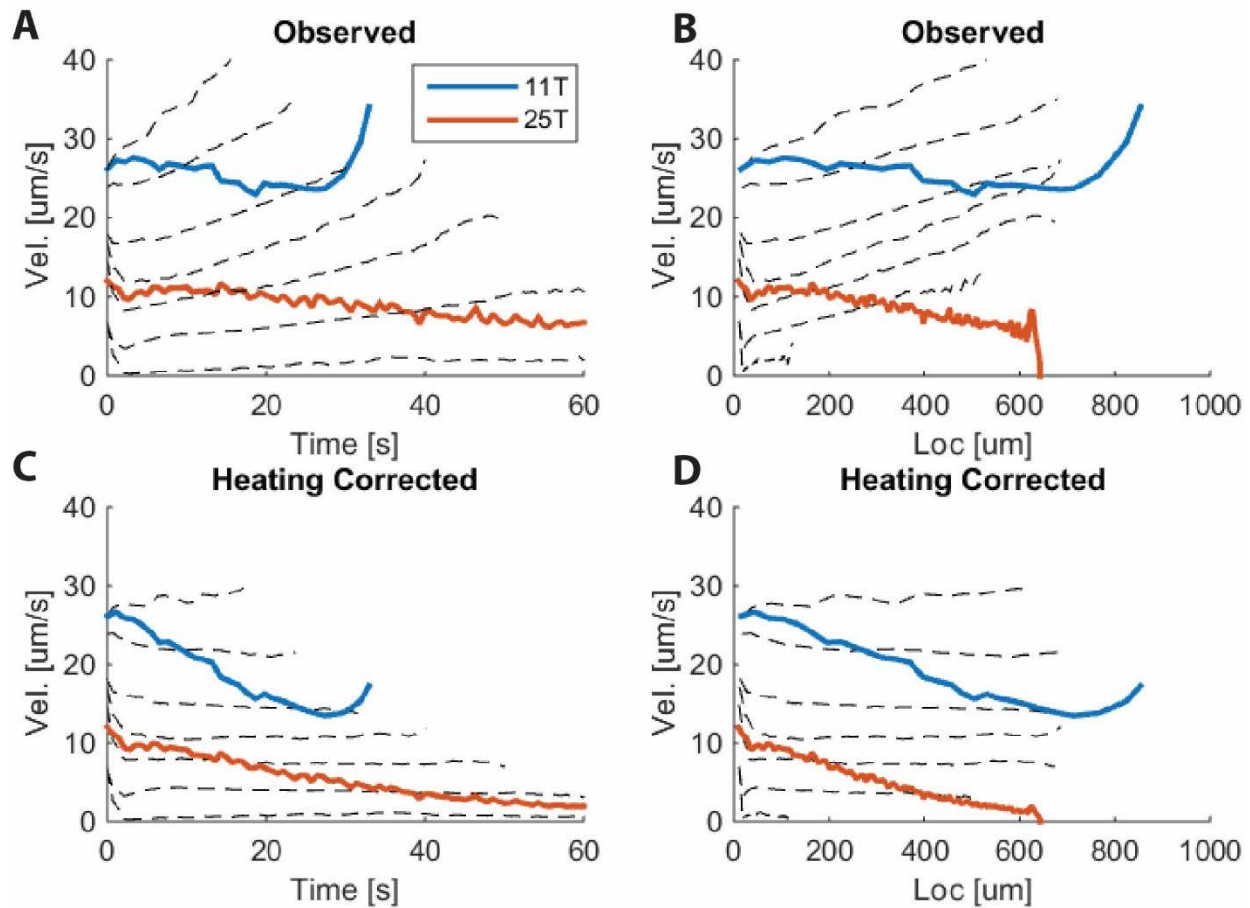


Figure 5.6 GFP Velocity Extracted from the scWB Platform. (A) Velocity versus time and (B) velocity versus location for the gradient gels with 11%T (blue) and 25%T (red) densities and uniform gel (dotted black) with the gel densities 4%T, 6%T, 8%T, 10%T, 12%T, 16%T and 25%T (from the highest to lowest velocities in the plots ordered respectively). In (C) and (D), we applied a heat correction to the velocity (described in Section 5.4.1) to remove the impact of joule heating in velocity. In the heat corrected plots, the uniform gel velocities are practically constant – consistent with their uniform sieving matrix – and the non-uniform velocity in the gradient gels can be clearly observed.

5.4.2. Estimating Effective Sieving Gel Density

To determine the effective gel density from measured velocity, we applied the Ferguson relationship¹⁸ (described in Section 1.3.1), $U = U_0 10^{-kT}$, where k is the retardation constant and U_0 is the free-solution electrophoretic velocity for a given protein. T is the density of a gel sieving matrix and U is the in-gel velocity. To eliminate the impact of joule heating, we used the heat corrected velocities from figure 5.6D for all analysis. The average heat corrected velocities for uniform gels (dotted black lines in figure 5.7A) were used to solve for U_0 and k for the GFP protein in our system. After establishing that relationship, we can describe the relationship between the effective gel density and the heat corrected velocity using Equation 1:

$$\text{Eq. 1} \quad T = \frac{\log_{10}(U_0/U)}{k}$$

We plotted this gel density and velocity relationship from Equation 1 in figure 5.7B for the U373-GFP protein. The corresponding uniform gel densities and the standard deviation of their mean heat corrected velocities are shown ($n=3$). Using this relationship we can solve for the gel density at different locations along the gradient by simply plugging the heat corrected velocity at each location. From this, we can determine that the 11%T gradient gel spans an effective gel density range from 5%T to 9.5%T and the 25%T gradient gel spans an effective gel density range from 10.2%T to 25.5%T. This tool was highly useful in optimizing the gradient gel to achieve the separation of interest.

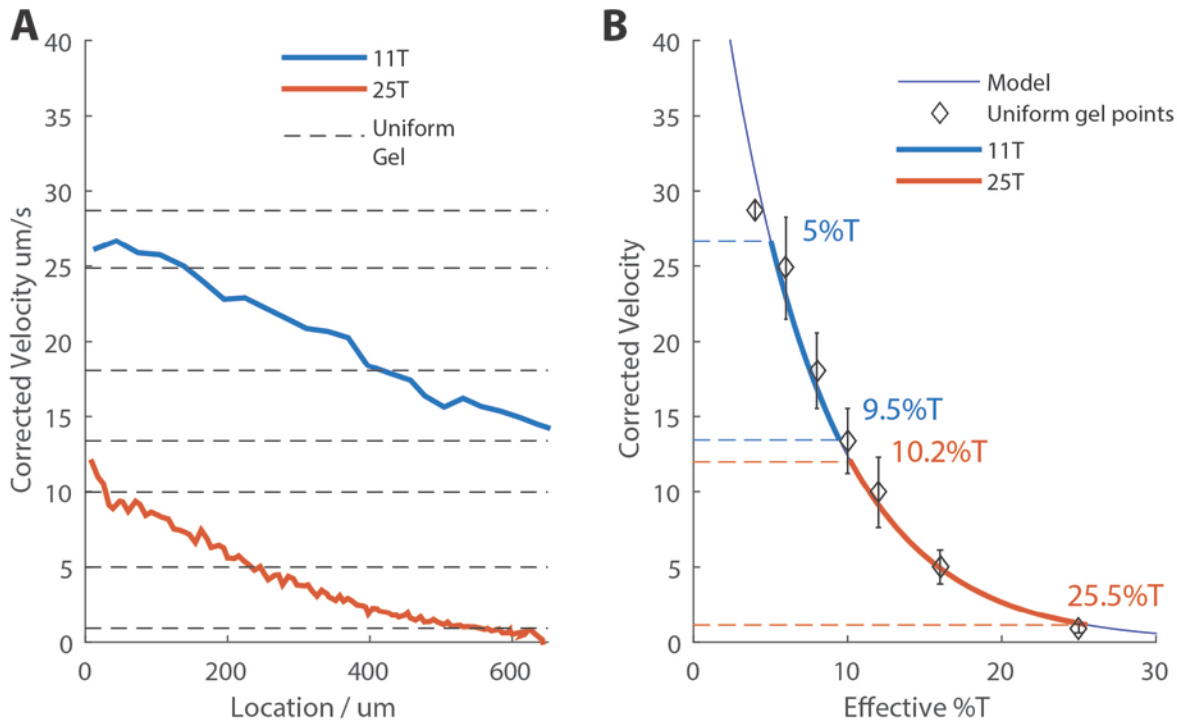


Figure 5.7 The Non-Uniform Heat-Corrected Velocity of GFP Reveals the Effective Gel Density (%T) along Gradient Gels. (A) The mean heat corrected velocities of uniform gels

(dotted black) are plotted with that of the gradient 11%T gradient gel (non-decrosslinking, blue), and 25%T gradient gel (with decrosslinking, red) over the location in the gel. Uniform gel densities included 4%T, 6%T, 8%T, 10%T, 12%T, 16%T and 25%T ($n=3$). (B) The heat corrected velocity versus gel density for U373-GFP migration is shown, from equation 1. This curve was determined using the Ferguson relationship¹⁹ and the velocities extracted from seven uniform gels (black diamond markers). The heat corrected velocity maps out the relative gel density along the axis of the gradient gel as described in equation 1. Using this relationship, we extracted the effective gel density for the 11%T and 25%T gradient gels.

5.5. Molecular Weight Dynamic Range of Gradient Gels

The scWB performance for analyzing proteins with a large molecular weight range differed in gradient and uniform gels. To observe this, we analyzed proteins in the Her2+ human breast cancer cell line, SKBR3. We evaluated four critical proteins in the Her2 signaling pathway that span an order of magnitude in molecular weight: elf4E (25 kDa), ERK (44 kDa), Her2 (185 kDa), and mTor (289 kDa). Separations were conducted in the 11%T gradient gel (shown to have an effective gel density range from 5% to 9.5%T, figure 5.7B) and uniform PAGs (4%T, 8%T and 10%T) in figure 5.8. The electrophoresis times were chosen based on the previous GFP mobility results, in an attempt to allow for injection into the gel of the largest protein, mTor, while preventing the smallest protein, elf4E, from overrunning the array period (1 mm). The electrophoresis times used were 15 s, 8 s, 25 s, and 30 s for the 11%T gradient gel and uniform gel densities (4%T, 8%T, and 10%T), respectively.

The average separation results for two array periods in a slide are shown in figure 5.8A for all gel conditions. Good protein injection is observed for all protein sizes in the 4%T uniform gel and the 11%T gradient gel. In contrast, for the 8%T and 10%T uniform gels, poor injection was observed for large proteins (Her2 & mTor) despite the long the electrophoresis time. This can be seen as the protein bands stuck on the edge of the reservoir and a high background around the reservoir itself. Further, these long electrophoresis times resulted in the elf4E protein bands overrunning the period length and contaminating the sequential separation. Therefore, to achieve a successful injection for proteins with large molecular weights, the gel density needs to be low (e.g. for mTor, < 6%T).

To measure separation performance we performed Gaussian fitting on protein separation results for three slides over all four gel conditions, extracting the mean location and standard deviation of proteins in each. The average Gaussian curves, shown in figure 5.8B, allow us to visualize protein migration patterns and ignore the injection issues with the dense PAGs discussed in the previous paragraph. Figure 5.8C reports the separation resolution (R_s , discussed in Section 1.2.2), for all protein pairs and gel conditions. In the dense gels, good separations were achieved between the smallest proteins ($R_s > 0.8$), elf4E-ERK, while little to no separation was achieved in the large protein pair ($R_s > 0.5$), Her2-mTor - despite the 50% longer separation length due to the array overrun. In contrast, the 4%T maintained injections but poor separation performance for the small and large protein pairs (all $R_s < 0.5$). The 11%T gradient had clean injections, reasonable separation performance for all protein pairs ($R_s > 0.65$), and stayed within the 1 mm gradient array length. Indicating that the gradient scWB expands the platforms compatible to a

broad molecular weight range, making it compatible to for a clean injection and sizing analysis of nearly 90% of the human proteome (figure 5.1A).

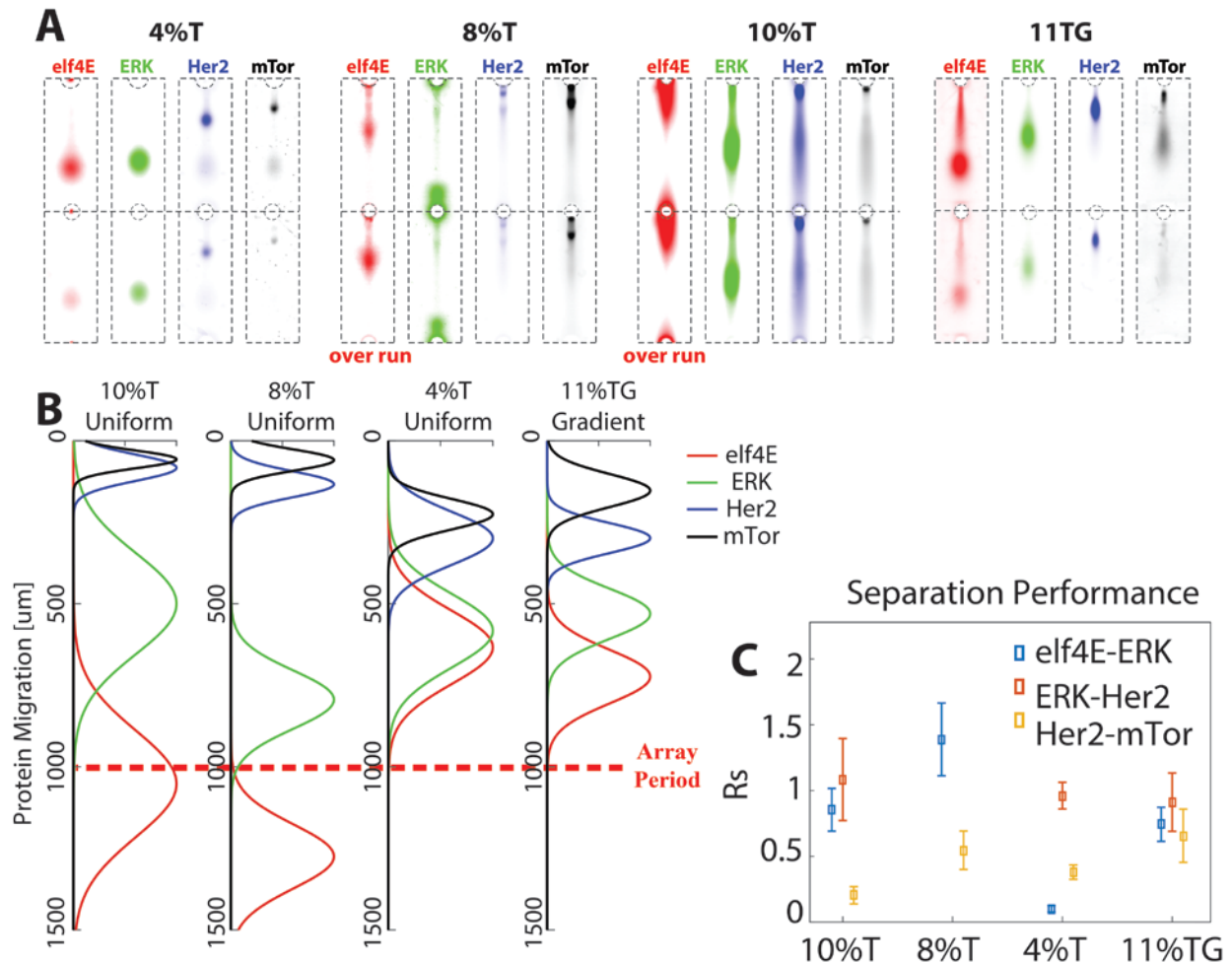


Figure 5.8 Gradient single-cell westerns achieves separations of four protein in 1 mm separation that vary from 25 to 289 kDa in size. (A) Average immune-probed images from a single slide are show for four protein targets separated in the 11%T gradient gel and 4%T, 8%T and 10%T uniform gels. The protein targets include elf4E(25 kDa, red), ERK (44 kDa, green), Her2 (185 kDa, blue) and mTor (289 kDa, black). All proteins are injected for the 4%T gel but it has but poor separations. For the 8%T and 10%T uniform gels, the gel is too dense for the large proteins to be injected. And the long electrophoresis time required to inject mTor and Her2 results in the smaller proteins overrunning the array and contaminating the next separation. Using the gradient gel, the four species are distinctly separated along the axis with in the 1 mm period of the array. (B) Average protein locations and standard deviations are shown for a one and a half array periods. The gradient gel is porous enough at the entry of the gel to allow entry for mTor and Her2, the gradually increasing gel density achieves separations for both the elf4E-ERK and the Her2-mTor separations. (C) The separation resolution (R_s) is shown for all protein pairs and gel condition. The gradient gel is the only condition that enables an $R_s > 0.65$ for a protein separations across the order of magnitude range in molecular weight.

5.6. Primary HER2+ Breast Cancer Tissue Analysis

Fresh primary HER2+ breast cancer tumor samples were analyzed in the gradient scWB platform to investigate the cell-to-cell heterogeneity in a patient samples. Previous researchers in our lab have shown that primary samples are more difficult to lyse than cell lines. In response, we adopted a harsher lysis buffer with 1% SDS and 0.5% TritonX-100, but continued to use the 25 second lysis time and 15 second electrophoresis times at 40 V/cm. Due to the small tumor size only 32 cells were settled into the micro-wells and analyzed. Each cell was probed for elf4E, ERK, Her2, mTor, and, as a loading control, bTub. To assess how protein expression was related across cells, we created a heat map of protein expression in figure 5.9A for all 32 cells. Proteins for each cell were first normalized by the bTub in the same cell. Then, proteins were normalized to their own maximum value. Setting the relative abundance for all proteins between 0 and 1. The heat map was organized by Her2 relative expression, high expression on the left (white) to low expression on the right (black).

Figure 5.9B displays the five protein separations for three cells at different points along the Her2 expression spectrum. These cells were chosen to illustrate both the dynamic range of Her2 and because their bTub expression varied by less than 20%, such that protein expression could be qualitatively compared to one another. The image contrast for the same proteins is constant. In figure 5.9C we show the relative protein abundance for Her2, mTor, ERK and elf4E. From this preliminary dataset, elf4E and mTor expression trend downward with a decreasing abundance of Her2. In contrast, ERK does not show a trend with Her2 expression. The most striking aspect of this data is the dynamic range of Her2 expression, which is reduced by 50-times from the most abundant to least abundant cells. In contrast, mTor, ERK and elf4E show a maximum expression range of 7-, 5-, and 4-times range across all 32 cells.

These results are preliminary and require a significant increase in replicates before solid conclusions can be formed. The population analysis shows the potential of the scWB platform for analyzing cell-to-cell protein expression heterogeneity in patient samples for proteins that range over an order of magnitude in molecular weight.

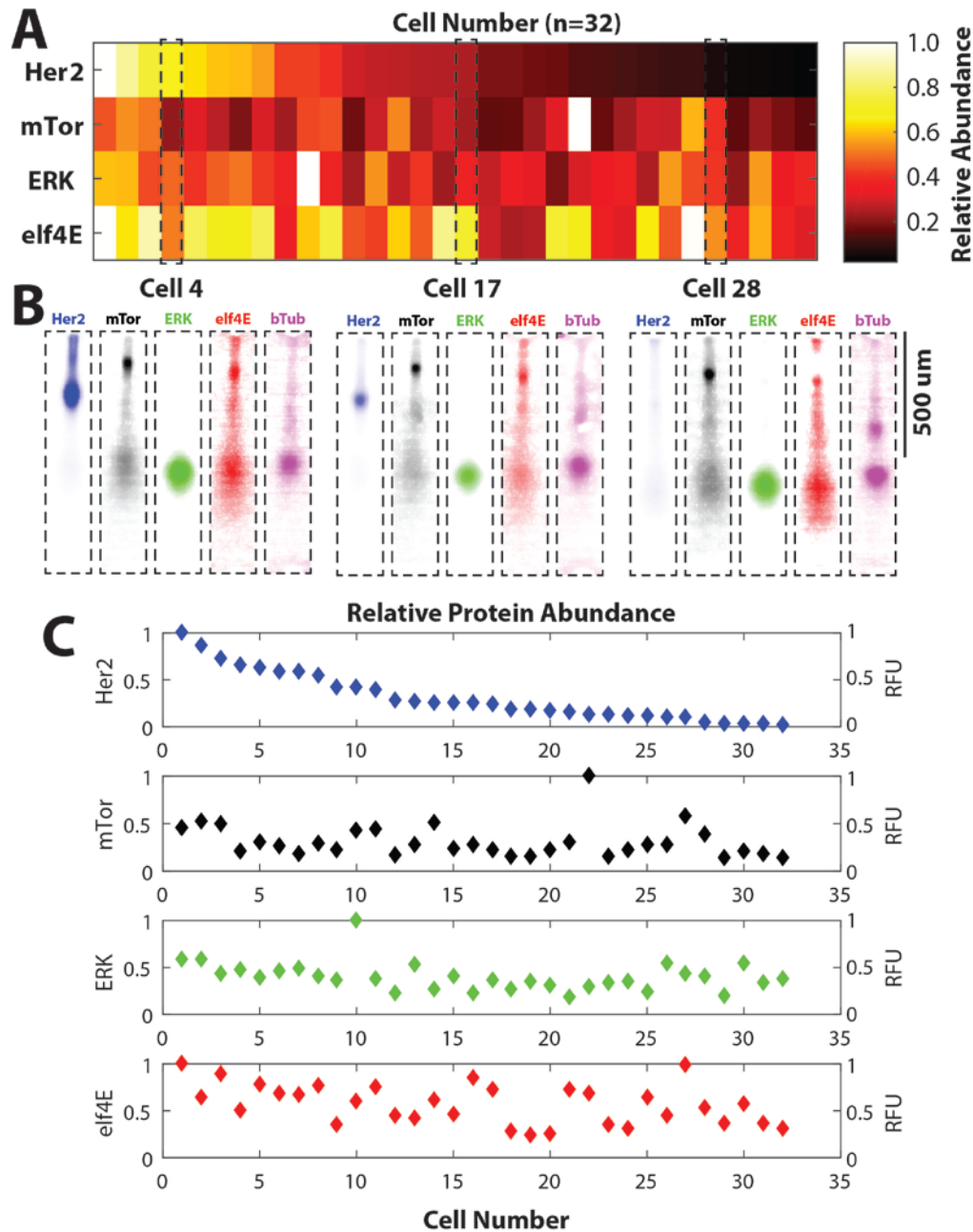


Figure 5.9 Gradient scWB Analysis of Primary Her2+ Breast Cancer Tissue Reveals an Order of Magnitude Variation in Her2 Expression. (A) Heat map of relative protein expression for Her2, mTor, ERK, and elf4E. Protein abundance was normalized by a loading control, bTub, and the maximum abundance for each protein. The heat map is organized by Her2 expression with high expression on the left (white) and low expression on the right (black). (B) Three separations are shown at a high, medium and low Her2 abundance. The contrast is constant for each protein, and the cells have similar bTub levels so that the separations can be compared qualitatively. (C) The relative protein abundance is shown for all 32 cells and protein targets. A slight downward trend can be seen for mTor and elf4E, but no trend is observed for ERK.

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Pore-Expansion of Polyacrylamide Gels for Large Reagent Delivery

6.1. Introduction

The unique physical advantages of microfluidic separations (discussed in Section 1.1.1) help to minimize dispersion (injection and diffusion based) to enable rapid high-performance separations over short separation distances. One challenge of using microfluidic separations is the post separation analysis. Due to the short separation distance between analytes, the separation performance can be quickly lost with dispersion after the separation, either through diffusion or other forms of dispersion (e.g. a transfer step^{1, 2}). Recently, to mitigate loss of separation resolution, researchers have adopted an alternative approach – photo-initiated immobilization of the separated species^{3, 4}. Photo-initiated capture (e.g. using a benzophenone capture agent⁷) offers the ability to immediately, and with spatial uniformity, immobilize analytes after the separation is completed. This approach has been utilized to realize automated high-throughput microfluidic immunoblotting for measuring protein size^{8, 9} or charge^{3, 4, 10, 11}.

For the high-efficiency photo-capture of species our group utilizes a functionalized hydrogel for immobilization of analytes within its aqueous volume. The volume-based capture³ has significantly higher capture efficiency than surface-capture⁴ strategies due to the reduced diffusion distances between immobilizing moiety and analytes. To further streamline the integration between separation and subsequent immobilization, the hydrogel can be used as the sieving matrix for performing protein sizing and the blotting membrane for immobilizing proteins. This low-loss integrated hydrogel system is essential for analyzing low abundance samples, for example single cell protein measurements in the single cell western blotting (scWB) platform. In scWB, proteins are fractionated from single cells, immobilized, and then probed with antibodies all within the same hydrogel. A drawback of complete assay integration is that each procedure has drastically different optimal conditions. Therefore a compromise in hydrogel composition is chosen between all three functions – each performing sub-optimally.

This chapter focuses on alleviating the tradeoffs between separation and antibody probing performance. Increased gel densities can enable high-resolution separations over short separation lengths (Section 1.2.4). Unfortunately, the maximum gel density that can be used is restricted by the accessibility of antibodies entering the gel - a considerable limitation due to their 150 kDa size. For antibody probing with active transport¹⁷, dense gels will lead to high background of non-specific antibodies stuck in the gel pores. In diffusive antibody probing¹⁸, antibodies are significantly partitioned out of dense gels such that the effective in-gel concentration can be orders of magnitude less than the in-solution antibody concentration, the partitioning factor scales with gel density¹⁹. To counter-act partitioning in the scWB assay, high-antibody concentrations are used and as a result antibody consumption is the expensive portion of the scWB assay workflow. In this work, we sought to develop an active hydrogel matrix that could maintain a dense form to achieve a high-resolution protein separation and immobilization, which then after a simulation, could transition to a lower-density form that is more accessible to antibody probing and the delivery of other large-reagents.

6.2. Pore-Expansion Design Rationale

Numerous strategies have been reported to accomplish pore-expansion. Commonly, reversible hydrogel swelling is controlled with temperature^{20, 21}, ionic strength²², or other solvent conditions²³. The reversible swelling approach is undesirable for our system for two reasons. First, shrunken gels are typically hydrophobic which could lead to protein adsorption during the separation stage. And second, the specific solvent or other conditions required for maintaining a swollen gel may impact the antibody affinity. Alternatively, we sought to develop a platform capable of irreversible pore-expansion that could be achieved through a transient stimulation. Our goal was to de-convolve the separation matrix from the probing matrix to allow for improved separations and subsequent probing²⁴.

An initial approach was to create a composite thermoset/thermoplastic hydrogel – specifically, a PAG/agarose composite gel^{25, 26}. After photo-initiated protein-immobilization to the PAG, the agarose was dissolved away at an elevated temperature. While this method showed some promise, the degree of pore-expansion was limited and the thermoset dissolution was a relatively slow process.

In a different approach, a dual crosslinked PAG with labile and stable crosslinkers was used. After protein immobilization, the labile crosslinkers were broken and the PAG expanded considerably due to the reduced crosslinking between fibers. This technique offers a significant transition in pore-size and can be accomplished in minutes. We demonstrate how partial gel decrosslinking can be used to enhance antibody probing in dense polyacrylamide gels, eliminate probing bias in single cell gradient western blotting (scGWB), and enable the introduction of large detection reagent into the gel - opening the possibility for new high-sensitivity protein detection strategies.

6.2.1. Selecting a Labile PAG Crosslinker

Decrosslinking strategies to solubilize PAGs have been around since 1976^{5, 27}. Typically after PAGE, a section of the gel containing the protein of interest is excised and isolated in a micro-centrifuge tube, then the gel is solubilized and protein of interest is extracted. Slab gels with a base-sensitive decrosslinker have been used to enhance the electrophoretic transfer of large proteins from a slab gel onto a blotting membrane²⁸. Composite crosslinked gels with a stable, methylene bisacrylamide (BIS), and labile crosslinker to expand PAG pore-size have been used to enhance peptide extraction for mass spectroscopy analysis²⁹. Similarly, for our application we sought to create a composite crosslinked PAG.

Researchers use both commercially available labile crosslinkers and synthesize their own with unique properties³⁰. The various labile crosslinkers we considered adopting are summarized in table 1. Commercially available examples include N,N'-(1,2-dihydroxyethylene) Bisacrylamide (DHEBA), N,N'-(diallyltartardiamide) (DATD), Ethylene Glycol Diacrylate (EDA) and N,N'-bisacrylylcystamine (BAC)³⁰. Researchers have synthesized N,N'-(7,7-dimethyl-3,6,8,11-tetraoxatridecane-1,13-diyl)diacrylamide (DOK)¹⁴ and N,N'-[(1-methylethylidene)bis(oxy-2,1-ethanediyl)]diacrylamide (DK)¹⁶ labile crosslinkers.

Table 1: Properties for a selection of common labile acrylamide crosslinkers for gel solubilization.

Acronym	Full Name	Dissolution Treatment	Reaction	Dissolution Kinetics	Solubility ⁺ (mol/L)	Irreversible	Non- Reactive Product	Chain Length	Molecular Weight
DHEBA ^{5,6}	N,N'-(1,2-dihydroxyethylene) Bisacrylamide	Periodic Acid	Periodate oxidation	Hours	0.14	Yes	Yes	10	200.19
DATD ⁵	N,N'-(diallyltartardiamide)	Periodic Acid	Periodate oxidation	Hours	0.26	Yes	Yes	12	228.25
BAC ¹²	N,N'-bisacrylylcystamine	Reducing Agent	Disulfide cleavage	Minutes	0.017	No	No	14	260.38
EDA ¹³	Ethylene Glycol Diacrylate	Base	Basic ester hydrolysis	Minutes	0.072	Yes	No	10	170.16
DOK ^{14,15}	N,N'-(7,7-dimethyl-3,6,8,11-tetraoxatridecane-1,13-diy)diacrylamide	Acid	Acidic acetyl hydrolysis	Minutes	0.073	Yes	Yes	21	358.43
DK ¹⁶	N,N'-[(1-methylethylidene) bis(oxy-2,1-ethanediy)] diacrylamide	Acid	Acidic acetyl hydrolysis	Minutes	0.042	Yes	Yes	15	270.32

Note⁺: Solubility was computationally estimated (Advanced Chemistry Development, Inc.).

Chapter 6

We considered the following criteria when selecting a labile crosslinker, listed in the order of importance:

1. Stable in common cell and electrophoresis buffers, for example in phosphate buffered saline (PBS, pH 7.4) and tris-glycine (pH 8.4).
2. Dissolution treatment does not damage protein-antibody epitopes.
3. Irreversible reaction.
4. Neutral, non-reactive product after decrosslinking.
5. Fast reaction, completed in less than one hour.
6. Minimal bond chain length, to maximize pore-size transition.

The commercially available labile crosslinker that best suited our objectives was EDA. While the composite crosslinked EDA/BIS gels showed rapid and significant pore expansion, the base hydrolysis of the ester produced an acetic acid product incorporated into the gel. The resulting charged gel responds with significant gel distortion under the presence of an electric field³¹, thereby ruling out the active transport method of antibody delivery, which we are interested in employing. The EDA/BIS gel testing is described in Appendix F.

The DOK crosslinker had been previously used in PAGE for the enhanced recovery of intact proteins and nucleic acids after a separation^{15, 32}. The acid hydrolysis of the ketal group is a rapid reaction at low pH values that results in an inert ketone product¹⁴. The ketal group should be relatively stable for days in the neutral to basic buffers used for electrophoresis¹⁶. This crosslinker meets the critical aspects of our assay the only drawback being the relative length of the DOK labile crosslinker (21 bond chain length) compared to the BIS stable crosslinker (9 bond chain length). We suspected that the pore-expansion would be limited when labile crosslinker is significantly longer than the stable crosslinker. As the stable crosslinker will maintain gel shape after decrosslinking and minimize the pore-expansion impact.

Alternatively we synthesized the DK crosslinker^{16, 33, 34}, which holds the same advantages as the DOK molecule but had a 15 bond chain length. We hypothesized the shorter chain length would enhance pore-expansion performance in comparison to DOK, although this was not directly tested. One possible drawback of DK over DOK is that the molecule is more hydrophobic, which could impact its effective incorporation rate during free-radical polymerization¹².

6.3. Diacrylamide Ketal PAG

The diacrylamide ketal used throughout this work was synthesized by our collaborator Dr. Santanu Maity, in Professor Niren Murthy's research laboratory at UC Berkeley. The synthesis protocol is shared in Protocol C.

Lyophilized DK product (figure 6.1A, 270.32 kDa molecular weight) was dissolved in a tris caps buffer pH 9.6 at a concentration of 500 mM at room temperature. Dissolution of the mixture was accelerated with a vortex mixer on high speed for five minutes. The final solution, shown in figure 6.1B, had a yellow color. The solution was placed in aliquots at -20°C for storage. Initially to test DK functionality, 100% DK crosslinked PAG was fabricated with 15%T, 5.82%C, 1% (w/v) VA086 photoinitiator, in a tris glycine buffer. The precursor solution was exposed for 30

seconds at 20 mW/cm² power. The resulting gel structure consisted of four 5 mm x 5 mm x ~300 µm height gel pieces attached to GelBond® as shown in figure 6.1C.

Previously Rachna Jain et. al. demonstrated that the half-life of DK was 0.03 days at pH 5 and 6 days at pH 7.4 (at 35°C)¹⁶. To qualitatively confirm decrosslinking functionality, four solutions of tris glycine were titrated to pH values of 8.3, 7.1, 2.6 and 0.07, with the addition hydrochloric acid. A piece of DK crosslinked PAG was placed into each of the separate solutions to assess dissolution at room temperature. Gels were periodically removed and photographs were taken to qualitatively assess how much gel remained. The gels soaking in 2.6 pH and 0.07 pH Tris Glycine were dramatically affected in two minutes, and completely dissolved in 5 minutes. The gels soaking in 8.3 pH and 7.1 pH were not visually affected even after overnight incubation. While highly qualitative, these results indicated that the DK molecule was as we anticipated in terms of photo-polymerization and decrosslinking, and we could proceed to studying composite crosslinked PAG.

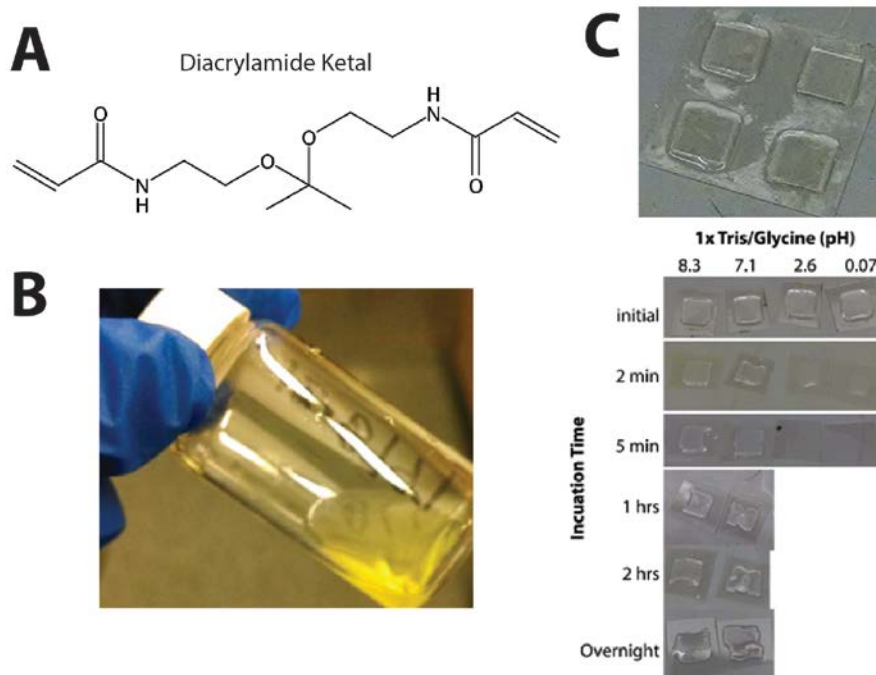


Figure 6.1 Diacrylamide Ketal (DK) (A) PAGs are compatible with conventional PAG polymerization protocols and solubilize quickly in the presence of an acid. (A) DK molecular structure. (B) DK solution at 500 mM has a yellow hue. (C) Photo-patterned 100% DK *fs*PAG structures were used to qualitatively assess decrosslinking in acidic conditions. The *fs*PAG morphology was monitored for the DK PAGs in tris glycine solutions titrated to 8.3, 7.1, 2.6 and 0.07 pH at time points 2 minutes, 5 minutes, 1 hour, 2 hours, and overnight (at room temperature). For gel structures in the 2.6 pH and 0.07 pH, complete dissolution was observed in the first five minutes.

6.4. Composite Crosslinked Diacrylamide Ketal PAGs

Substitution of crosslinkers in PAG was performed at a mol per mol basis, which is the more comparable substitution³⁵ than on a gram per gram basis. For each 1 mg BIS we substituted 1.75 mg of DK (molecular weights 154.17 Da and 270.32 Da for BIS and DK, respectively). The descriptions for PAGs are typically mass based. The gel density, %T, is the mass percentage of all the monomers in the precursor solution. Gel crosslinking is described by %C, which is the ratio of the crosslinker mass to the all monomers in the gel. As BIS is the predominant crosslinker used for PAGs, we sought to maintain the effective BIS PAG %T and %C so that our experimental conditions would be comparable. We defined as %C* as the BIS effective %C, in equation 1. Similarly we defined %T*, such that the changing the ratio of DK and BIS did not impact the total amount of linear acrylamide in the PAG. Equation 2 defines %T*.

$$\text{Eq. 1} \quad \%C^* = \frac{BIS_{mg} + \frac{154.17}{270.32}(DK_{mg})}{Acrylamide_{mg} + BIS_{mg} + \frac{154.17}{270.32}(DK_{mg})} * 100$$

$$\text{Eq. 2} \quad \%T^* = \frac{Acrylamide_{mg} + BIS_{mg} + \frac{154.17}{270.32}(DK_{mg})}{Volume_{\mu L}} * 100$$

The design values for %T* and %C* in all gels tested in this chapter, the corresponding masses in the precursor solution and their actual %T and %C values are all shown in table 2. For each gel composition, we described the molar percent of DK crosslinker as: %DK = $100 * DK_{mol} / (DK_{mol} + BIS_{mol})$.

Table 2: PAG composition in composite crosslinked gels for all gels discussed in this chapter. The metric %C used to describe the crosslinking of a gel is defined as the ratio of the mass of the crosslinker to the total monomer mass in the gel (including the crosslinkers). The effect of crosslinking in a gel is more closely related to the number of crosslinks (i.e. mols of crosslinker) as opposed to the mass of a crosslinker³⁵. We maintained the acrylamide monomer content such that for a given %T, a BIS gel and a DK gel would have the same amount of acrylamide monomer. We refer to these design values, %T* and %C* (equation 1 and equation 2, respectively), throughout this chapter. The actual %T and %C are shown below.

<u>Design Values</u>		Molar Cross-linker Percent	Volume	Acrylamide	BIS	DK	<u>Real Values</u>	
%T*	%C*						%T	%C
16	5	100	1000	152.00	0.00	14.00	16.60	8.43
6	6	99	1000	56.40	0.04	6.24	6.27	10.01
12	6	99	1000	112.80	0.07	12.47	12.53	10.01
16	6	99	1000	150.40	0.10	16.63	16.71	10.01
18	6	99	1000	169.20	0.11	18.71	18.80	10.01
20	6	99	1000	188.00	0.12	20.79	20.89	10.01
12	6	98	1000	112.80	0.14	12.35	12.53	9.97
16	6	98	1000	150.40	0.19	16.46	16.71	9.97
18	6	98	1000	169.20	0.22	18.52	18.79	9.97
20	6	98	1000	188.00	0.24	20.58	20.88	9.97
16	6	90	1000	150.40	0.96	15.12	16.65	9.66
11	3.33	0	1000	106.34	3.66	0.00	11.00	3.33
12	3.33	0	1000	116.00	4.00	0.00	12.00	3.33
10	3.33	0	1000	96.67	3.33	0.00	10.00	3.33
8	3.33	0	1000	77.34	2.66	0.00	8.00	3.33
6	3.33	0	1000	58.00	2.00	0.00	6.00	3.33

To determine the kinetics of DK decrosslinking when polymerized into a PAG, we evaluated the mechanical properties of a DK/BIS gel after exposure to buffers of varying acidity. 16%T* 6%C* 98%DK composite crosslinked gels were fabricated as 8 mm diameter, 300 μ m thick discs such that they would be compatible with an MCR Rheometer (Anton Paar). The storage and elastic modulus was measured at 1 Hz for gels exposed to tris caps, 1% HCl, 0.1% HCl, 0.01% HCl, and PBS (pH values of 9.6, 1.1, 2.2, 3.6, and 7.4, respectively) after 10 minutes and again after 1 day of exposure. The results, shown in figure 6.2A, indicate that the vast majority of PAG decrosslinking occur in the first 10 minutes of exposure to 1% HCl. Therefore we used between 10 minutes and 30 minutes exposure to 1% HCl for all decrosslinking steps.

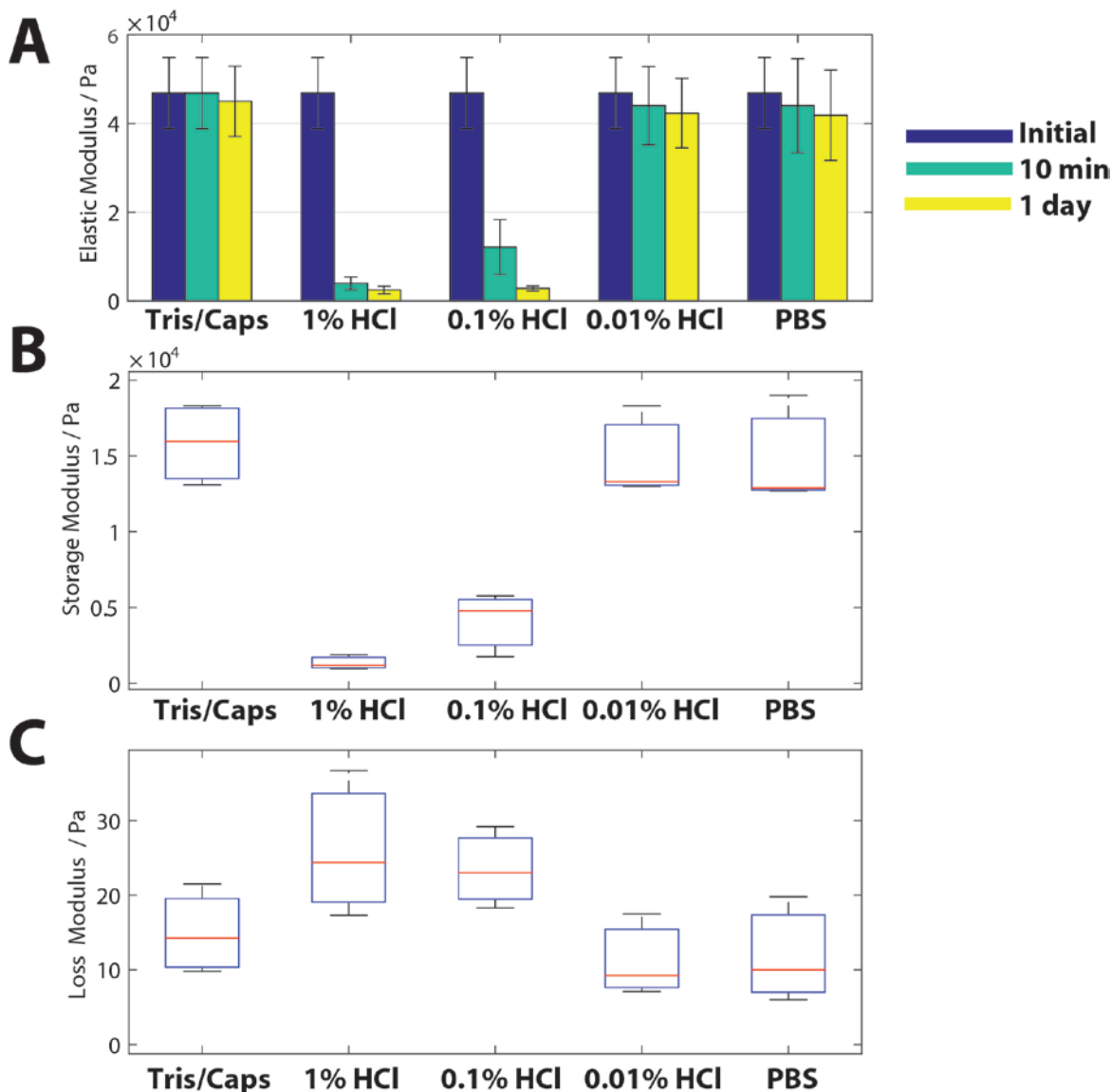


Figure 6.2 Decrosslinking Kinetics of DK in composite PAGs is largely completed in 10 minutes for the 1% HCl solution. Composite DK and BIS crosslinked PAGs with 16%T*, 6%C*, and a 98% DK crosslinker molar abundances were fabricated in the shape of small gel discs with an 8 mm diameter and a 300 μ m thickness. Gel discs were placed in solutions of tris caps, 1% HCl, 0.1% HCl, 0.01% HCl, and PBS which had pH values of 9.6, 1.1, 2.2, 3.6, and 7.4, respectively. (A) The elastic modulus for the PAG after exposure to the various buffers for 10 minutes and 1 day. The initial elastic modulus for all conditions stiffness was determined as the initial measurement done for the tris caps gels. For tris caps, PBS, and 0.01% HCl the gel is stable over the course of day. In contrast exposure to 1% and 0.1% HCl resulted in a dramatic shift in average elastic modulus from 45 kPa to 2.5 kPa and 2.8 kPa, respectively. In just 10 minutes of exposure, the majority of gel alteration was completed for 1% HCl condition which had average elastic modulus of 4.0 kPa. (B) The storage and (C) loss modulus for the various conditions after a 10 minute exposure. The increased loss modulus for the 1% HCl and 0.1% HCl is consistent with a highly disordered and poorly crosslinked PAG that we would expect after decrosslinking DK.

6.4.1. Probing Efficiency in Decrosslinked Composite Gels

We adopted the composite crosslinked PAGs to scWB assay. The fabrication and operation of the scWB platform is described in Section 5.2, we describe the antibody probing procedure below. Initially we tested a 16%T* 6%C* 98%DK PAG using GFP transfected MCF7 cells. After UV immobilization, the gels were scanned for their GFP signal. This step must be performed before acid exposure, which eliminates GFP fluorescence. Gels were either decrosslinked in 1% HCl for 10 minutes or left soaking in PBS (control). After decrosslinking, gels were allowed to equilibrate in TBST for 15 minutes. The probing with a primary antibody was performed in TBST with 2% BSA in a 1:20 dilution anti-GFP (ab6673) for 2 hours. A 1 hour wash is then performed on the shaker. Probing with a secondary antibody at a 1:20 dilution of secondary antibody (A21432, anti-goat Alexa Flour 565) was performed for 1 hour, and then was followed by another 1 hour wash. Afterwards the gels were scanned on a GenePix 4300A.

For the 16%T* 6%C* 98%DK PAG shown in figure 6.3A no antibody signal was observed for the non-decrosslinked gel. In contrast, significant probing was observed in the decrosslinked gel. We quantified the probing efficiency, defined as the antibody signal normalized by the GFP signal for each cell, for 12%T*, 16%T*, and 18%T* in figure 6.3B. We observed that despite decrosslinking dense gels – there still existed a gel density probing bias, increasing as gel density is reduced. To determine the optimal %DK, we fabricated 16%T* 6%C* gels, which varied from 90%DK, 98%DK, 99%DK, and 100% DK, and tested their respective probing efficiency. The resulting probing efficiency, displayed in figure 6.3C, showed an order of magnitude increase from 90%DK to 99%DK, which was found to be the maximum. The 100%DK had a lower probing efficiency than the 99%DK test case - we hypothesized that this is caused by diffusive losses of GFP which are no longer covalently bound to the hydrogel backbone.

Interestingly, the 100%DK case did not completely solubilize, as we previously observed in figure 6.1C. The critical difference is the substrate the PAG was polymerized on, which was GelBond[®] and methacrylate functionalized glass slides, and their thickness, ~300 μ m and 30 μ m, shown in figure 6.1C and figure 6.3C, respectively. While more testing is necessary, the 100% DK gel may be entangled linear acrylamide chains still bound to the glass surface. This could be highly advantageous for pore-expansion if an alternative immobilization strategy for proteins could be achieved, such that they were not released after decrosslinking.

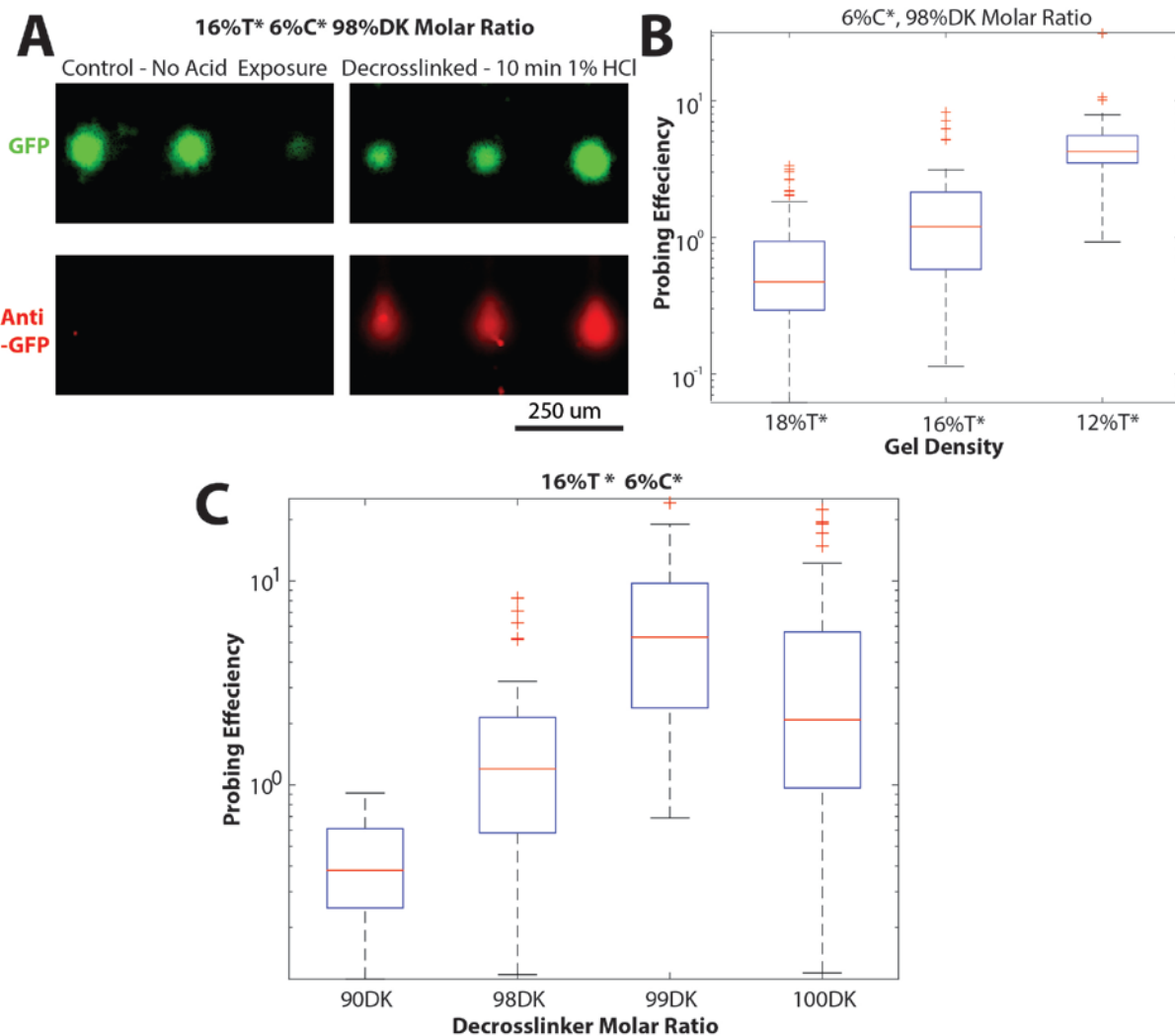


Figure 6.3 Decrosslinked composite PAG enables probing in dense gels that are not accessible without decrosslinking. (A) The scWB assay was run using a DK and BIS crosslinked 16%T*, 6%C* and 98%DK PAG for MCF7-GFP expressing cells. The GFP (green) and probed-GFP (red) signals are displayed for a decrosslinked and a control gel for three adjacent wells that contained cells. The microarray scanning conditions and image contrasts are constant for each color channel. Clear antibody probing is seen in the decrosslinked gel, while no signal is observed in the control. (B) The probing efficiency, defined as the fluorescent integral of the antibody probed signal divided by the GFP signal, was evaluated for decrosslinked gels with the densities 12%T*, 16%T* and 18%T*. We observed a decrease in signal with an increasing gel density, which was not unexpected. (C) To determine the optimal decrosslinking ratio for antibody probing we tested 16%T* PAGs with 90%, 98%, 99% and 100% DK crosslinker abundances shown in a Box plot. The maximum probing efficiency was determined to be 99% DK, which we adopted for all conditions gel compositions going forward. To our surprise, the 100% DK crosslinked gels were not completely dissolved in the acid treatment. This may be related to side reactions during polymerization or impurity in our DK sample, this requires further investigation. We hypothesize that the lower probing efficiency observed in the

100% DK gel is caused by diffusive loss of GFP after decrosslinking has broken their connection with the gel matrix.

6.4.2. Single Cell Gradient Western Blotting

To evaluate the impact of gel density on probing efficiency without decrosslinking, we measured uniform PAG with 6%T, 8%T, 10%T and 12%T gel densities, 3.33%C with 100% BIS. The U373-GFP was probed with antibody dilutions of 1/10 and 1/20 in figure 6.4A. As gel density is increased two-fold, from 6%T to 12%T, probing efficiency plummets two orders and three orders of magnitude for the 1/10 and 1/20 antibody dilutions, respectively. This is of particular importance for gradient gels (Chapter 4 & 5), which have a spatial variation in gel density along the separation axis. Along with the non-uniform gel density comes biased antibody probing for proteins at different locations along the gradient. We tested probing uniformity with the 11%T gradient gel, previously used Section 5.5 for a large molecular range protein separations. GFP was electrophoresis for different times – 10s, 15s, 20s and 30s – to sample different regions of the gradient and then immobilized. Their respective migration distances are shown in figure 6.4B. Probing efficiency was evaluated for each location along the gel in figure 6.4C (1/10 primary antibody dilutions were used). As expected, the probing efficiency reduces as proteins advanced into denser regions of the gel. Along the 11%T gel, it reduced exactly an order of magnitude from a 13.6 probing efficiency to 1.36.

To address this issue we fabricated a similar gradient gel with 99% DK. Specifically, a 12%T* 6%C* gel polymerized at 26.5 mW/cm² (measured prior to long-pass filter) for 140 seconds through the 70% to 1% grayscale mask. The resulting gel had an effective gel density range of 4.7%T to 8.5%T, as compared a 5.2%T to 9.1%T for the 11%T gradient gel (method of estimating effective %T is described in Section 5.4.2). The 12%T* 6%C* 99%DK gradient was analyzed similarly to the 11%T in figure 6.4. In contrast to the 100% BIS gel, the probing efficiency along the 99%DK gel was not significantly varied finding the initial efficiency of 2.69 at the beginning of the gel and 2.33 at the end of the gel. The decrosslinking step eliminates probing bias for the scGWB platform. The average antibody signal is shown in for the 11%T and 12%T* gradients are shown in figure 6.4D and 6.4E, respectively.

To test a more difficult gradient condition, we fabricated a gradient gel that is not accessible to antibody probing at any location along its separation axis, shown in figure 6.5. The 20%T* 6%C* 99%DK* gel had an effective gel density range from 12.4%T to 21.3%T. We electrophoresed U373-GFP for 30 s, 50 s, and 75 s. Antibody probing in a decrosslinked and non-decrosslinked gel in figure 6.5A demonstrate that excellent antibody probing can be achieved in the decrosslinked gel and no signal is seen for any electrophoresis time in the control case. These preliminary results (n=1, technical replicates for each condition), show a similar probing efficiency across the gradient and suggest that axial bias does not exist. More experiments on this dense gradient gel condition will be required to confirm if probing bias is truly eliminated. This result suggests that this technology could be used to resolve small proteins that require a dense sieving matrix (e.g. 6 – 20 kDa) and have not previously been detectable in the scWB platform.

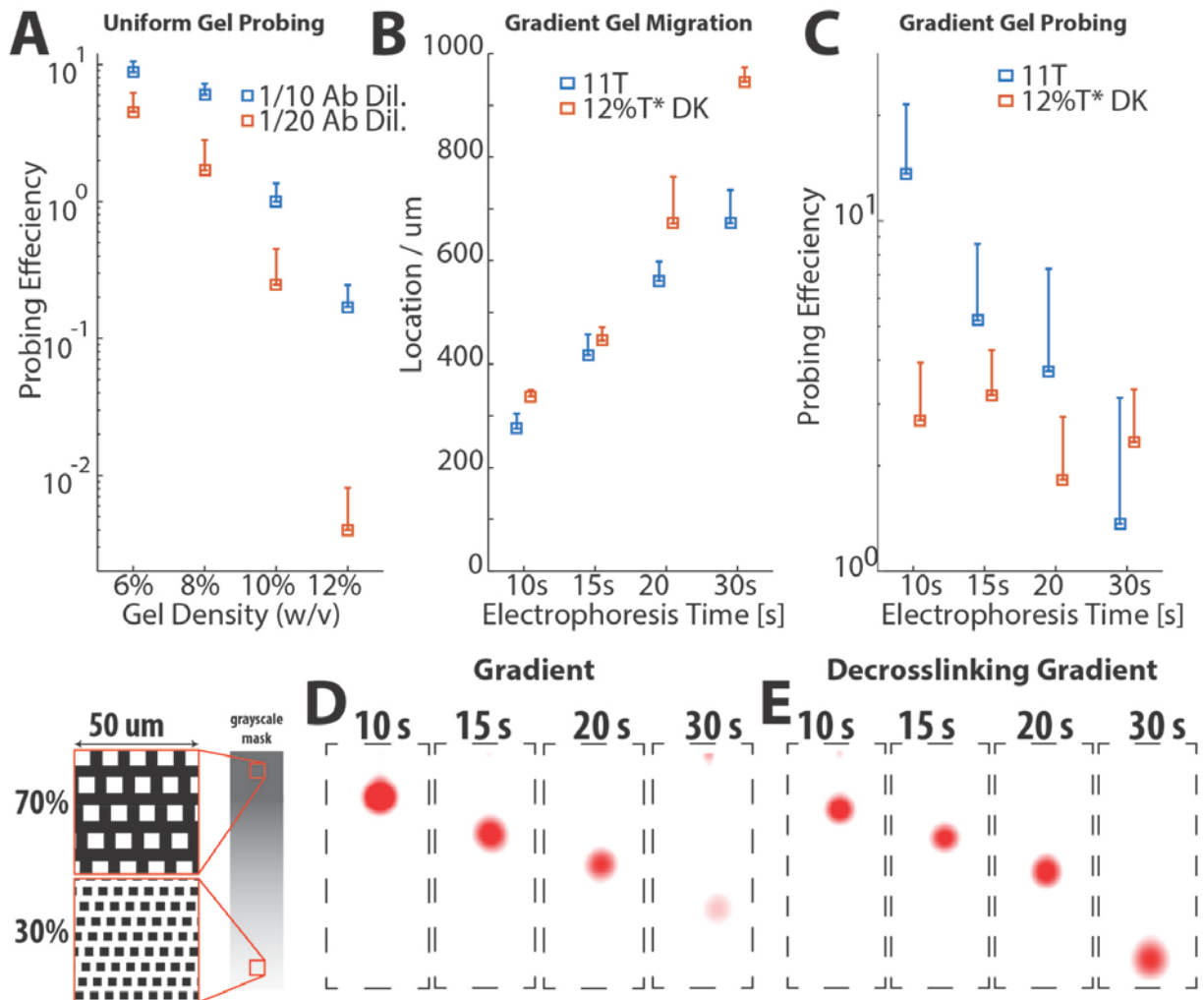


Figure 6.4 Expandable-pores mitigates antibody probing bias along the separation axis. (A) Antibody probing efficiency, calculated by normalizing the RFU from the secondary antibody probe with the initial turboGFP signal for each analyzed lane, was shown to decrease as gel density is increased from 6%T to 12%T by 10^2 at a 1/10 antibody dilution and 10^3 at a 1/20 antibody dilution in uniform polyacrylamide gels (no decrosslinker). (B) Assessing probing bias along the separation axis in gradient gels, turboGFP was electrophoresed for 10 s, 15 s, 20 s or 30 s to sample different locations along the gradient in BIS and DK/BIS crosslinked gradient gels. (C) Along the separation axis, the BIS gel showed a reduction in probing efficiency from 13.6 to 1.36 from the 275 μm to 673 μm migration distances, respectively. In contrast, in the decrosslinked gel probing efficiency was minimally impacted along the separation axis, from 2.69 to 2.33 from the 338 μm to 946 μm migration distances, respectively. The averaged antibody probed signal is shown for the (D) BIS and the (E) DK/BIS gradient gels. *Note: The experiments used to produce the data shown in this figure were run by a colleague, Dr. Chi-Chih Kang. We collaborated on the experimental design. I conducted data processing, analysis, and presentation.*

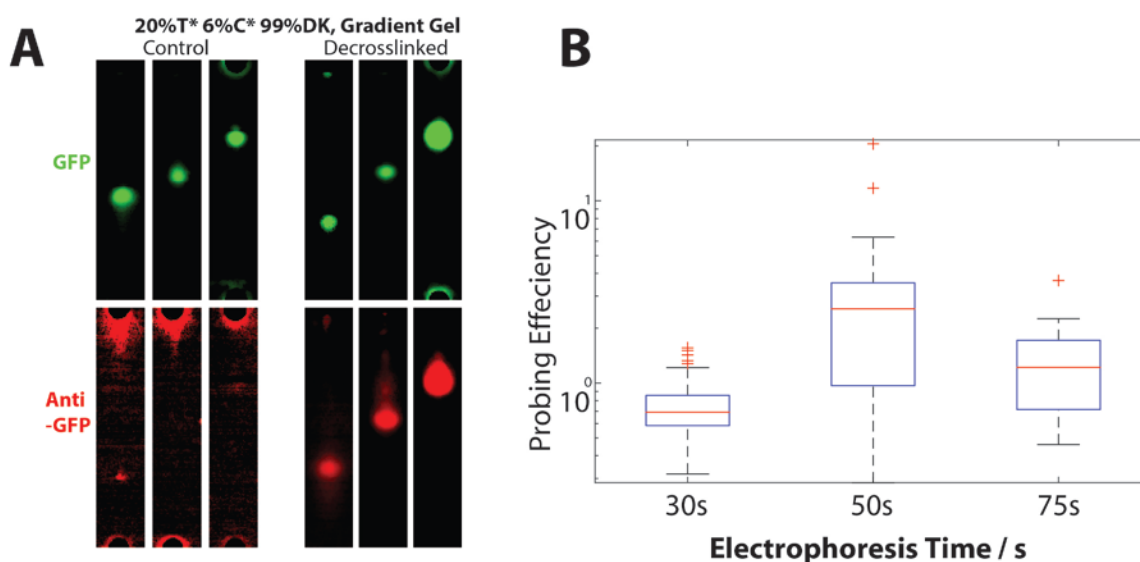


Figure 6.5 Preliminary results (n=1) indicate pore-expansion mitigates probing bias in a dense 99% DK composite crosslinked PAG. A gradient gel fabricated with a precursor solution of 20%T*, 6%C*, 99%DK produced an effective gel density range from 12.4%T to 21.3%T (effective density measurements described in Section 5.4.2). (A) The scWB assay was run with a U373-GFP cell line for different time electrophoresis time points – 30s, 50s, and 75s. In the non-decrosslinked control no probing was observed at any point along the gradient – high contrast images for the anti-gfp (red) are shown to highlight that no signal was observed at the location of the immobilized GFP (green). For the decrosslinked gel, excellent antibody probing was observed for all points along the gradient. (B) Probing efficiency for each electrophoresis time in the decrosslinked gel are similar, suggesting no probing bias in the gradient gel. Only one slide replicate has been performed for each electrophoresis time, more replicates are required to confirm these results.

6.4.3. Large Reagent Delivery

The readout for in-gel assays are, by and large, restrained to using antibody labeled with small fluorescent molecules. We sought to utilize the pore-expansion technology to introduced larger particles that could be advantageous for higher sensitivity assays. As a proof of principle, in figure 6.6 we demonstrate that decrosslinking PAG can be used to probe with quantum dots. A 6%T* 6%C* 99%DK gel was used to run MCF7-GFP cells. The decrosslinked and the control gels were probed as usual, except a secondary antibody labeled with a 20 nm QDOT (Q22084, Life Technologies, Carlsbad, CA, USA) at a dilution of 1/5 was used for probing. In the control, no signal can be seen but the QDOT can be clearly seen on the decrosslinked gel. This demonstrates the utility of decrosslinking to introduce much larger reagents. This ability could open the door for potentially new and high sensitivity in-gel assay.

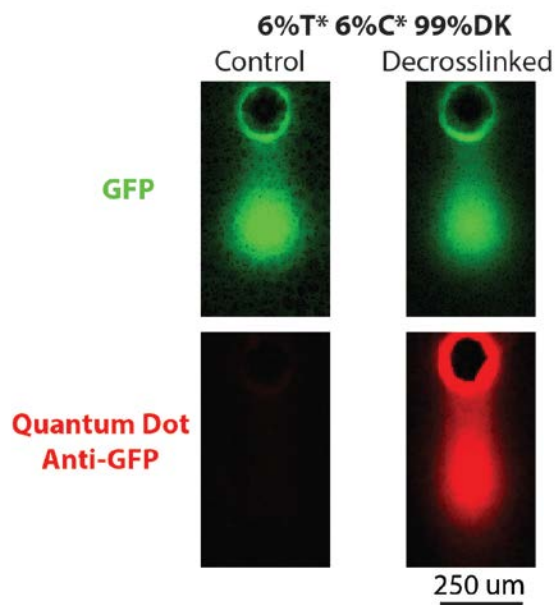


Figure 6.6 Decrosslinking PAG enables the use of large quantum dots potentially for enhanced protein readout. A 6%T* 6%C* and 99DK PAG was used to run a scWB in MCF7-GFP expressing cells. The immobilized GFP was probed with a primary antibody then a secondary antibody attach to a quantum dot (QDot, 20 nm in diameter) for fluorescent readout. The large QDot dot signal could not be observed for the non-decrosslinked PAGs, but was easily seen in the decrosslinked gels.

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Appendix

A. Anomalous Diffusion

Protein dispersion is typically described in hydrogels as a time based process following the relationship, $\sigma^2 = Dt$, where σ^2 describes the variance of a protein distribution, D is the diffusion constant of the protein in the gel, and t is the diffusion time. While this relationship holds when diffusion is the only force acting on the protein, it does not hold when an electrophoretic force is applied to achieve protein transport in the gel. In practice, the non-time based (anomalous) dispersion that occurs during protein transport is commonly ignored in literature, including throughout my own PhD work, due to the assumption that it is small. A rarely cited study, titled “Protein Bandwidth in Gel Electrophoresis: A Primary Function of Migration Distance” from 1998 (8 citations) directly monitored protein transport in slab PAGE and their findings directly contradict this assumption¹. The authors used different electric fields (5 to 40 V/cm) to control the relative protein speed through the gel and monitored protein migration and band broadening. As the title suggests, they found that bandwidth was closely tied to migration distance and has little relationship with time. The mechanism for migration based dispersion could be in-part related to the advantages and unique features seen in protein pulse-field electrophoresis^{2,3}.

To confirm anomalous diffusion in microfluidic systems which typically use higher electric fields, we fabricated gels in T-channel microfluidic devices (10%T 3.33%C polyacrylamide gel)⁴. Using epi-fluorescence microscopy, we monitored the migration and band width expansion of a 200 nM Alexa Fluor 488 labeled bovine serum albumin protein (figure A1A). We used several constant electric field conditions: 30, 50, 102, 200, and 300 V/cm as well as pulsed electric fields at 300 V/cm and 0 V/cm when off. The electric field was periodically turned on/off at periods of 0.8 s/ 0.2 s and 2.0 s/ 0.2 s. When no electric field was applied (figure A1B), we observed the expected band growth pattern in time. Band growth during electrophoresis was measured for all electric field conditions over time and location, in figure A1C and A1D, respectively. Our findings confirm the observations of the 1998 study; dispersion is significantly higher during in electromigration. Additionally, protein bandwidth growth has little to no relationship to time and has a strong relationship to distance migrated – largely irrespective of the applied electric field.

This observation is a compelling demonstration that anomalous diffusion is a dominant factor in protein PAGE. Unfortunately, there is neither a mechanistic explanation that is experimentally supported nor an empirical model that can be used to predict how band growth proceeds given the set of experimental conditions (e.g. protein size, electric field, gel density, etc.). Understanding the anomalous diffusion phenomena in gel electrophoresis and its contributing factors is essential for the future development of gel-sieving based analytical tools.

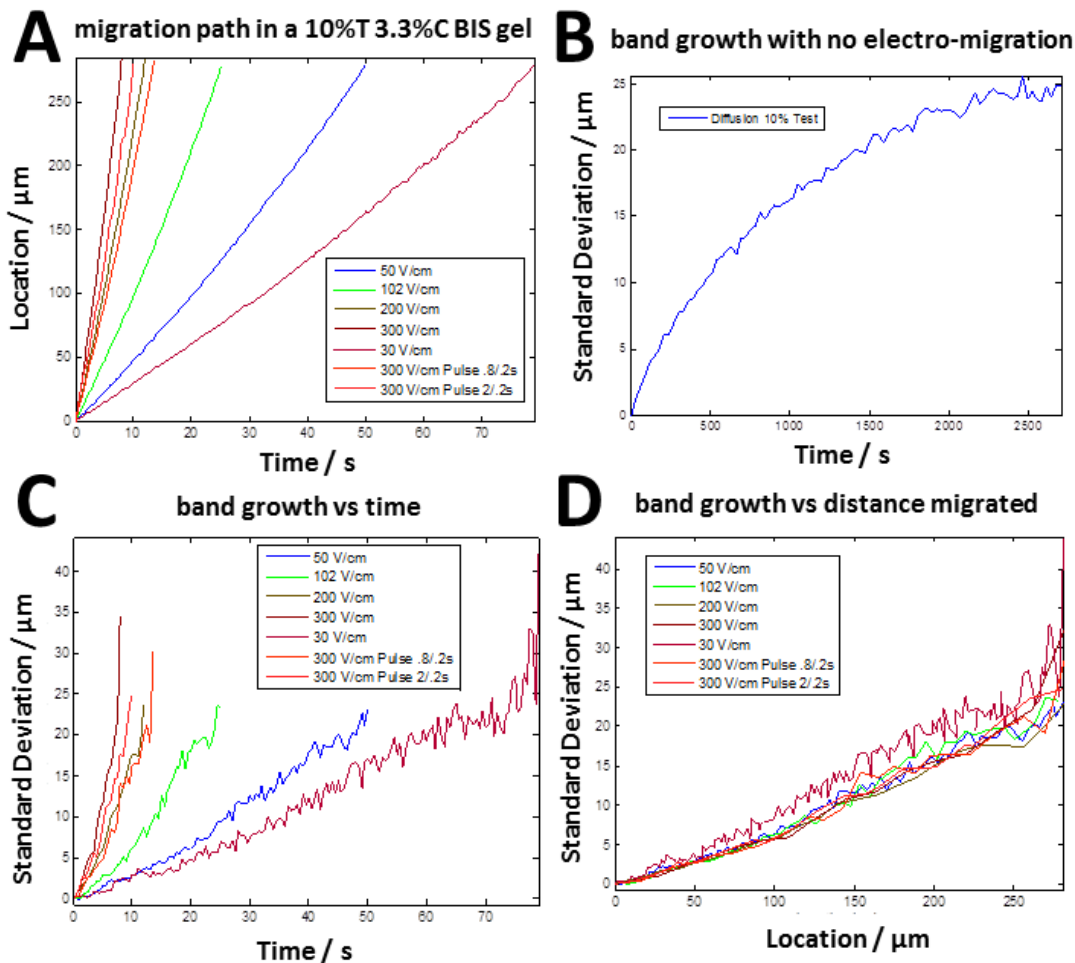


Figure A1 Protein diffusion during electromigration through PAG is anomalous in nature.

Protein electrophoresis of 200 nM Alexa Fluor 488 labeled BSA was monitored in a T-channel microdevice as it migrated through a 10%T 3.3%C polyacrylamide gel. Electrophoresis was conducted at various constant electric fields 30, 50, 102, 200, and 300 V/cm and at pulsed electric fields of 300 V/cm that was on/off for 0.8 s/0.2 s and 2.0 s/ 0.2 s. (A) The location versus time is plotted all conditions, and as expected, their relative velocities vary significantly over a 250 μm length of the channel. (B) To assess the rate of diffusion in this matrix, we tracked band growth over time for a protein band that was not being electromigrated. As expected with Brownian diffusion, the standard deviation of the band grew with a square root relationship with time. (C) We plotted the band growth versus time of electromigrating BSA species. If diffusion were time based, band growth would be independent of the applied electric field and we would observe overlaid trends for each condition. Instead, the rate of band growth was increased with migration speed. For the 300 V/cm case, band growth proceeded over 100-times faster than in the static case. (D) We plotted the migration distance and band growth for all electrophoresis conditions. For all conditions, the same linear trend was observed between band growth and migration distance. This demonstrates that anomalous diffusion is the driver of protein dispersion in PAGE.

B. Discontinuous buffers for *fs*PAGE Arrays

Typically in protein slab PAGE a discontinuous conductivity buffer system is used that functions to stack proteins (Section 1.2.3) using transient isotachopheresis prior to an electrophoretic separation^{5,6}. We attempted to use discontinuous electrophoresis in our 96-plex *fs*PAGE gradient gel array format to enhance protein separations in figure B1. In figure B1A, we show three rows - the first two contain a protein ladder in discontinuous buffer (150 mM tris HCL) while the central row contains a homogenous run buffer (tris-glycine). For the homogenous buffer row, we observe repeatable separations for all columns along the row (figure B1B). In contrast, we observed inconsistent separations along the rows contained discontinuous buffers. Specifically, proteins in the separation lane closest to the cathode traveled the furthest and the lanes approaching the anode had progressively reduced migration distances. The cause of this lane-to-lane variation is contamination of highly charged Cl^- ions from the proceeding reservoir which alters the local electric field. Unfortunately, this contamination prevents the use of discontinuous buffers in the current *fs*PAGE array format.

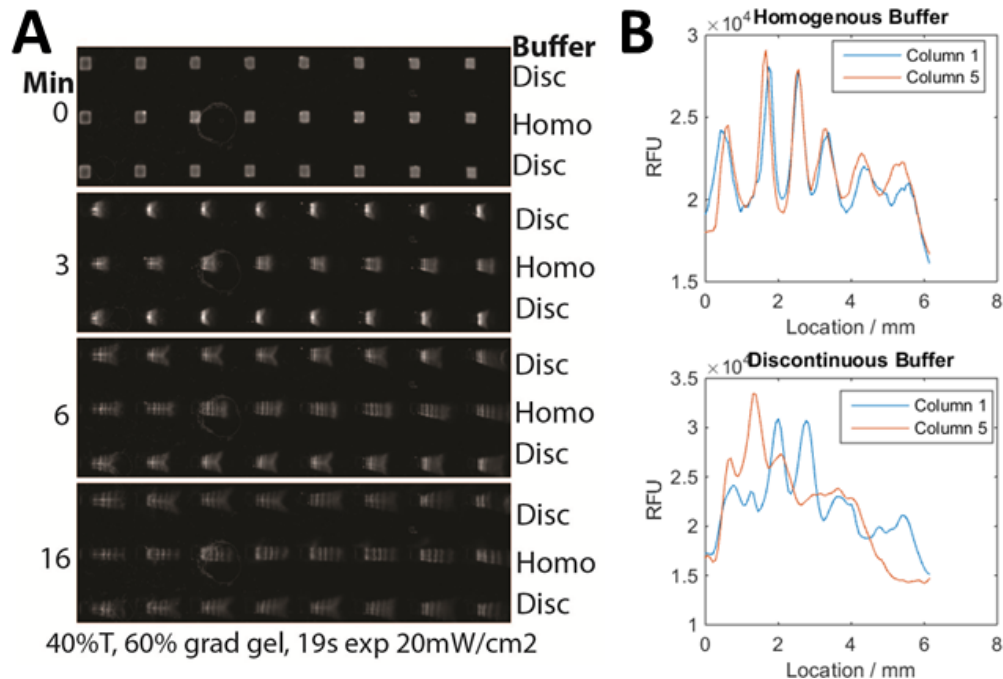


Figure B1 The *fs*PAGE arrays are incompatible with discontinuous buffers. A protein ladder was separated in a 96-plex *fs*PAGE array. (A) Three rows and eight columns are displayed from the arrayed separation initially and at 3, 6 and 16 minutes. The top and bottom rows contain a discontinuous buffer and the central row contains a homogenous buffer. (B) Protein separations were conserved for the homogenous buffer row, while they varied in the discontinuous buffer rows. This is the result of ionic contamination from the proceeding lanes that effectively reduces the local electric field.

C. Multistage in-gel chemistry patterning

The development and optimization of *fsPAG* fabrication has enabled the rapid prototyping of new polyacrylamide gel geometric microstructures to achieve specific assay objectives. This ability has been taken advantage to create large scale arrays^{7, 8} and for other currently unpublished geometries for specific assay applications. Similar prototyping advantages could be gained in developing novel analytical assays with the direct photo-patterning of chemical moieties into pre-existing *fsPAG* structures. A potential application could be large scale arrayed isoelectric focusing of proteins by using grayscale photo-patterning of pH gradients in gels⁹. In this section, we demonstrate a simple approach for multi-stage photo-patterning to patterning chemical moieties into a pre-existing photo-patterned gel. For this approach to work, the desired chemical group must contain an acrylamide, methacrylate, acrylate, allyl or another group that reacts in a free-radical reaction. There are hundreds of commercially available chemistries that can be immobilized using this strategy including buffers⁹, hydroxyl groups¹⁰, glycidyl groups¹¹, primary amines¹², chlorine groups¹³, fluorine groups¹⁴, and many more.

First, *fsPAG* fabrication is performed to create a gel as described elsewhere (Chapter 3 & Protocol A). For consistency, we let the *fsPAG* structure soak in DI water for at least 1 hour before use (it can be stored in water for days to weeks). A wait time is not required between polymerization steps and there is anecdotal evidence that eliminating the wait time could enhance *PAG* incorporation in the second stage. We chose to include this incubation step to improve gel to gel reliability in fabrication. In the results shown in figure C1 a 3%T acrylamide solution was used - in figure C1A we used 1% (v/v) allyamine and in figure C1B we used 2% (v/v) glycidyl methacrylate (Sigma Aldrich PN 145831 and PN 151238, respectively). Excess solution was removed from the *fsPAG* structure - then it was placed in a small bath of solution containing the precursor solution for 15 minutes. UV polymerization was then performed using a grayscale mask (figure C1A) or simple photomask (figure C1B).

Here, we demonstrate grayscale allylamine patterning in a uniform and microstructured *fsPAG* regions (which is then labeled with FITC¹²). The acrylamide concentration is low enough such that incorporation only occurs where an *fsPAG* was previously localized – such that the structural definition step is de-coupled from the chemical patterning step. From protein electrophoresis studies in which proteins travel between regions into and out of moiety patterned regions (data not shown), the effective pore-size was not appreciably impacted. In Figure C1B, we immobilized a glycidyl group in a V shaped pattern. Glycidyl groups react with primary amines to form a covalent bond, and are commonly used to immobilize proteins¹¹. We soaked the gel in 0.1 mg/mL IgG-FITC overnight in a tris HCl buffer to demonstrate protein immobilization.

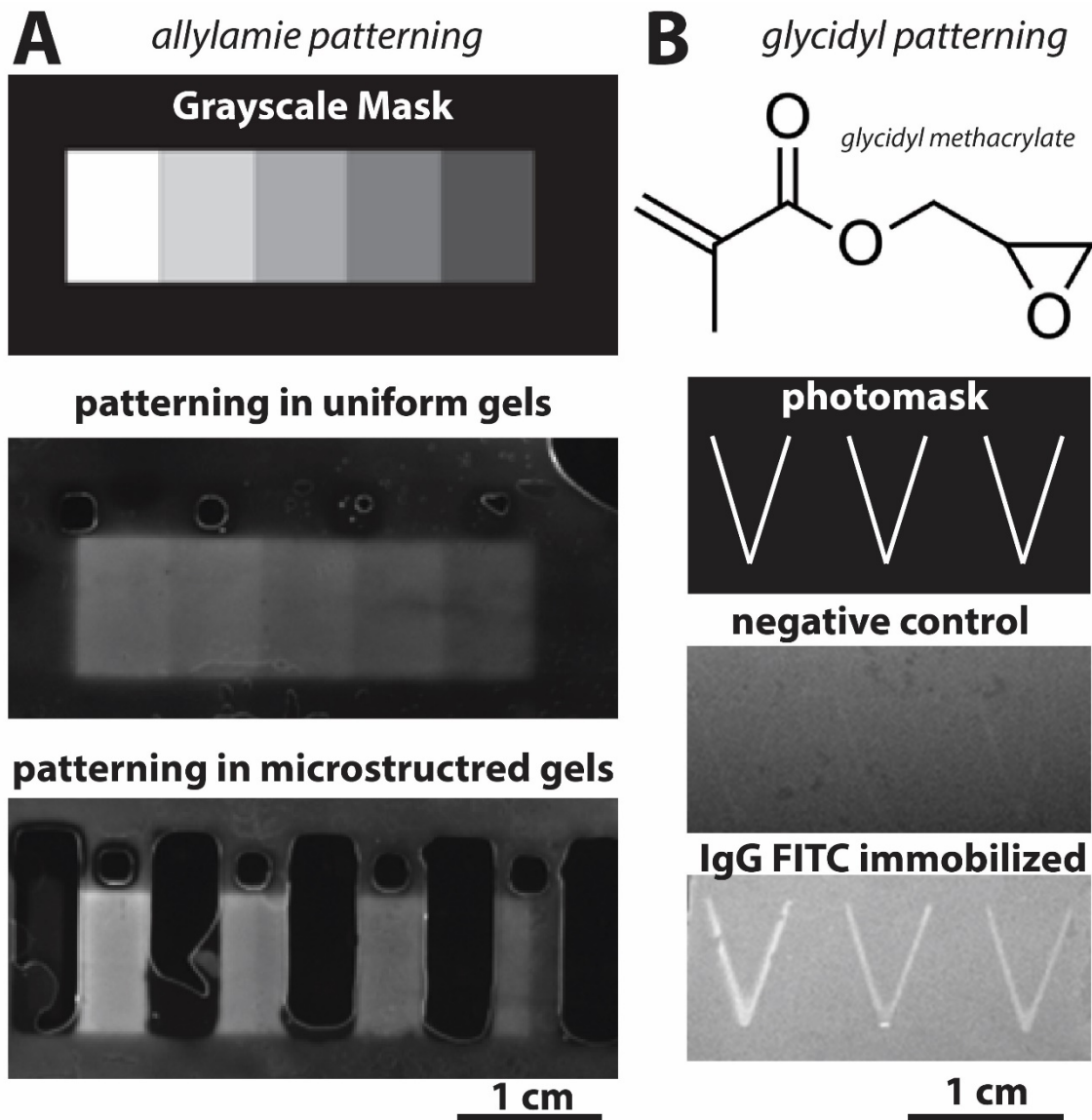


Figure C1 Chemical groups can easily be photo-patterned directly into *fsPAG* structures. Pre-fabricated *fsPAG* structures were soaked in a precursor solution containing the photo-initiator, a low concentration of bis/acrylamide, and a chemical group that contains an acrylamide, methacrylate, acrylate, or an allyl portion. Photo-polymerization through a photomask spatially patterned the chemical groups within the *fsPAG* structure. (A) Allylamine incorporation into a PAG is spatially and density photo-patterned directly into a PAG using a grayscale mask. The low acrylamide concentration prevents incorporation in regions where the *fsPAG* structure is not present. (B) Glycidyl groups are patterned within a PAG and shown to be active in immobilizing IgG proteins.

D. Violet-light polymerization of UV active *fsPAG*

In recent work out of the Herr lab¹⁵⁻¹⁸, benzophenone methacrylate groups have been incorporated into PAG for photo-immobilization of species after electrophoresis for western blotting applications. Benzophenone is a UV active molecule that forms a covalent bond during UV exposure¹⁹. Blue light and riboflavin was previously used to achieve gel photo-patterning in a glass-channel without UV activation of the benzophenone¹⁶. Unfortunately, the blue-light riboflavin free-radical initiation process is slow and we were not successful in adopting it for *fsPAG* fabrication. Benzophenone absorbance predominantly occurs between 200 nm and 365 nm. In contrast, the peak absorbance of the VA086 photo-initiator occurs at 374 nm. At 400 nm, VA086 maintains ~30% of its peak activity. We utilized a 390 nm long-pass filter (Edmund Optics, PN 39-426) to directly pattern the *fsPAG* structures with 390 nm to 410 nm light (violet light) while preventing the activation the benzophenone group. Typically, the UV exposure times were increased by 33% to achieve similar *fsPAG* structures.

In Figure D1, we fabricated benzophenone incorporated gels with and without the use of the longpass filter and calculated their relative capture efficiency in retaining protein. We observed a 25% increase in protein capture efficiency in the longpass filtered gels over the broadband fabricated gels for long UV capture steps (5 minutes), and a 75% increase for short UV capture steps (1 minute). This demonstrates that direct violet-light photopolymerization of UV active *fsPAG* maintain high photo-capture activity.

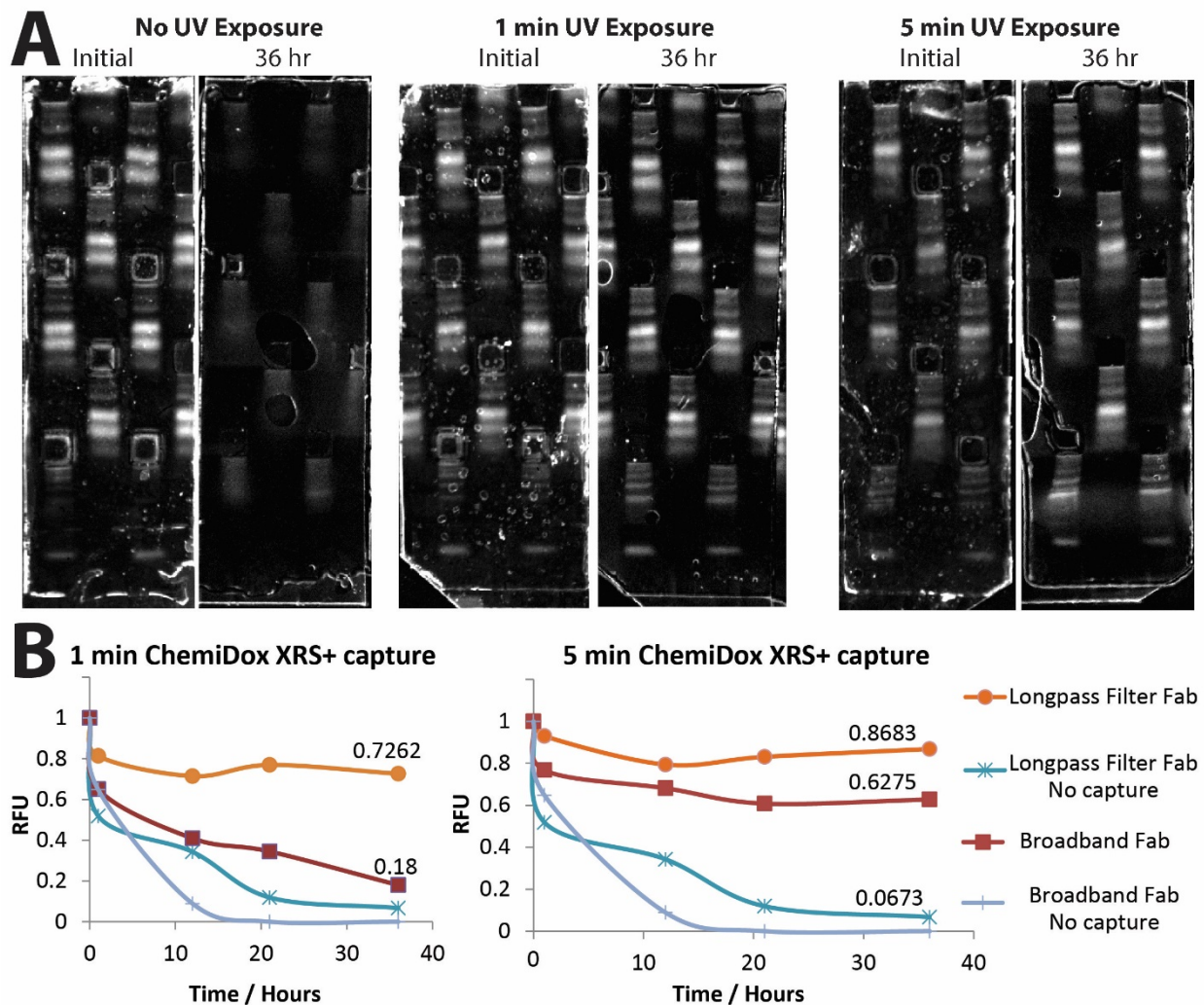


Figure D1 Violet-light photo-patterning of benzophenone incorporated *fs*PAGs maintain UV activity for in-gel protein photo-capture. (A) A 390 nm long pass filter was used for the direct photo-patterning of a 15%T 3.33%C PAG containing 1.6 mM of benzophenone methacrylate. Separation of fluorescently labeled protein ladders was conducted followed by a UV photo-capture step for 0 minutes, 1 minutes or 5 minutes. Gels were stored in tris glycine buffer, periodically imaged over a 35 hour period to assess the UV capture efficiency. As expected, 5 minute photo-capture condition had the highest capture efficiency. (B) Capture efficiency was compared between gels that were polymerized with the long pass filter and with broadband UV. Gels fabricated with a long pass filter had improved capture efficiencies compared to the broadband fabricated gels. This demonstrates that the use of a long pass filter during fabrication helps maintain benzophenone activity – and should be used when possible in the photo-polymerization of UV active PAG fabrication.

E. Grayscale mask MATLAB® code

MATLAB script used in CleWin to create a 200 μm stacking region (at 70 gray) with an 800 μm gradient (70% to 30% gray) that is 505 μm wide. After being produced, it is cropped to 500 μm wide, then arrayed in CleWin to create the desired pattern size. The mask is fabricated as a negative of the design, such that 30% gray in the code and CleWin design, will signify 70% gray in the actual mask.

```
%MATLAB script for generating grayscale patterns in CleWin.  
%Set location & size of the array  
x0 = 0; %initial x position  
y0 = 0; %initial y position  
  
w = 505; %Width in microns  
l = 1000; %Length in microns  
  
l_stack=200; %Length of Stacking Region in microns  
  
%Set grayscale gradient, g1 to g2  
g1 = 30; %Starting gray value of gradient  
g2 = 70; %Ending gray value of gradient  
grad = double((g2-g1)/(l-l_stack));%Gradient slope  
  
% Set minimum feature size: e.g 3 um for chrome mask  
minsqside=3; %Set the minimum feature size for mask production  
minline=minsqside; % minimum line in mask fabrication  
minsq=minsqside*minsqside; % minimum square in mask fabrication  
  
x=x0;  
y_stack=l_stack;  
y0=0;  
y=0;  
  
done=0;  
while done ~=1  
    if y<=l_stack;  
        g=g1;  
    else  
        g = double((y-y_stack)*grad+g1);  
    end
```

Appendix

```
syms xa;
if g>0 && g<25
    c=solve(minsq/xa^2==g*.01, 'Real', true);
    array = double(c(1));
elseif g<45
    c=solve((((xa-minline)^2))/xa^2==g*.01, 'Real', true);
    array=double(c(1));
elseif g<75
    minside = -10*(g*.01)+10.5;
    c=solve(((xa^2)-minside*minside)/xa^2==g*.01, 'Real', true);
    array=double(c(1));
elseif g<100
    c=solve(((xa^2)-minsq)/xa^2==g*.01, 'Real', true);
    array=double(c(1));
end

for x=x0:array:(x0+w)
    if g>0 && g<25
        rectangle(x,y,x+minsqside,y+minsqside);
    elseif g<45
        rectangle(x, y, x+array-minline,y+array-minline);
    elseif g<75
        rectangle(minside+x, y, x+array, array+y);
        rectangle(x, y, x+array, y+array-minside);
    elseif g<100
        rectangle(minsqside+x, y, x+array, array+y);
        rectangle(x, y, x+array, y+array-minline);
    end
end

y = y+array;
if y>=(y0+l)
    done=1;
end
clear c array g;
end
```

F. Dual crosslinked ethylene glycol diacrylate gels

To achieve pore-expansion of PAGs, we utilized a composite crosslinked gel containing the stable methylene bisacrylamide (BIS) and the base-labile ethylene glycol diacrylate (EDA). When placed in a high pH solution, base hydrolysis occurs on the ester groups resulting in rapid and significant pore expansion. To qualitatively assess the rate of dissolution, we fabricated 100% EDA crosslinked PAG and observed the dissolution rate in buffers of various basic pH values in Table F1. We observed rapid dissolution in less than 30 minutes for buffers with a pH value of 11.6 and over.

Table F1: Qualitative assessment of 100% EDA PAGs dissolution in different basic buffer conditions. A 15%T 3.3%C 100%EDA crosslinked gels were fabricated atop a GelBond® backing. They were placed in solutions with pH values 13.1, 12.4, 11.6, 10.9, and 9.2 and were qualitatively monitored over time.

Dissolution Experiment pH Incubation time	15%T, 3.3%C, 100% EDA cross linker				
	13.1	12.4	11.6	10.9	9.2
5 min	Dissolved	partially	partially	OK	OK
10 min	x	Dissolved	partially	OK	OK
15 min	x	x	partially	OK	OK
20 min	x	x	Dissolved	OK	OK
65 min	x	x	x	partially	OK
95 min	x	x	x	Dissolved	OK
120 min	x	x	x	x	OK - soft

To test composite crosslinked EDA/BIS gels, we fabricated a 15%T, 3.3%C, 80% EDA 20%BIS crosslinked gel in figure F1. When decrosslinked, the gel was noticeably swollen in the regions where EDA crosslinker had been. This extreme swelling is in part due to the presence of a charged group in the gel. After EDA base hydrolysis, an acetic acid product is incorporated into the gel. The resulting ionic gel responds with excess swelling²⁰ and under the presence of an electric field, may lead to severe gel distortion²¹. The resulting charged gel could result in non-specific background during in-gel antibody probing and eliminates the future possibility of using electrophoretic active antibody introduction – which is a future direction for enhanced antibody delivery. For these reasons, we decided to abandon the EDA/BIS gels for western blotting applications.

Appendix

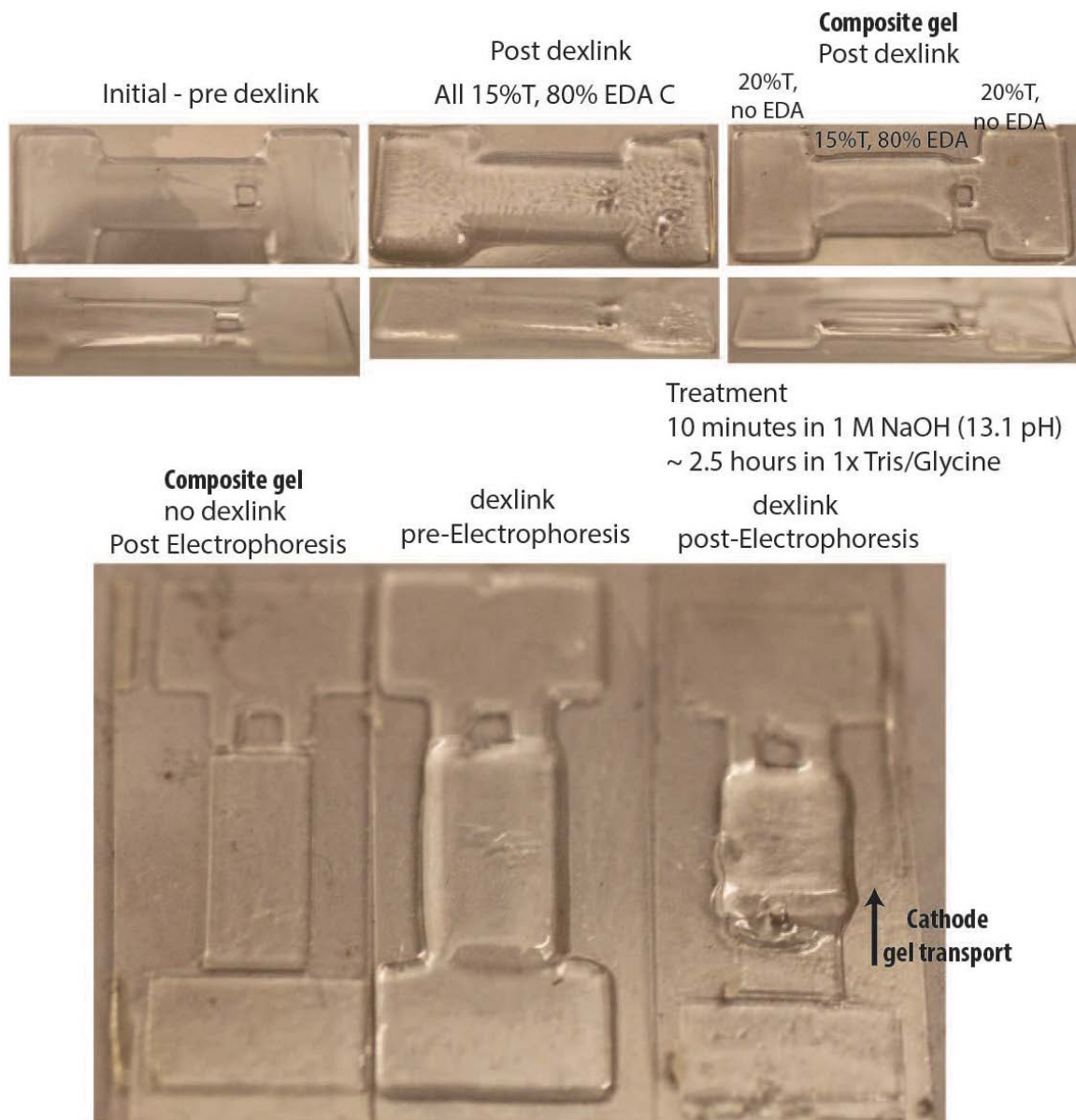


Figure F1 Dual crosslinked EDA/BIS gels contain charged moieties that make it incompatible for electrophoresis after decrosslinking. *fsPAG* were fabricated with 15%T, 3.3%C, 80% EDA, and 20% BIS. The EDA/BIS gels showed dramatic swelling after decrosslinking for 10 minutes in 1 M NaOH (pH 13.1). The gels were then washed in tris-glycine for 2.5 hours to remove any excess Na ions from the solution. We observed dramatic migration of the gel itself during the application of an electric field. During electrophoresis of proteins from the reservoir, the proteins were unable to enter the decrosslinked gel (data not shown).

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Protocols

A. Free-standing polyacrylamide gel (fsPAG) fabrication protocol

Todd Duncombe

11/5/2013

PPE

- Safety Goggles
- Lab coat
- Gloves

Equipment

- UV light source
- UV light meter
- Sonicator / Vacuum
- Pipette

Materials

- GelBond®
- VA-086
- Acrylamide
- Water
- UV Mask
- Scissors /guillotine cutter
- Glass plates
 - o 3 mm thick carrier plate
 - o 1 mm thick mask plate
 - o cover plate (any thickness)
- Spacers
- GelSlick®

PAG Fab Reservoir Setup

1. Cut out GelBond pieces in the desired size.

Note: GelBond is light sensitive and should be not left uncovered for an extended period of time, though it will remain stable for several hours.

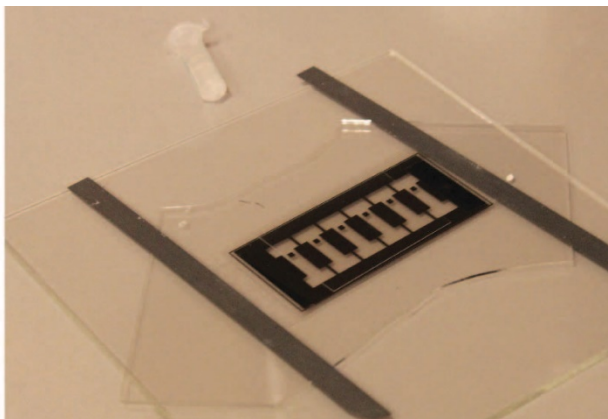
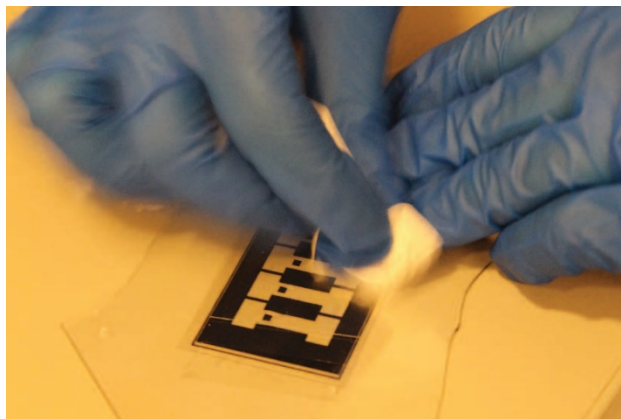
2. Determine the hydrophilic and hydrophobic side of GelBond but pipetting a droplet of water.



Protocols

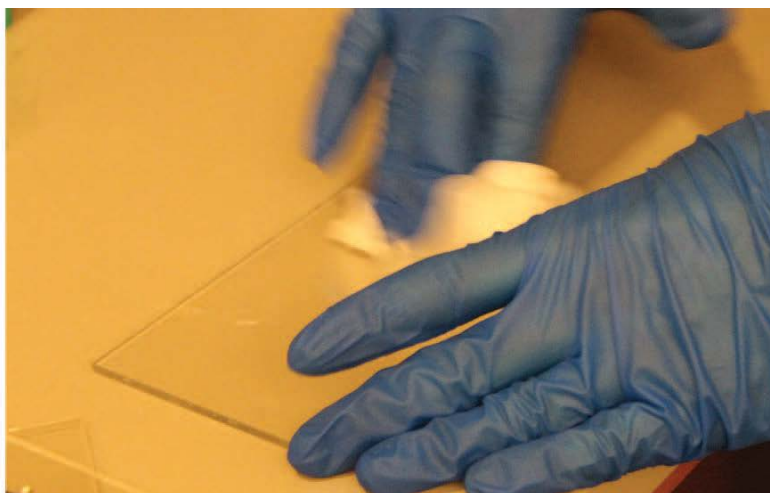
3. Pipette large droplets of water onto the 1 mm borosilicate plate and place the GelBond's hydrophobic surface face down atop the water. Using a Kimwipe to touch the GelBond, push it flat against the borosilicate surface such that it is strongly adhered to the glass by the small meniscus of water.

Ideally, the meniscus should cover the entire GelBond piece. This step ensures the piece is flat and that when adding precursor, it does not wick onto the backside of the GelBond.



4. Apply GelSlick® on cover piece. Pipette a small amount of GelSlick (~100 μ L) on surfaces and buff it in using a kimwipe for several seconds (~3 seconds).

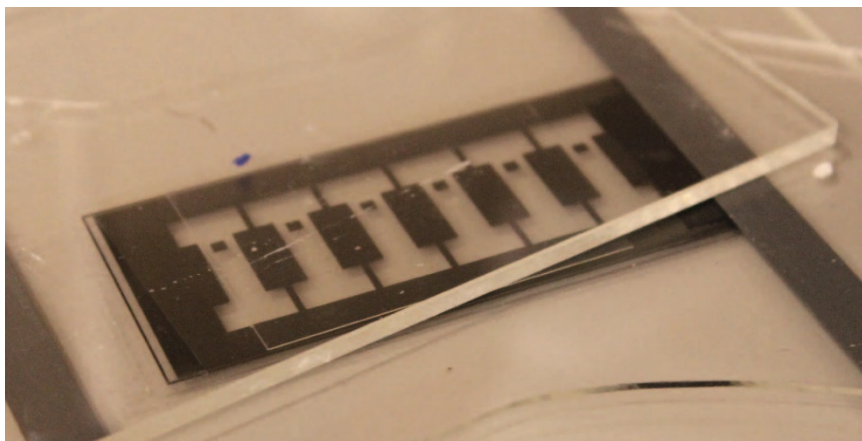
This should be repeated for every other use of the cover plate.



Protocols

5. Assemble PAG reservoir, from bottom to top
 - a. 3 mm back plate
 - b. UV photomask
 - c. 1 mm mask plate with adhered GelBond piece
 - i. Place desired spacers adjacent to GelBond on mask plate
 - d. Place the cover plate atop the spaces – the GelSlick treated side facing down

Leave a small corner of GelBond exposed such that you can introduce the precursor solution



Precursor Preparation

1. Estimate the amount of precursor required for fabrication – then add 25%
 - a. Height (spacer height – GelBond thickness (~120 μm)) x Width x Length x # number of desired gels
 - b. $1 \text{ mm}^3 = 1 \text{ microliter}$
2. Create a 2x solution of photoinitiator (VA-086) at 2% w/v in water.
 - a. Vortex / sonicate it as necessary until no residual VA-086 is visible
3. Prepare a 2x solution acrylamide / crosslinker in water

OAI Setup – Always use UV protective Safety goggles (e.g. polycarbonate)

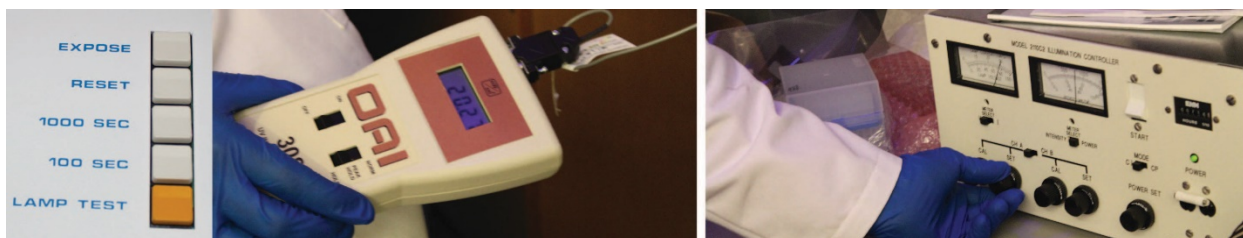
1. Turn on the fan, Power Source, and light controller – in this order. **The fan must be turned on before turning on the power source.**



2. Depress the 'start' switch on the power source until the light is initiated. You will hear a high pitch sound when it is initiated and both the voltage/current and intensity/power indicators will be non-zero.
3. Wait for the OAI to warm up. The power meter should reach 1000 Watts, ~10 minutes warm-up.

Warning: Ensure that the UV shield atop the OAI is closed whenever the shutter is opened – otherwise potentially harmful UV exposure will result.

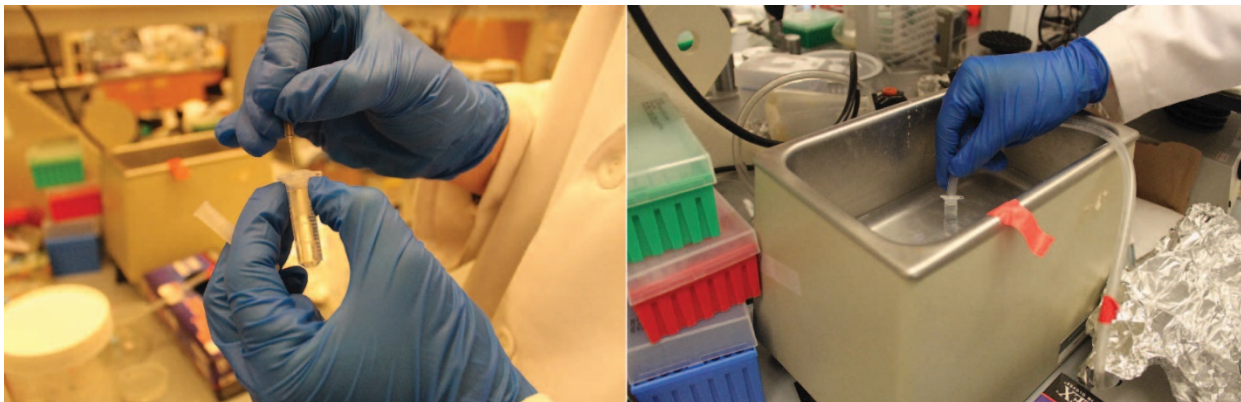
4. Once warmed up, open up the UV shield atop the OAI, place the head of the UV meter into the center of the OAI platform, **close the UV shield**. Press 'lamp test' on the light controller to open the shutter and initiate UV exposure.
 - a. Measure the UV intensity and adjust as necessary using the set dial on the power source. The OAI can range between $\sim 18 - 30 \text{ mW/cm}^2$.
 - b. Please try to keep setting A at 20 mW/cm^2 , and setting B at 30 mW/cm^2
 - c. Hit "reset" on the light controller to close the shutter.



If at any time you hear a loud beep coming from the OAI, close the shutter and reduce the UV intensity with the 'set' dial.

fsPAG Fabrication

1. Create PAG precursor solution by mixing 2x photoinitiator solution and 2x PAG solution 1:1 – vortex briefly.
2. Using a needle create hole at the top of the precursor solution container, apply house-vacuum and sonicate until little or no bubbles are seen. This step will often take ~1mn.



3. Using a pipette, introduce the sample carefully atop the GelBond and below the cover plate until it is full.
 - a. Be careful not to spill over to the top of the GelBond – the precursor can wick under the GelBond and create height non-uniformity in addition to a mess.
 - b. This step should be done nearby the OAI / light source – such that it can be easily moved to the exposure location.
4. Align GelBond piece above the UV mask design as desired by moving the mask plate.
5. Once satisfied with the position, open up the UV shield atop the OAI. Pick up the carrier plate and carefully transfer the PAG precursor / UV mask setup atop the UV the light. Close the UV shield.
6. Select the desired time in the light controller and hit expose to initiate exposure.
7. Once exposure is complete, open the UV shield and remove the PAG precursor / UV mask setup.

Note: There are likely regions of unexposed acrylamide – which is highly toxic. Be careful to dispose of the acrylamide accordingly.



Protocols

8. Pick up the mask plate, cover plate sandwich. Tip the sandwiched plates such that the unpolymerized acrylamide falls away from the acrylamide features to a paper towel for Acrylamide collection.

Note this paper towel should be disposed of in chemical waste at the end of fabrication.

9. Carefully remove the glass cover plate from the sandwich – the GelBond / *fs*PAG piece may stick to the mask plate or the cover plate.
10. Remove the GelBond *fs*PAG device and place it into a large container of water for at least 5 minutes to remove excess photoinitiator and unpolymerized acrylamide.



Shutdown, Cleanup and Disposal

OAI Shutdown

- Turn off shutter controller, Power source. **LEAVE THE FAN ON FOR 30 MINUTES TO ALLOW THE UV LIGHT TO COOL DOWN.**
DO NOT TURN ON THE LIGHT FOR AT LEAST 20 MINUTES AFTER IT IS TURNED OFF.

Cleanup & Disposal

- **Wipe down all glass plates, spacers used for fabrication and rinse them in water.**
- Dispose of all paper towel / kimwipe waste in chemical waste. **Please take out chemical waste bins when they are full.**
- Put away all extra GelBond pieces in a sealed bag/box out of the light.

B. Glass-SU8 fabrication Protocol

SU8 2025 on glass fabrication protocol – updated September 22nd 2014
Todd Duncombe

If wafer is not new:

Wafer solvent clean – soak in acetone (5 min), IPA (5 min), N₂ dried

- a. Note: N₂ dried immediately after IPA, no contact with water

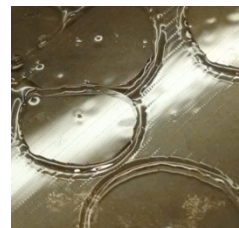
New wafer:

1. Prepare adhesion primer - 2% titanium acetylacetonate in anhydrous isopropanol.
2. Wafer dehydration - 10 min @ 110°C
3. Place wafer on chuck.
4. Coat wafer by placing adhesion primer on it such that the wafer is completely covered.
5. After 30 s in contact with the adhesion primer, the adhesion primer is spun off with the following protocol: (1) 500 rpm, 100 rpm/s, 5s. (2) 3000 rpm, 500 rpm/s, 30s.
6. Immediately after the spin is complete, SU8 2025 is added and spun at: (1) 500 rpm, 100 rpm/s, 31s. (2) 2500 rpm, 500 rpm/s, 30s.
7. Soft bake: 110°C for 3 min. Allow the wafer to cool to room temperature (~2 min).
8. Exposure (no mask!): 250 mJ/cm²
9. Post exposure bake / Hard bake: 110C for 5 minutes. Allow the wafer to cool to room temperature (~3 min).
10. Place the wafer onto the chuck.
11. Add SU8 2025 to the wafer, spun at: (1) 500 rpm, 100 rpm/s, 31s. (2) 2500* rpm, 500 rpm/s, 30s.
12. Soft bake: 65°C for 6 min**. Allow the wafer to cool to room temperature (~2 min).
13. Exposure: 250 mJ/cm² (through the desired mask)
14. Post exposure bake. Afterwards, allow the wafer to cool to room temperature.
 - a. 65°C for 3 min
 - b. 90°C for 7 min
15. Develop for 2-2.5 min while being swirled (setting #3 in BNC rotator). After development, place the wafer directly into an IPA bath for 1 min. Rinse with water, then inspect. If additional development is required, perform development in 20s increments.
16. Soak in water: >2 min
17. Dry with N₂
18. Hard bake: 110C for 15 - 60 minutes

*Peak spin speed dictates layer thickness, from previous experiments 3000 rpm ~ 25 um, 2500 rpm ~ 34 um, and 2000 rpm ~ 40 um. Typically the layer thickness for the 2nd SU8 layer is lower than expected (compared to the spec sheet). Make sure to verify feature heights.

Protocols

**If the soft bake for the second SU8 layer is done $>80^{\circ}\text{C}$, the top SU8 layer can easily reflow, as seen in this photo. Reflow after exposure (i.e. during the post exposure bake) is OK, and does not appear to disrupt the final device structure or robustness.

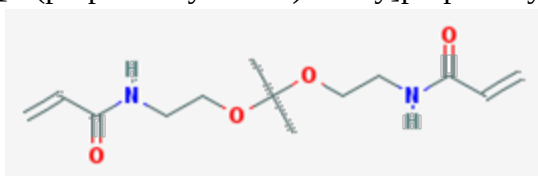


Protocol was adapted from Professor Stephen Jacobson's labs method¹, described in DOI: 10.1021/ac301565g

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C. Diacrylamide Ketal Synthesis

Desired product: N-[2-[2-[2-(prop-2-enoylamino)ethoxy]propan-2-yloxy]ethyl]prop-2-enamide



Reaction Mechanism: NHS-ester activated crosslinking

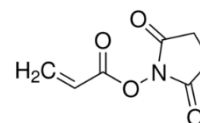
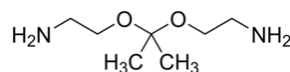
Purification: Thin Film Chromatography

Product verification: NMR

Reaction

Reagents

- Ketal-diamine (2,2-Bis(aminoethoxy)propane)
- Acryloxysuccinimide (AA-NHS)
- Dichloromethane (DCM)
- Anhydrous DCM
- Ice
- Triethylamine (Et_3N)



Equipment

- Round bottom flask + flask cap
- Cooling bath – “crystallizer”
- Magnetic stirrer + stir bar
- Laboratory stand
- Flask clamp
- N_2 source
- Syringe with short needle / tubing that can interface with N_2 source
- Syringe with long needle

Synthesis Procedure

1. Determine the desired reagent usage. Example table below.

Note: use a 1:1.1 (mol:mol) for the primary amine to activated NHS ester

Chemical Name	MW	Amount Added	mmol	eq
Ketal-diamine	162.2	100 mg	0.616	1
NHS-Ester	169.1	229.35 mg	1.356	2.2
DCM	-	5 mL	-	-
Et ₃ N (0.725 g/mL)	101.19	(1.246g, 1.718 mL)	12.32	20

2. Assemble the experimental setup for performing the reaction at 0°C. Place a cooling bath atop a magnetic stirrer. Using a laboratory stand and clamp, insert the round bottom flask into the cooling bath. Place a cleaned stir bar into the round bottom flask. Cap the round bottom flask then add ice to the cooling bath.
3. Wash a chemical spatula with acetone and wipe it dry with a paper towel, then with N₂.
4. Weigh the appropriate amount of NHS-ester, and add to the round bottom flask. Insert flask cap. **Note: error on too much NHS ester.**
5. Weigh the ketal-diamine with a plastic pipette. **Note: as the ketal-diamine is eq 1, make sure it is very accurate.**



Anhydrous DCM extraction protocol:

- a. Open lid of anhydrous DCM
 - b. DCM container and syringe N₂ purge
 - i. Attach short needle on a syringe to the N₂ source. Turn on N₂ source.
 - ii. Insert short needle syringe into the DCM container's vapor (NOT LIQUID)
 - iii. Purge long needle syringe by inserting it into the vapor section of the anhydrous DCM container and extract nitrogen. Do this several times (~4).
 - c. Insert purged long needle syringe into the DCM and extract desired volume. Once desired DCM volume is extracted, move the tip of the needle to the vapor and extract ~1 mL of nitrogen.
6. Following the anhydrous DCM extraction protocol detailed above, extract 3 mL of DCM. Add ½ of the Et₃N volume to the DCM. Add the solution to round bottom flask / NHS-ester. Initiate magnetic stirrer.
 7. Following the anhydrous DCM extraction protocol detailed above, extract 2 mL of DCM. Add ½ of the Et₃N volume to the DCM. Add solution to the ketal-diamine container.
 8. Once the NHS-ester solution is clear, add the ketal-diamine solution **very slowly (drop by drop)**.
 - a. Using bulb/plastic pipette works well.

9. Allow reaction to progress for approximately 40 minutes, monitor with thin film chromatography.
10. Once the reaction is complete, run TLC to purify the product (described below).

Thin Film Chromatography (TLC)

Reagents

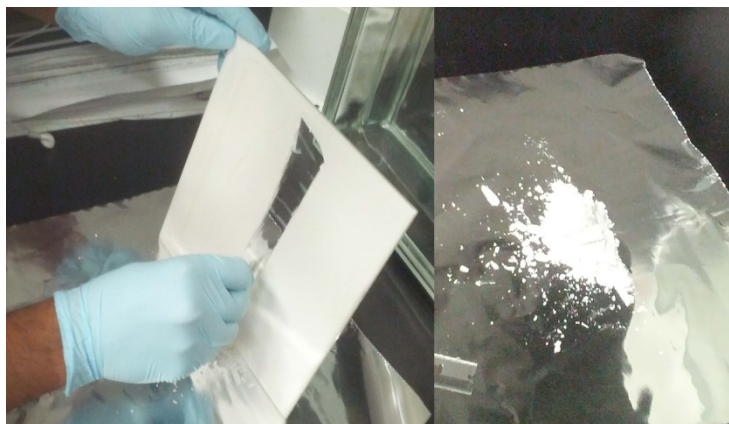
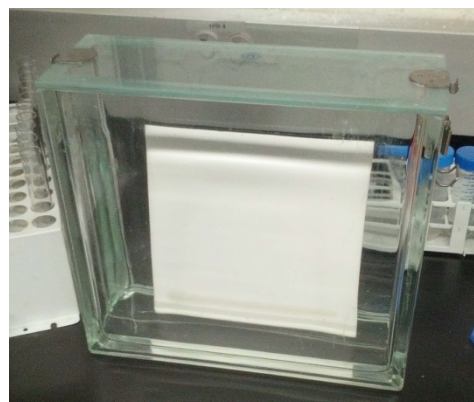
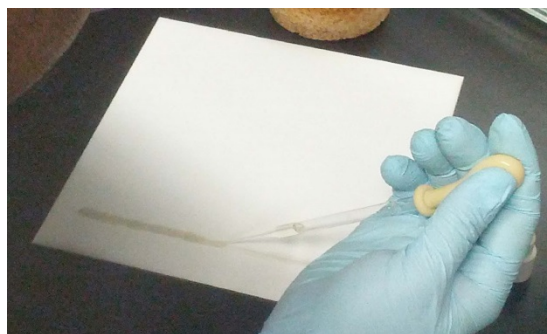
- Ethyl Acetate
- Triethylamine (Et_3N)

Materials/Equipment

- Millipore Silica Gel GF TLC plate (2000um thickness, 20 cm x 20 cm, catalog #:02015)
- TLC chamber
- Pencil
- Flasks (1 for each band)

TLC Protocol

1. Make Mobile Phase: 0.1% Et_3N in Ethyl Acetate.
2. Using a ruler and pencil draw a line along the base of the TLC plate at $\frac{3}{4}$ inch above base.
3. Add solution along the drawn line. For each 20cm plate add no more than 250 mg of product.
4. Let the TLC plate dry ~ 1-2 minutes.
5. Fill the base of the TLC chamber with mobile phase ~ $\frac{1}{3}^{\text{rd}}$ inch (must be below the height of where the sample was deposited).
6. Add TLC plate to the TLC chamber. Close the chamber. Allow the TLC to run (~1 hour).
7. Once the TLC is complete, use a UV light to determine the bands. Circle them with a pencil.
8. Separately for each band you're interested in collecting - use a razor to remove the silica gel of a desired band and collect silica pieces it atop aluminum foil.
9. Dissolve the silica gel pieces in 0.1% Et_3N in Ethyl Acetate in a flask. Sonicate as necessary.
10. Once dissolved, filter silica (as described next).



Silica Gel Filtration / Organic Solvent Removal

Materials

- Sand
- Celite 545 (filter agent)

Equipment

- Buchner funnel
- Vacuum
- Round bottom flask (1 for each band)
- Lab stand / clamp
- Rotary Evaporator

Silica Bead Filtration

1. Assemble the Buchner funnel above a round bottom flask, attach house vacuum.
2. Add sand, Celite 545 layers in the funnel.
3. Pour in the Ethyl Acetate, Et₃N and Silica gel solution atop the Buchner filter slowly. Allow ~5 minutes for the filtration to complete.
4. Once completed, remove the excess organic solvent using a rotovape (described below).



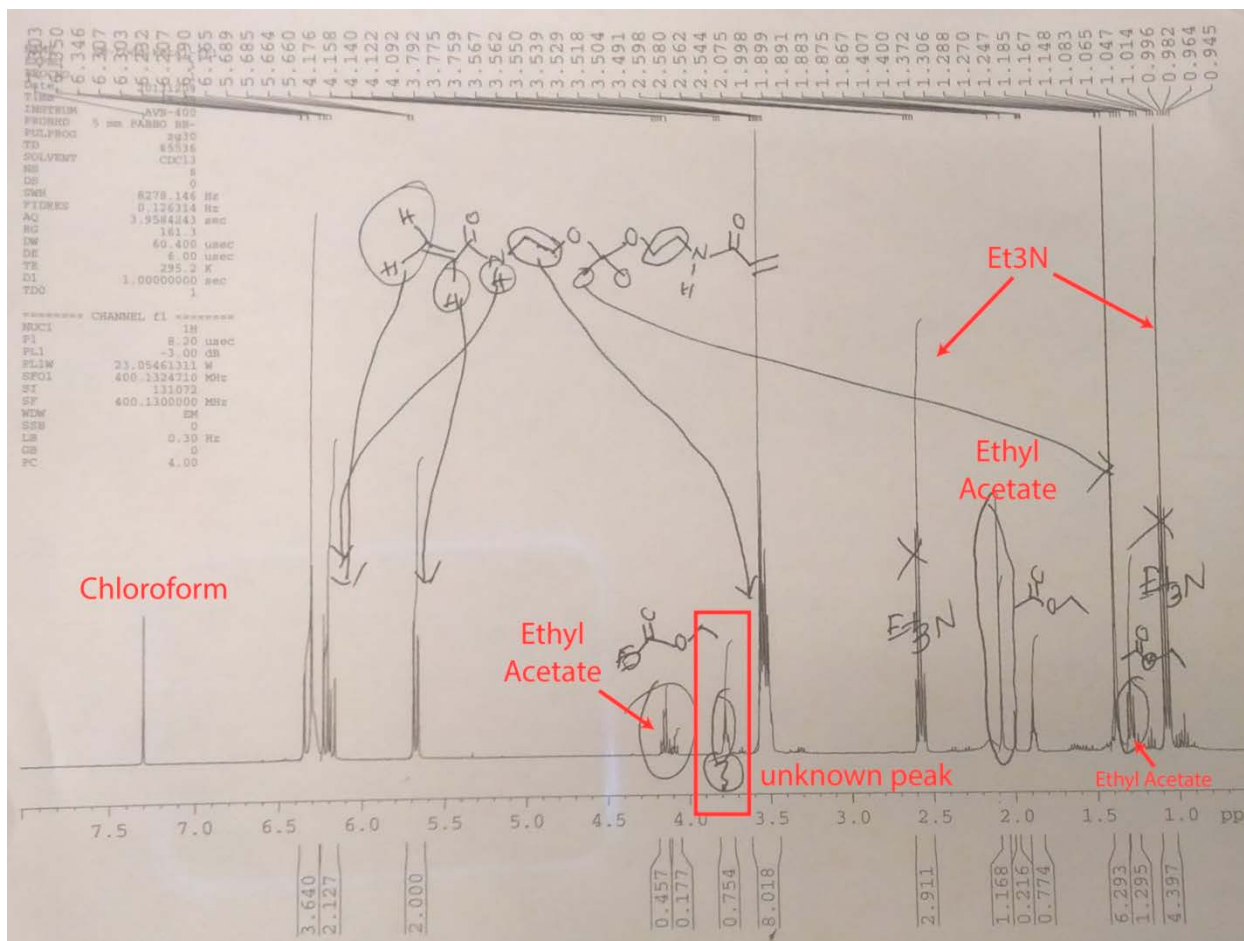
Organic solvent evaporation – rotovape

1. Attach the round bottom flask to the rotovape.
2. Allow it to rotate at 25°C or less. Elevated temperature can lead to premature polymerization.
3. Set rotovape to a pressure of 123 Torr and spin at 75 RPM.
4. Once evaporated, add ~1 ml of Et₃N. Cap, wrap in aluminum foil. Store at -20°C.



Verification: NMR

1. Add chloroform to the round bottom flask.
2. Extract __ amount? And add to a NMR tube.
3. Perform NMR and identify proton peaks. NMR below is ~ 90% pure.
4. Set calibration peak then integrate other peaks of interest.



Dissolution

1. Measure empty vial weight and write it down.
2. Dissolve chloroform Et₃N product in rotovape in the vial.
3. Measure final mass of the vial to determine mass. (~15-20% yield)