OPTIMIZATION OF PEGDA HYDROGEL PLATFORM FOR GEL ELECTROPHORESIS APPLICATION

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Presented to

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In Partial Fulfillment

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Master of Science

in Biomedical Engineering

by

Christopher Candelari

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ABSTRACT

We have developed a micrometer scale thick electrophoresis system based on polyethylene glycol dimethylacrylate (PEGDA, Mn = 750) to separate nucleic acids. Using photo-polymerization, we fabricated PEGDA hydrogel attached to cover glass that is robust, easily handled and comparable to agarose gels in DNA band detection. Optimization parameters including PEGDA concentration, UV exposure time, voltage, run-time and hydrogel thickness were determined and optimized for PEGDA-750 to enable the separation of standard DNA ladder bands ranging from 250 to 1000 base pairs.

Our optimized method for creating PEGDA hydrogels is capable of performing DNA electrophoresis and holds promise for being further developed as a platform for detecting specific ranges of nucleic acids. This study enhanced parameters surrounding DNA band separation and brightness, and refined fabrication methods. PEDGA hydrogels hold potential to be a highly customizable alternative to agarose gels because of their distinct photo-polymerization fabrication method and attachment to cover glass.

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CHAPTER 1: INTRODUCTION

Gel electrophoresis is a useful technique for separating nucleic acid fragments and for identifying target genes after amplification via polymerase chain reaction (PCR). It is a widely used, standard technique that has been progressively developed since the 20th century, and even has roots from the earliest experiments in electrochemistry in the 19th century by scientists such as Michael Faraday. Arne Tiselius described the first form of electrophoresis in his seminal work from 1937, in which he describes moving boundary electrophoresis and his own Tiselius apparatus (Tiselius, 1937). Oliver Smithies revolutionized the field with his 1955 paper introducing starch gels as a medium for electrophoresis separation for the first time, opening the door for many others to advance this technique during the remaining half of the century (Smithies, 1955). The work of these two great scientists enabled countless others to discover new methods for optimizing electrophoresis for new applications and using new techniques.

Gel electrophoresis works on the principle of separation by charge. DNA and other nucleic acid molecules contain a negatively charged sugar-phosphate backbone, which allows them to migrate toward the positively charged anode. By creating an electric field current between a positively charged anode and a negatively charged cathode over some distance, DNA fragments placed in between will migrate through a gel matrix and be separated based on the mobility of the different sizes of the fragments through the pores of the substance.

The process of preparing the agarose gel has changed over this time and new techniques have been continually adapted to incorporate more ways to identify nucleic acids and proteins. Several parameters including gel concentration, running voltage, running time, and nucleic acid type and fragment size need to be optimized when conducting gel electrophoresis (Guttman & Ronai, 2000). Several groups have proposed miniaturized electrophoresis systems (Guttman & Ronai, 2000; Li et al. 2010; Demianova et al., 2008; Ma et al., 2001; Ogita & Markert, 1979; Kenyon et al., 2011; Zheng et al., 1999) or the use of poly (ethylene glycol) dimethacrylate (PEGDA) in capillary electrophoresis (Rogers et al., 2011; Slater et al., 2000; Schulze & Belder, 2012; Nagata et al., 2005) which is a growing research area due to the attractiveness of quick and accurate results. However, capillary electrophoresis requires a high voltage power supply which is generally packaged as a costly and specialized piece of equipment.

PEGDA is an attractive material due to its FDA approval and ability to be custom fabricated in a variety of ways. It has become increasingly popular as a synthetic hydrogel polymer material and is used in various biomedical applications such as microparticle drug delivery carriers, micropatterning, and bioassays (Bae et al., 2010; Subramani & Birch, 2006; Secret et al., 2014). For many applications, PEGDA can be fabricated on nanometer scales that allows for novel uses in engineering very small and intricate structures. PEGDA hydrogels can also be fabricated across a large range of molecular weights allowing for the control of specific characteristics of the hydrogel for a desired

application. Additionally, PEGDA has an advantage of being able to incorporate various molecular structures into its polymer chain, making it highly customizable.

The goal for our PEGDA platform is to create a robust micro scale system that can be easily fabricated and customized for specific applications for gel electrophoresis. This could allow our system to be used in field settings as a portable all in one device for gel electrophoresis. This study demonstrates the steps taken to optimize fabrication of this platform utilizing a PEGDA hydrogel for gel electrophoresis tested in a lab setting. PEGDA is the candidate material because it is customizable and easily fabricated using photo-polymerization on mini- and micro-scales (Ban & Nanyang, 2011; Datta, 2007). In this study, we have begun the systematic optimization process for the fabrication of PEGDA-750 as well as the protocol for its use in gel electrophoresis. The hydrogel is shown to reliably separate DNA fragments of specific base pair (bp) ranges after optimizing numerous parameters of this system. In our protocol for DNA electrophoresis we have chosen to use tris-borate-EDTA (TBE) buffer, instead of tris-acetate-EDTA (TAE) buffer, to separate DNA fragments smaller than 1 kb (Lonza).

This report focuses on the development of parameters involved in the fabrication and optimization of PEGDA hydrogel for gel electrophoresis. Major parameters in our study used for the fabrication of our hydrogels include PEGDA concentration, mold design, and UV exposure time. For the optimization of our system for gel electrophoresis we have tested parameters such as running time

and running voltage to determine the characteristics of our hydrogel. We will discuss how our hydrogels are made and the procedures we have taken to improve our system's performance. Systematically measuring the results of our hydrogel platform during the optimization process will allow us to continue refining the protocol for our system and develop new methods for fabricating PEGDA hydrogels with desired characteristics.

CHAPTER 2: MATERIALS AND METHODS

2.1 Cover Glass Modification

One of the distinguishing factors of our PEGDA hydrogel platform is that during our fabrication process we are able to attach our hydrogel to cover glass. We prepare our cover glass slides with a chemical treatment, 3-(trimethoxysilyl)propyl methacrylate (TMSPMA, Sigma Aldrich, St. Louis, MO), before the fabrication of our gel. Then, after the gel fabrication process, our hydrogel is able to stick to the glass. Doing so greatly increases the ability to easily handle the hydrogel, and provides necessary support for hydrogels of millimeter scale thickness that would otherwise fall apart under their own weight.

When working with a free hydrogel less than 5 mm in thickness it becomes increasingly hard to prevent the hydrogel from tearing apart during the steps necessary to run a sample. Additionally, while free hydrogels that are larger than 5 mm in thickness tend to have increased structural stability, the increased weight of the gel can cause them to fall apart under their own weight when picking up the hydrogels. There are three critical steps in the procedure for running hydrogels that requires the transfer of the hydrogel from one container to another, where damaging the hydrogel can most commonly occur.

The first step occurs when placing the newly made hydrogel into the running apparatus before loading the samples. After the hydrogel has been cross-linked and removed from the mold, it must be transferred into the running trey where the running voltage can be applied to migrate the DNA samples. The second step occurs when moving the hydrogel from the running apparatus to be

stained. Removing the hydrogel involves picking it up from the running trey where it is submerged in buffer solution and moving it into a petri dish with staining dye. The third step occurs when moving the hydrogel from the staining dye bath to the surface of the imager so that the DNA bands can be visualized. Having our hydrogel attached to a cover glass provides much needed structural support when picking up the hydrogel from these containers and placing them into another. Due to the design of our molds, we are able to create a working space when we attach our hydrogels to the cover glass. This working space is approximately 5 mm above and below the ends of the hydrogel, where the cover glass extends out. Having this additional area for picking up our hydrogels without actually touching the hydrogel is critical for our system. When working with a hydrogel that is less than 1 mm in thickness, any touching of the hydrogel where the sample with run will cause a defect and can prevent the DNA sample from separating reliably.

Although agarose hydrogels are typically handled by simply picking up the entire hydrogel, for our system there is a need to avoid touching the running area where the sample will migrate. The difference is that agarose hydrogels are typically more than 1 cm in thickness, allowing the samples to run within the hydrogel deep enough that touching the hydrogel will most likely not interfere with the running area. In our system, we are attempting to decrease the thickness of our hydrogel to below 1 mm in thickness. Understandably, as the hydrogel's dimensions decrease, there is less room for error when accidentally damaging the hydrogel. The addition of the modified cover glass allows for the

ease of handling of our hydrogel system, adds necessary structural support, and allows for a working area where the hydrogel can be picked up without damaging the running area. Traditional agarose gels are not attached to a cover glass, so this step in the fabrication of the hydrogel is distinct.

We can modify the width of our gels using different cover glass dimensions. The molds we have created to fabricate our hydrogels have been custom made to fit distinct cover glass sizes of dimensions 1.42 in by 2.36 in and 24 mm by 60 mm. We have also found that we can use the wider 1.42 in by 2.36 in cover glass with our smaller molds to prepare hydrogels with a working area of glass free of hydrogel approximately 5 mm wide around the parameter of the hydrogel. This additional working space allows for great ease of handling, and demonstrates one way that we are able to customize our system without modifying the hydrogel. By finding new ways to customize the use of our attached modified cover glass on our hydrogel, we can continue to build onto our system and develop it towards a portable, point-of-care tool.

In order to facilitate the attachment of poly(ethylene glycol) dimethacrylate (PEGDA, Sigma Aldrich, St. Louis, MO) to the cover glass, the surface was modified with 3-(trimethoxysilyl)propyl methacrylate (TMSPMA, Sigma Aldrich, St. Louis, MO), as described in the literature (Fan et al., 2015). First, cover glasses (1.42 in x 2.36 in, Ted Pella, Inc., Redding, CA and 24 mm x 60 mm micro cover glasses, VWR, Radnor, PA) were added to a beaker containing 10% (w/v) NaOH solution and left overnight. Each cover glass is individually placed into the NaOH solution to ensure maximal surface area interaction and to prevent

cover glasses from sticking together. Next, the cover glasses were washed with distilled water and three consecutive baths of 95% ethyl alcohol before being air dried and finally baked at 80°C. After baking for at least an hour, TMSPMA was administered over the sides of a stack of cover glasses to coat them, and then continued to bake. The cover glasses were rotated periodically until TMSPMA had been applied over the entire surface of the cover glasses. We allow our cover glasses to bake with the coated TMSPMA for at least 24 hours. Finally, the cover glasses are washed again with three consecutive 95% ethyl alcohol baths and then let to air-dry. By carefully preparing our cover glasses in this way, we can ensure that the hydrogels will form correctly, attaching to the modified cover glass and enabling easy removal from the molds they are fabricated in. In the next section, we will discuss the various molds used and go into detail of how the hydrogels are formed.

2.2 Fabrication of PEGDA Hydrogel Platform Molds

During our process of creating custom sized PEGDA hydrogels, various molds were hand-made having differing dimensions and qualities that would allow us to assess which proved to form the best hydrogels. In our attempt to create molds that could optimally fabricate our hydrogels to specific dimensions, we focused on designs that would also allow us to easily remove the hydrogels from the molds without having to worry about damaging the hydrogels in the process. Attaching our hydrogels to cover glass allowed us to reliably remove the hydrogels from the molds after cross-linking. We also focused on designs that would allow us to customize the dimensions of the wells for loading the

sample DNA. It was crucial to fabricate molds that not only allowed us to specifically control the dimensions of the loading wells, but that also allowed us to safely remove the hydrogels while maintaining the integrity of the loading wells. We focused on controlling the dimensions of our molds such as width, length, volume, and thickness while making sure that the molds were the proper fit for the cover glasses we utilize. The first three of our molds were constructed by stacking micro slides (75 mm x 25 mm x 1 mm, Corning, VWR, Radnor, PA) onto a plastic square dish to create the desired hydrogel thickness (Table 1).

Table 1. Comparison of molds for PEGDA hydrogel fabrication.

Mold	Gel Volume (mL)	Gel Thickness (mm)	UV time (sec)	Gel Clarity
Mold A	2	0.5	360	Clearest
Mold B	3	1	360	Clear
Mold C	5	2	600	Clear
Mold D	12	10	720	Opaque

These three molds (A, B, and C) were glued together using Krazy glue, which was also used to seal all edges of the mold. Mold A and mold B were identical, except for the depth of the mold that correlates to the hydrogel thickness. Mold A was constructed by stacking Corning micro slides where the distance between the top of the mold and the modified cover glass was approximately 0.5 mm in thickness. This design allows for a volume of liquid hydrogel precursor of approximately 2 mL to be dispensed between the two glasses. This mold was designed to fit 24 mm by 60 mm cover glasses, which

could be placed on top of the mold to create a space for the liquid hydrogel precursor to fill. Mold B was constructed to allow for a distance of about 1 mm between the top of the mold and the modified cover glass, also utilizing the 24 mm by 60 mm cover slides. Mold C was designed for the wider 1.42 in by 2.36 in cover glasses to allow for more loading wells, and was created for a thickness of 2 mm. During our optimization, we designed molds A and B at a width that allowed for two to three loading wells, however, mold C was designed with a width that could fit between three and five loading wells.

A fourth mold was created using the lid of a glass slide box and a comb used for creating the wells of an agarose gel. This design was chosen due to its geometry and volume fitting our desired characteristics to be tested. This fourth mold was much deeper than the previous three, and allowed us to place a standard agarose comb inserted to create standard dimensioned wells. Because this fourth mold involved filling a container with hydrogel precursor, rather than sandwiching the hydrogel precursor between two sheets of glass and allowing hydrostatic forces to hold the liquid hydrogel precursor in place, we could fill the fourth mold to our desired volume. Controlling the volume of liquid hydrogel precursor that we dispensed into this mold allowed us to dynamically control the width of these hydrogels. With the other three molds, the spacing left between the glass slides when the mold was designed predetermined the end width of the hydrogel. So for our fourth mold, mold D, we tested a range of volumes and corresponding hydrogel thicknesses.

In Table 1, we have listed a 12 mL volume with 10 mm thickness which required a 720 second UV exposure time and resulted in an opaque gel. This volume of 12 mL was chosen because it allowed us to produce a hydrogel approximately 10 mm in thickness, where the standard agarose comb we used to create the loading wells was positioned optimally for creating standard dimension loading wells. At volumes larger than this, the hydrogel was visibly opaque and would not be suitable for imaging. At volumes lesser than this, the standard agarose comb would not reach deep enough into the hydrogel to form proper loading wells. At this 12 mL volume, the hydrogel was visibly clear, however we determined it to be opaque relative to the imaging process after the DNA sample had been run. DNA samples that were run and stained in these thick hydrogels could not be properly imaged due to the thickness of the hydrogel. In our efforts to optimize our hydrogel fabrication process, mold D represents our intention of designing the system based on the ability to easily create loading wells of the same dimensions as the standard agarose. While this mold was much thicker than the others, our testing was aimed at optimizing the loading well dimensions during this experimentation. The goal of decreasing our hydrogel thickness is apparent in the design of the other three molds. All of the characteristics of our molds listed in Table 1 correspond to the optimized hydrogel concentration discussed later in this paper.

Our molds were created with thicknesses of about 0.5 mm, 1 mm, 2 mm and 10 mm (molds A, B, C and D respectively). We could also control for the

number of wells created in our hydrogels, ranging from one to five wells. We used a variety of methods to create wells in our PEGDA hydrogels.

For the first two molds, A and B, we utilized two methods for creating the wells. Also, because each of these hydrogels were designed to be less than 2 mm in thickness, we created a novel way to maintain the necessary dimensions of the loading wells while decreasing the thickness of the running area. To accomplish this, we allowed for the top portion of our hydrogels, where the loading wells would be constructed, to remain thicker than the rest of the hydrogel. By keeping the loading well portion of the hydrogel at a thickness of about 2 mm, we could create loading wells deep enough for sample DNA. As the sample DNA is loaded into the wells and settles to the bottom, it will run across the hydrogel that is designed to have decreased thickness. The design of our molds, along with the UV photo-polymerization fabrication method for cross-linking our hydrogels allows us to customize the thickness of our hydrogels at different points. Maintaining a certain thickness at the top of our hydrogels allows for the proper loading dimensions, while a decreased thickness along the rest of the hydrogel allows for clearer imaging. The highly customizable nature of the fabrication methods for our hydrogels allows for a great degree of freedom to design our hydrogels in ways that standard agarose are not suitable for.

First, we designed photo-masks that we could place over the desired well location on top of the cover glass during UV excitation. These photo-masks were hand made from plastic and aluminum foil in a rectangular shape and were reusable. We produced varying sizes of these photo-masks with dimensions of 2

mm x 1.5 mm and 3 mm x 1.5 mm approximately. Each of these photo-masks consisted of two rectangles separated by approximately 2 mm of space between them to allow for the hydrogel to crosslink and form a wall to separate the wells. The second method we used was to hand cut wells in the desired location after the hydrogel had formed under UV excitation. Using a surgical blade, we hand cut the wells in the exact same location and dimensions as the photo-mask and then removed the excess hydrogel leaving behind very clean cut wells. This method allowed us to fabricate wells of desirable dimensions with very straight lines and fine edges.

While both methods of forming loading wells for the molds were limited by the ability to consistently form loading wells of the exact same sizes between samples, we noticed that by hand-cutting the wells we could more accurately remove the desire amount of hydrogel leaving behind a loading well with sharper edges. While the photo-mask method is ideally more standardized than the hand-cutting method, due to the room for human error in cutting out the loading well shapes, more often than not the photo-mask was less reliable than the hand-cut loading wells. As we continued to optimize our system during our experiments we tried a variety of custom made photo-masks of varying dimensions, as well as varying the placement of the photo-mask in relation to the UV excitation laser. By placing the photo-mask directly under the UV excitation laser, we attempted to prevent cross-linking of the hydrogel directly under the photo-mask which would give allow for the formation of rectangular loading wells once the hydrogel is removed from the mold. The surrounding hydrogel not

covered by the photo-mask would provide the structure of loading wells after they had cross-linked, and once the uncross-linked hydrogel remaining was washed out.

For the third design, mold C, the impression for the loading wells were cut out of plastic with a dimension of 2 mm x 2 mm x 1.5 mm and glued into the mold, which enabled a maximum sample volume of 5 µL to be loaded into each well. The number of desired wells determined the width of the mold. Utilizing this design, we created hydrogels that had either three or five loading wells. The method for removing these hydrogels from their mold was different than the methods used for the first two because of the design of the wells. Additionally, because this mold used nearly twice the volume of liquid hydrogel precursor, photo-polymerization required 600 seconds of UV exposure time to ensure optimal cross-linking.

The PEGDA hydrogel was produced according to the literature (Fan et al., 2015; Avci et al., 2015). A schematic of the fabrication process of the PEGDA electrophoresis platform is shown in Figure 1.

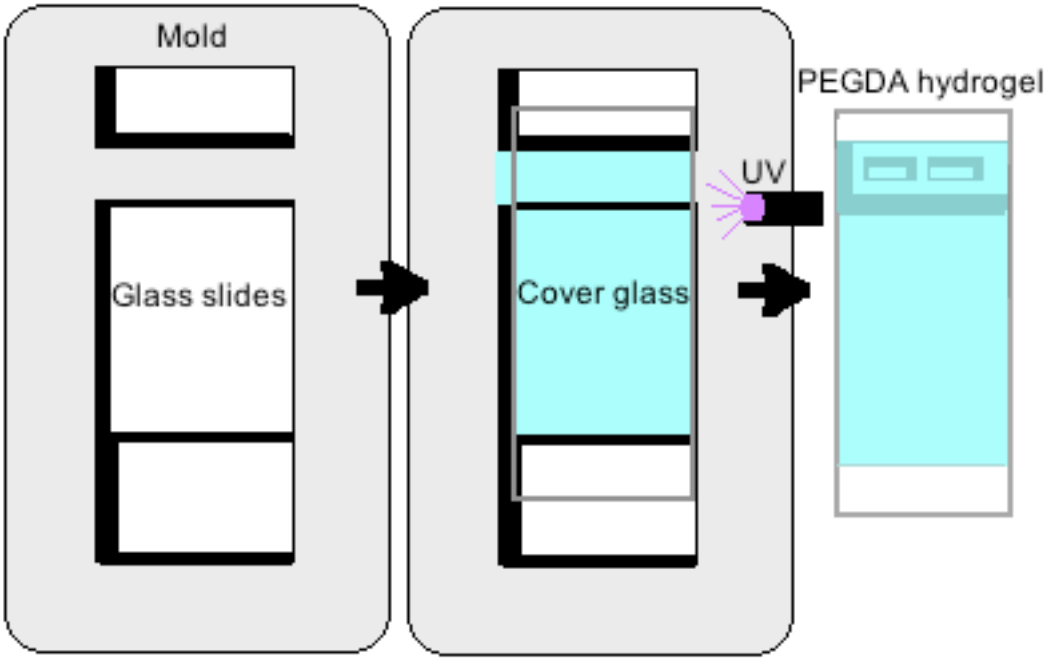

Figure 1. Schematic of the fabrication process. 1) Design of the mold. 2) Cover glass placed and the hydrogel precursor pipetted under**.** 3) PEGDA hydrogel attached to the cover glass once removed from the mold.

First, the photo initiator (PI, 2-hydroxy-4'-(2-hydroxyethoxy)-2-methylpropiophenone, (Sigma Aldrich, St. Louis, MO) was dissolved in PEGDA-750 by vortexing, then 1 x TBE buffer (Bio-Rad, Hercules, CA) was added to the mixture and vortexed again until thoroughly mixed. Preparing this mixture before hand, and allowing it to dissolve overnight resulted in hydrogels that were clearer and that would form more consistently during the UV cross-linking exposure. The PEGDA hydrogel was prepared at 8% (v/v) concentration of PEGDA and 0.1% (w/v) concentration of PI based on the findings of our optimization experiments. When ready to begin electrophoresis, the hydrogel precursor was pipetted into the mold, taking from the top in order to avoid undissolved PI. A TMSPMA-coated cover glass was placed over the top of the hydrogel across the

mold. Additionally, depending on the design of the mold, we could first place a modified cover glass onto the mold and subsequently pipette liquid hydrogel precursor between the glass slides. The hydrostatic forces would hold the liquid hydrogel precursor in place for small volumes, allowing us to design molds that did not completely wall in the hydrogels. The hydrogel was cross-linked using an OmniCure S2000 lamp (Lumen Dynamics, Mississauga, ON, Canada) at 100 W/cm^2 for a range of seconds described here depending on the mold thickness and size and the PEGDA concentration. This process of pipetting the hydrogel into the mold and polymerization takes less than 15 minutes.

After our PEGDA hydrogels are cross-linked via UV photo-polymerization, we remove them from the UV OmniCure system and prepare them for removal from the molds. For molds A and B, after the liquid hydrogel precursor had cross-linked and attached to the gel, we could simply apply pressure from left to right across the cover glass in a smooth motion to slide the hydrogel out of the molds. The hydrogel would remove easily from the molds because of the smooth glass surfaces they were prepared on. Here, modifying the cover glass so that the hydrogel is attached prevents the hydrogel from preferentially adhering to the mold. The hydrogels could be removed with relative ease and with a high degree of fidelity because of the structure added by the cover glass. For mold C, we could lift the hydrogel out of the mold by peeling upward from the bottom area of the cover glass. Because this mold used plastic pieces to create impressions for the loading wells, and because this mold had four surrounding walls built to contain the hydrogel, you could not slide these hydrogels out of the mold in the

manner used for the previous. By lifting the cover glass upward from the bottom, these hydrogels could easily be removed from this mold with relative ease and still maintain the fidelity of the loading wells. Similarly, hydrogels formed in mold D could be removed by lifting upwards to pull the hydrogel from the mold.

2.3 Preparation of 1% Agarose Gel

The agarose gel was prepared according to standard procedures. To create a 1% agarose gel, 0.5 g of agarose powder (Bio-Rad, Hercules, CA) was dissolved in 50 mL of 1 x TBE buffer by bringing the solution to a boil using a hot plate and stir rod. After boiling, the mixture was removed from the hot plate and 5 µL of SYBR Safe (S.S.) (Thermo Scientific) was added and mixed. Then, the gel was poured into a casting tray (7 cm x 10 cm) and a 12 well comb was inserted. The gel was left to cool and solidify. The comb was removed and the gel and tray were then placed in the electrophoresis chamber. This process of gel fabrication takes about 30 minutes.

The DNA ladder (1 kb DNA ladders, Promega, VWR, Radnor, PA) was prepared with the DNA samples using a 5:1 dilution of the DNA sample in 6x sample loading dye (Blue/Orange Loading Dye, Promega, VWR, Radnor, PA). The results after running electrophoresis on the PEGDA hydrogel were compared to the results obtained using the 1% agarose gel.

2.5 Running the Gel

After removing either our PEGDA hydrogel or the agarose hydrogel from their mold, we placed them in the electrophoresis chamber (Mini-Sub Cell GT Horizontal Electrophoresis System, Bio-Rad, Hercules, CA) and 1 x TBE was

poured until the gel was completely submerged. Both our PEGDA hydrogel and the standard agarose gel were run using the same system and could be run under the same parameters. Typically, we would run two PEGDA hydrogels per chamber because they could fit side by side and allow us to run more samples. While our hydrogels were being cross-linked under UV excitation, we could simultaneously prepare our standard DNA ladder for loading. The prepared DNA ladders were loaded into the wells with caution. Using the proper amount of 6 µL of DNA ladder was crucial to ensuring the samples were run correctly. Additionally, we had experimented with larger wells that could hold samples as large as 12 µL, but increasing the amount of the loading sample did not improve the band resolution. Also, we tried loading as little as 1 µL of DNA ladder into our system and found that this amount was not enough to be detected consistently. After placing the hydrogel into the running tray and loading the samples, the top of the electrophoresis chamber was carefully fastened to the chamber and plugged into the power source (PowerPac Basic, 400mA, 75W, Bio-Rad, Hercules, CA).

The voltage was set between 70 and 110V for testing the optimal voltage to run the PEGDA hydrogel. Based on previous experiments conducted in our lab, we had evidence that our hydrogels could be run between 40V and 140V. Hydrogels run at less than 70V typically took much longer than an hour to separate bands. Hydrogels run at more than 110V typically had less consistent band separation, where smearing became a common issue. Although using a higher voltage corresponds to shorter running times, there can be a decrease in

the hydrogel performance in separating bands, as well as the ability to separate them in straight lines. Voltages of over 110V often resulted in misshapen curved bands. Knowing previous data from our lab experiments, we concentrated on a more focused range of voltages that also corresponded well with the standard range of voltages used in the agarose hydrogels.

For running time we tested times between 45 minutes, 60 minutes, 75 minutes, and 90 minutes. Previous experiments in our lab had tested our hydrogels at times ranging from 30 minutes to 120 minutes. Based on these initial findings, we refined the range of running times to correspond properly to the selected running voltages in our focused range. During our experimentation, we also tested running times as short as 15 minutes for proof-of-concept findings as we optimized our well geometries and loading sample volumes. Typically, each time we started to use a new type of loading well design in our molds, we would make sure that the loading wells had the right shape to concentrate the loading sample so that it would run properly. If a well geometry was incorrect, the loading sample would disperse from the loading well before it had a chance to run in the gel. Testing our hydrogels at systematic time intervals gave us evidence to support the optimization of our well geometries. For our results in this research, we focused on the range of running times that best corresponded to the range of voltages we were interested in.

2.6 Staining the Gel

In our protocol, we used Thermo Fischer SYBR Safe (S.S.) dye over Ethidium bromide because of the increased safety advantages of using this dye.

This dye is a commonly used DNA stain that provides for a lower mutagenicity upon accidental contact or exposure and has comparable band detection when imaging. A limitation of previous experiments in our lab was that in using Ethidium bromide any material that came in contact with the hydrogel must be treated as contaminated. By switching to S.S. dye, we mitigated the risk of exposure to harmful substances for those involved in our experiments. This was an important change for our experiment because in order to obtain enough data to support the optimization of our system, we needed to fabricate a large number of hydrogels, each needing to be subsequently bathed in a dye for imaging. While a typical agarose hydrogel could be made with a dilution of Ethidium bromide stain during the fabrication process and then carefully handled afterwards, we chose to use S.S. dye to prevent unnecessary exposure.

For agarose gels, S.S. dye could be premixed within the hydrogel or bathed after running. For PEGDA hydrogels we only tested bathing the hydrogels in a 1:10k concentration of S.S. to TBE buffer because premixing the dye into the hydrogel was not possible because of the photo-polymerization method of fabrication. In our study we tested both staining before and after running the gel electrophoresis. We also tested a variety of bathing times between 30 minutes and 3 hours and overnight staining, with shaking speeds between 40 and 120 rpm. Early on, we compared the hydrogels of both PEGDA and agarose using S.S. and Ethidium bromide and found no significant difference in the ability to detect the bands or the brightness of the bands during imaging. Agarose and PEGDA hydrogels were visualized using the FluorChem 8000 UV

Transilluminator (Alpha Innotech, San Leandro, CA). Using the provided computer software, we were able to set the detector options specifically for S.S. dye.

CHAPTER 3: RESULTS AND DISCUSSION

Previous work towards this project from members of our lab, combined with the knowledge of standard agarose concentrations ranging between 0.5-2% in concentration, allowed us to set the range of PEGDA hydrogel concentrations for this study. We began by forming standard agarose at a 1% concentration. We began PEGDA at an 8% concentration based on anecdotal findings on the stability of the hydrogel and the comparison in resolution to the 1% agarose.

3.1 Optimization of PEGDA concentration

In agarose gel, a variety of pore sizes allow smaller fragments to pass more easily through the gel and larger fragments to take a longer time to find paths through the pores of the gel thus separating the fragments by size. When choosing the concentration of the agarose gel for DNA electrophoresis, lower concentrations are recommended for the separation of smaller DNA fragments. Applying this principle to the PEGDA hydrogel, by lowering the concentration as much as possible, we would be able to separate smaller DNA fragments. We tested different concentrations of PEGDA to find which concentration resolved a 1kb DNA ladder best. The PEGDA concentrations of 8%, 6%, and even 4% were tested using various molds (Table 2).

Table 2. Comparison of fabrication results.

Mold	PEGDA Concentration	UV time (sec)	Gel Formation	Removes from Mold
D	8%	720	Consistent	Yes
D	6%	960	Inconsistent	Somewhat
D	4%	960	None	Never
A	8%	360	Consistent	Yes
A	6%	360	Inconsistent	Somewhat

An 8% gel was preferred because it showed the most promise for consistent fabrication as well as ability to resolve bands. As we decreased the PEGDA concentration to create a softer hydrogel, we encountered problems with handling.

In previous attempts from members of our lab, higher concentrations of PEGDA than 8% created solid slabs that could be handled easily, but these resulted in very poor band separation and would also bend over time. The bending of hydrogels is most likely attributed to the absorption of water from the air and buffer. Although we attempted up to a 4% PEGDA hydrogel in our thickest mold, this hydrogel could not be successfully removed from the mold. When using a 6% PEGDA hydrogel, the results were too inconsistent to use reliably. We encountered problems extracting these lower concentration gels from the molds, and they did not demonstrate the robustness or durability in handling that an 8% PEGDA hydrogel could support. Additionally, at an 8% PEGDA concentration, we found the optimal combination of thickness, clarity, and ease of handling.

Table 2 shows some of the data from our optimizing fabrication parameters regarding PEGDA concentration, UV exposure time, and which mold we found could produce the best results. After initial concentration findings, we focused on mold A and mold D. Mold A gave the advantage of using the least volume of hydrogel, which corresponded to lesser UV exposure times and allowed us to try to fabricate hydrogels at concentrations lower than 8% PEGDA. Also, because the hydrogel volume was so low, we could typically remove these low concentration hydrogels from the mold with more ease because there was a smaller amount of hydrogel that could stick into the mold. Mold D was an attractive option for using lower PEGDA concentrations, however, the large volume of hydrogel needed for the mold required longer UV exposure times and also caused problems when trying to remove the hydrogel from the mold. When using PEGDA concentrations lower than 8%, typically not all of the hydrogel would fully remove from the mold. This also happened with molds B and C, where part of the running lane or loading wells would be left stuck in the mold. Mold A consistently allowed for using smaller volumes of hydrogel and lower UV excitation times, and formed hydrogels that were the easiest to remove from the mold without regularly causing damage to the running area or loading wells.

3.2 Optimization of UV Exposure Time

The PEGDA hydrogel used in this study is polymerized via UV exposure. The length of UV exposure time as well as the concentration of PEGDA determines the rigidity of the resulting hydrogel. Increasing the length of UV exposure also results in more rigid and durable hydrogels. As the concentration

or thickness of PEGDA was increased, a longer UV exposure time was required to produce solid hydrogels; however, as the thickness of the hydrogel was decreased, the UV exposure time required also decreased. It was important to find the optimal UV exposure time to minimize the fabrication time, but also as not to over-crosslink the hydrogel which would lead to poor band resolution. Additionally, there was an upper limit to the amount of UV exposure time we could set for any single hydrogel in order to prevent damage to the plastic molds but also to prevent damaging the laser by overuse. This upper limit was about twenty minutes of constant UV exposure, and in our experiments we were careful not to go over sixteen minutes of UV exposure per hydrogel. At most, we would subject our hydrogels to two consecutive exposures of eight minutes, but generally as we began to optimize our system and move towards using mold A the most, we began to utilize UV exposure times less than six minutes.

Based on our findings, the PEGDA hydrogel with a concentration of 8%, PI 0.1% fabricated in mold A produced the most consistent results. Using this mold enable us to lower the UV exposure time to 360 seconds. We tested UV exposure times as little as 200 seconds for this mold and concentration, and found that at least 300 seconds could effectively cross-link the hydrogel. Increasing the UV time to 360 seconds for this mold and concentration was determined to produce the most consistent conditions for accurately running our DNA samples. As we systematically tested the UV exposure time parameter for each PEGDA concentration and our various molds, we began to refine our range based on the ability for the hydrogel to fully cross-link and remove from the mold

consistently, while also trying to decrease the overall fabrication time as well as prevent over cross-linking.

3.3 Optimization of Hydrogel Thickness

During the optimization of the PEGDA hydrogel, different molds were created. The standard agarose gel used in DNA electrophoresis was about 10 mm thick, so we designed and tested a variety of molds with varying thicknesses. Our goal was to minimize the necessary thickness of the PEGDA hydrogel needed to run the same sample DNA as the standard agarose. This would allow us to have a faster fabrication time, and would work towards making our system smaller and more efficient.

We found that decreasing the PEGDA hydrogel thickness improved many qualities of our system. First, it decreased the amount of UV exposure time necessary to cross-link the hydrogel. Second, it allowed better visualization of the DNA bands after running. Using the 10 mm thick mold D, we encountered problems detecting the DNA bands using the imager because of the gel thickness preventing the detector light to pass through and also be absorbed. The hydrogels we fabricated using mold C and B had similar clarity, and the bands in these hydrogels could also be consistently imaged, however there was still less band brightness than we expected. We noticed that the 0.5 mm thick gels produced by mold A gave us the best band detection capabilities and also gave us the brightest band strength. These hydrogels were easier to remove from the mold than the other designs that we tested, provided the best results

from the imager, and supported our goal of fabricating hydrogels with dimensions less than 1 mm.

3.4 Optimization of Running Voltage and Time

In gel electrophoresis, the running voltage is determined by a number of factors. Increasing the voltage generally requires less time for the samples to run, but also results in less resolution and can smear the DNA bands together. Decreasing the voltage requires more time to run the sample, but can result in higher resolution to an extent. Another factor is the size of DNA fragments to be separated—general guidelines require higher voltage for smaller DNA fragments. For smaller fragments (less than 1 kb), it is recommended to use 5-10V/cm to run the sample (Thermo Scientific). Based on these observations, in our PEGDA electrophoresis, we used 1kb DNA ladders and tested voltages between 70V and 110V for various lengths of time to find the optimal voltage to run small DNA fragments as shown in Figure 2 and Figure 3.

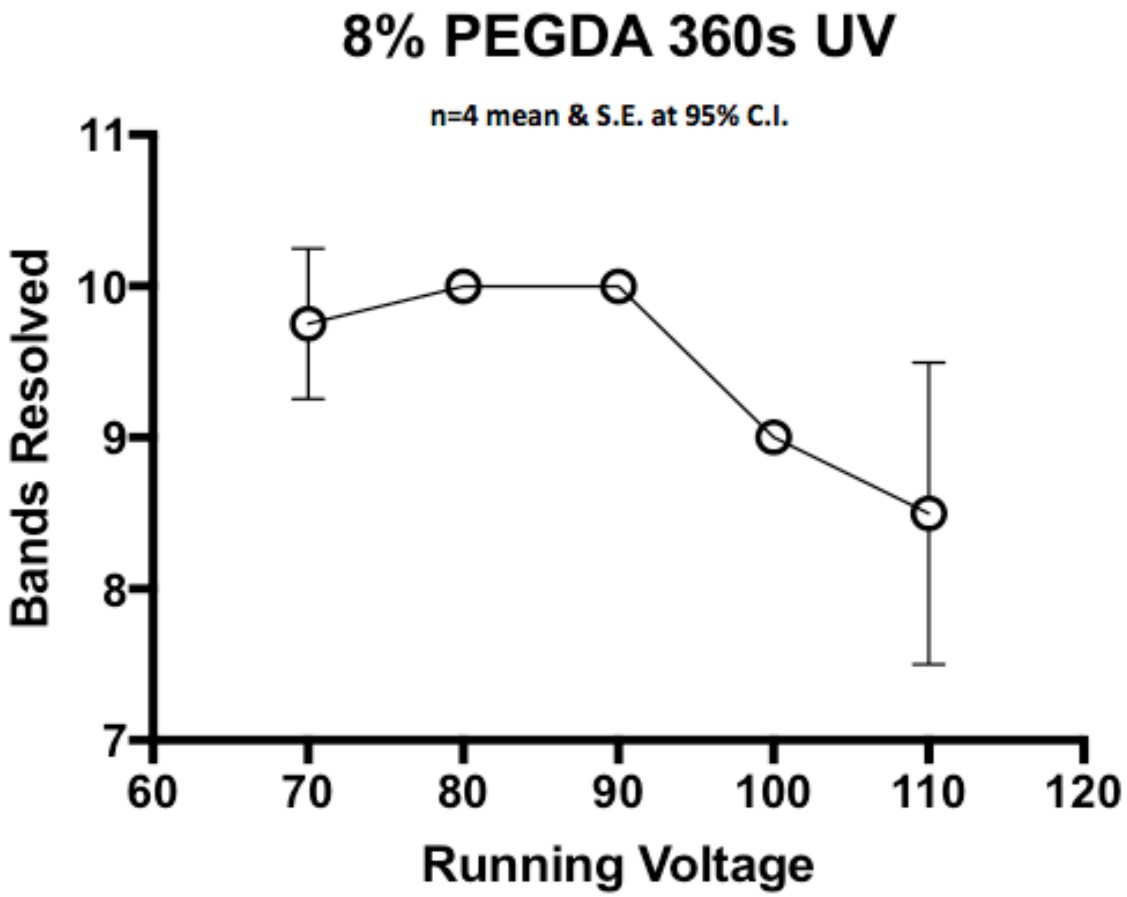

Figure 2. Average DNA band resolution of 1kb DNA ladder at varying running voltages. For all experiments, 8% PEGDA hydrogels containing 0.1% PI were photo-polymerized after 360 seconds of UV exposure.

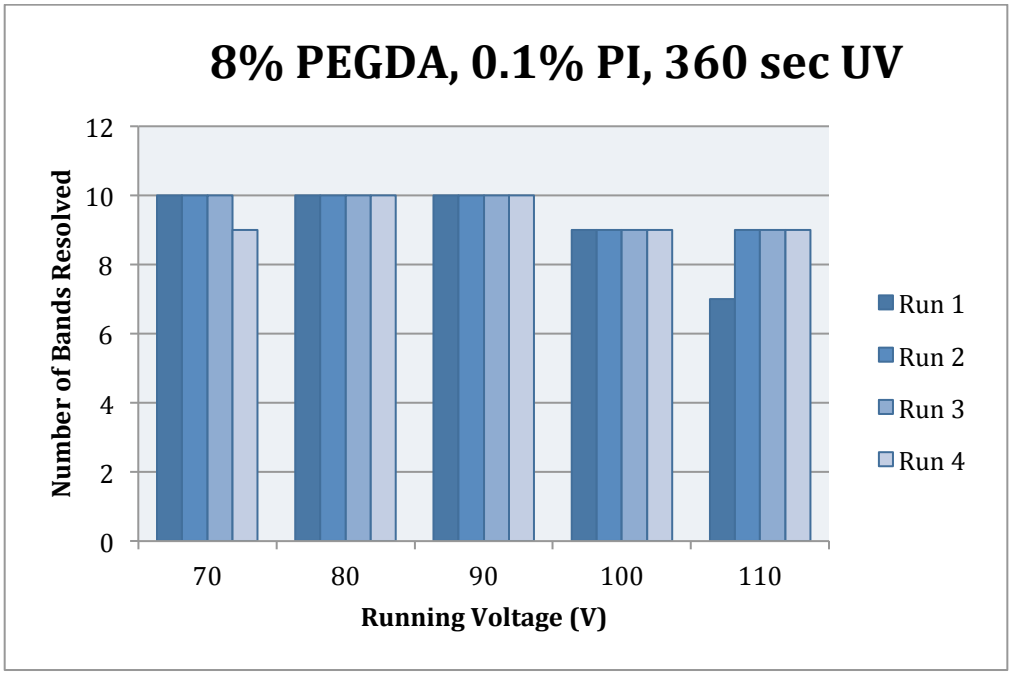

Figure 3. DNA Band Visualization of 1kb DNA ladder at varying running voltage across four separate runs.

Our results show that the optimal running time for our 8% PEGDA hydrogels falls between the range voltages between 70V, 80V, and 90V. For these results, we ran 8% PEGDA, 0.1% PI hydrogels for one hour after 360 seconds of UV exposure time. Each hydrogel was loaded with a 1kb standard DNA ladder. Running the hydrogel at higher voltages resulted in smearing of the bands and a lower detection threshold. Typically, we were able to resolve ten bands of our standard DNA ladder at voltages between 70V and 90V. These results are based on the optimization of many parameters of our system including the mold design, the loading wells, PEGDA and PI concentrations, UV exposure time, and bathing time in the S.S. DNA stain.

We also took a systematic approach at determining the optimal running voltage for our hydrogel system by running a number of PEGDA hydrogels at

various times ranging from 45 minutes to 90 minutes to determine the band separation capabilities of our system. From our data, we found that band separation occurred as early as 45 minutes (Figure 4).

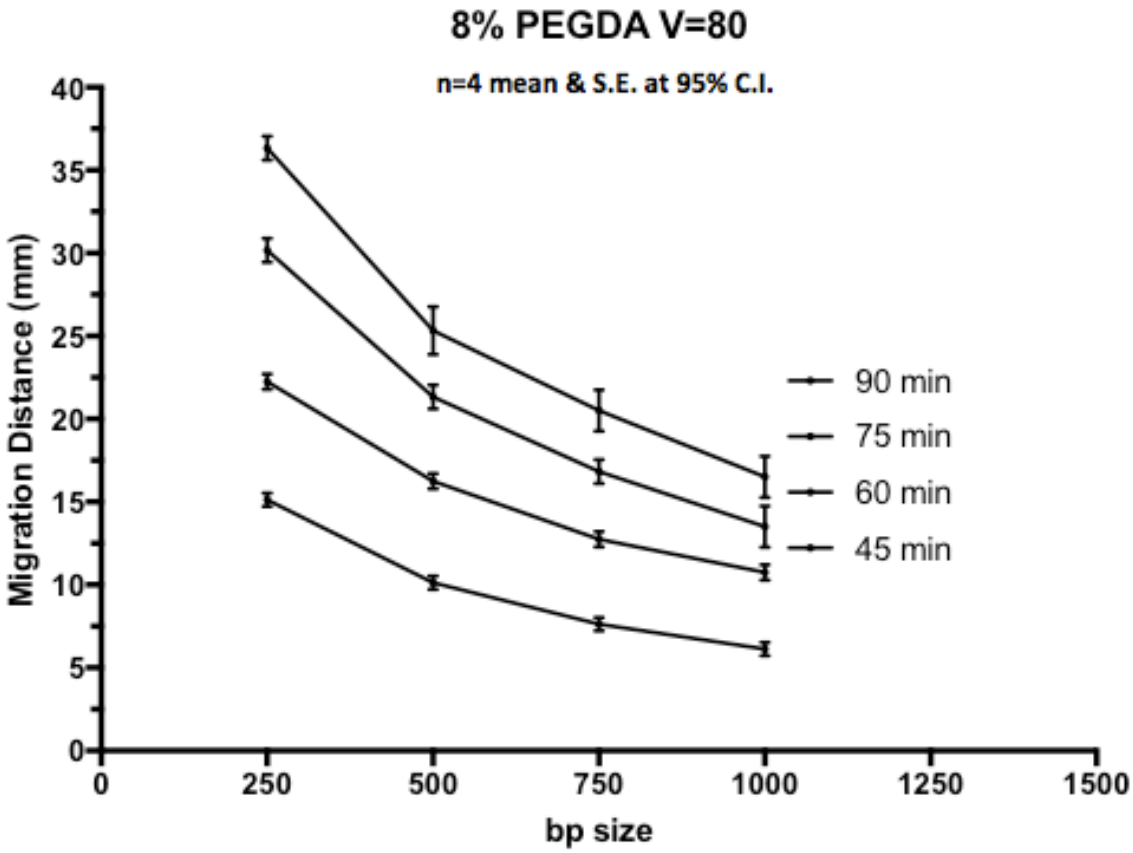

Figure 4. DNA Band Visualization of 1kb DNA ladder at varying running times across four separate runs focusing on bp range of 250-1000 bp.

In combination with our voltage data, we were able to determine using run time the average migration distances of DNA fragments in the 250-1000 base pair (bp) range. This data shows that running times as long as 75 minutes and 90 minutes are not necessary as the DNA fragments show good separation at running times in the range of 45 minutes to 60 minutes. Typically, 60 minutes proved to be a reliable run time to ensure good band separation and migration

distances at 80V for the entire DNA fragment range of bp sizes. The results in Figure 4 focus on the smallest DNA fragment sizes in our standard DNA ladder because these fragments showed the best separation relative to the other larger base pair lengths.

3.5 Electrophoresis of 1kb Ladder After Optimization

Using the data we obtained from the various running times and running voltages for our 8% PEGDA hydrogel, we found that running the gel for 60 minutes at voltages between 70-90V resulted in the most consistent DNA band resolution. Using these ranges, we characterized the expected DNA migration distances of our 1kb ladder in the 8% PEGDA hydrogel (Figure 5 and 6).

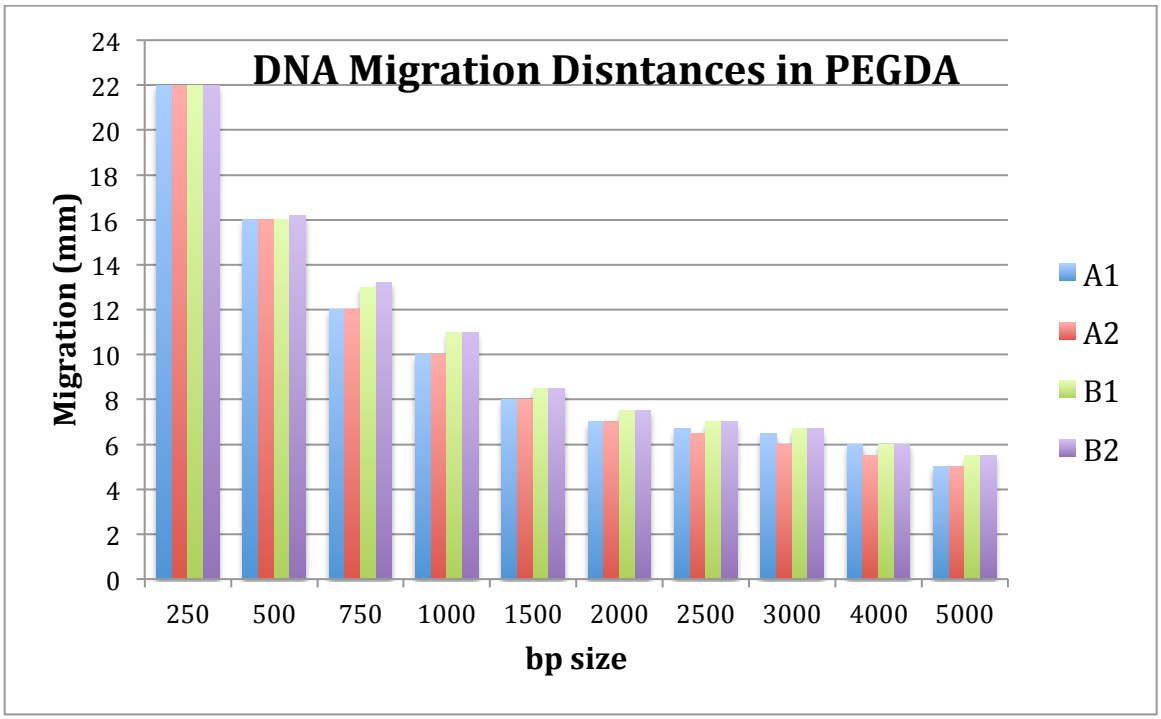

Figure 5. 1kb DNA Band Migration (mm) across 4 runs at V=80 and t=60.

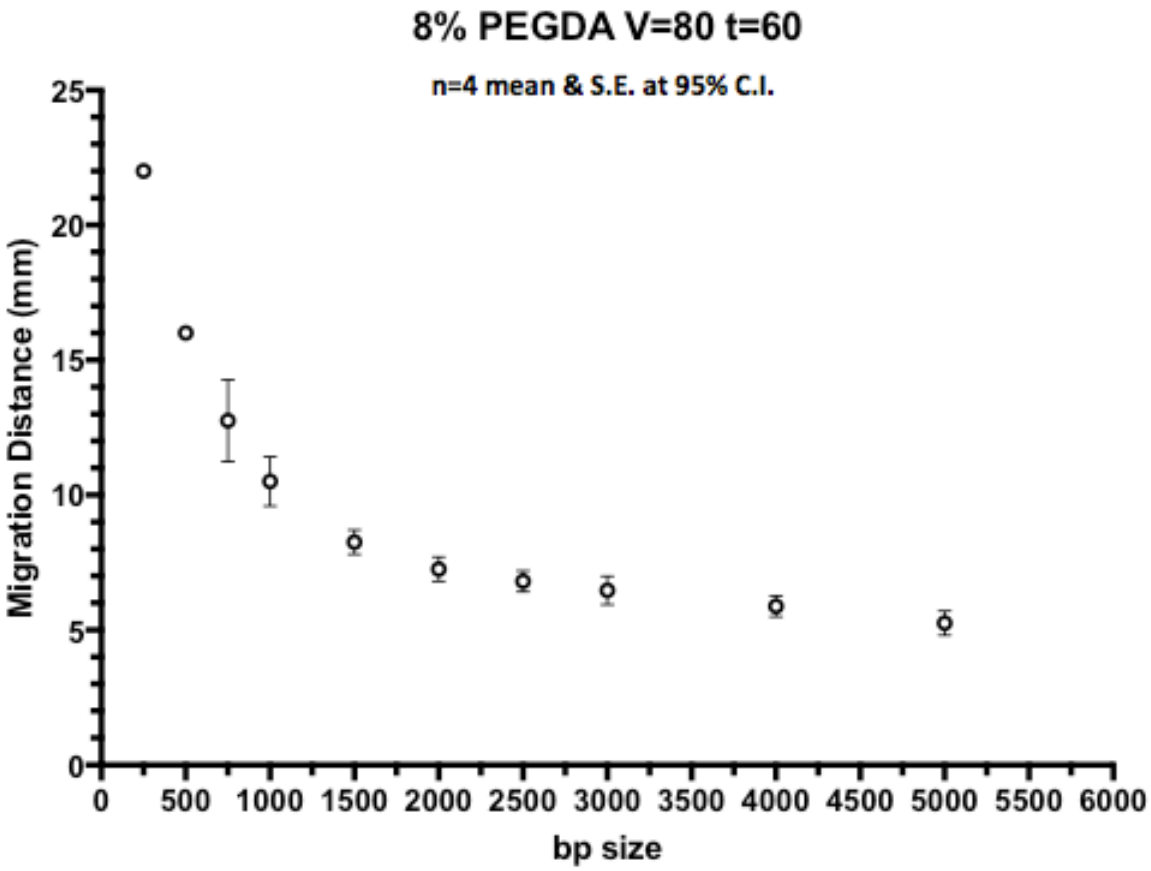

Figure 6. Average 1kb DNA Band Migration (mm) across 4 runs at V=80 and t=60.

Our results quantifying DNA band migration distances show us that for PEGDA-750 hydrogel the highest area of band separation and detection occurs between 250-1000 bp. Our 8% PEGDA data can be compared to 1% agarose in Figure 7.

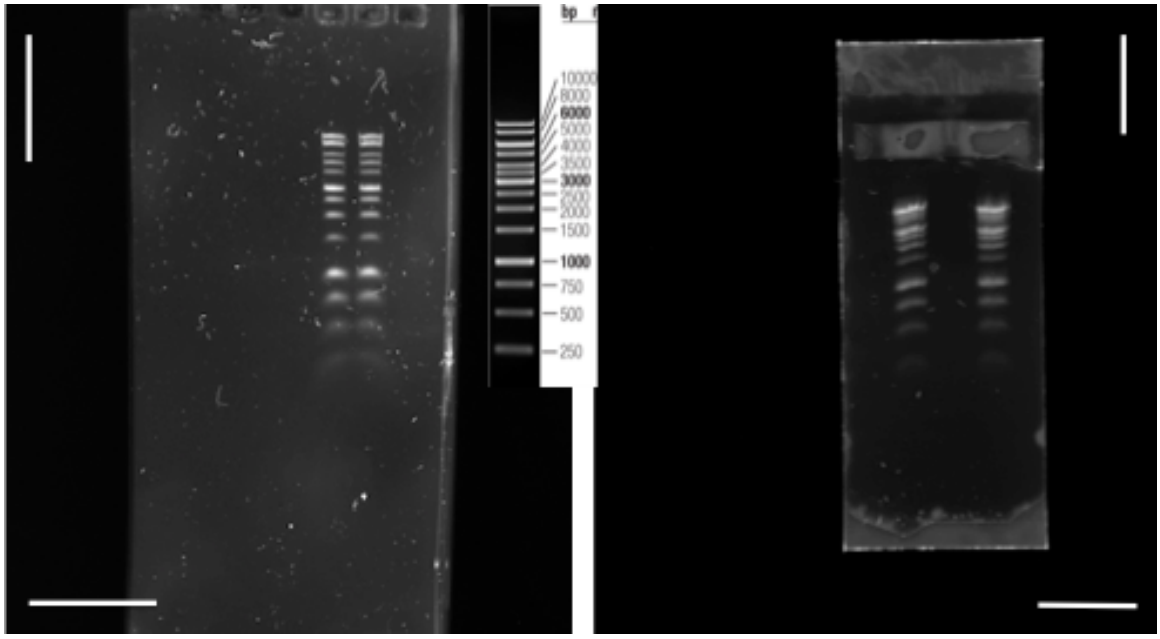

Figure 7. Comparing results obtained using 1% agarose gel electrophoresis (a) versus 8% PEGDA electrophoresis (b). Scale bar = 10 mm.

These gels show a 1kb DNA ladder run under the same conditions comparing our PEGDA hydrogel to the standard agarose gel. Each gel was run for a time of 60 minutes at 80V. In our results we can see that 8% PEGDA hydrogel performs most comparably to 1% agarose in the 250-1000 bp range, which is the smallest DNA fragments that travel the furthest in the gels. In Figure 8 we can see the separation of this range of DNA fragments relative to the range of voltages we tested across our hydrogels.

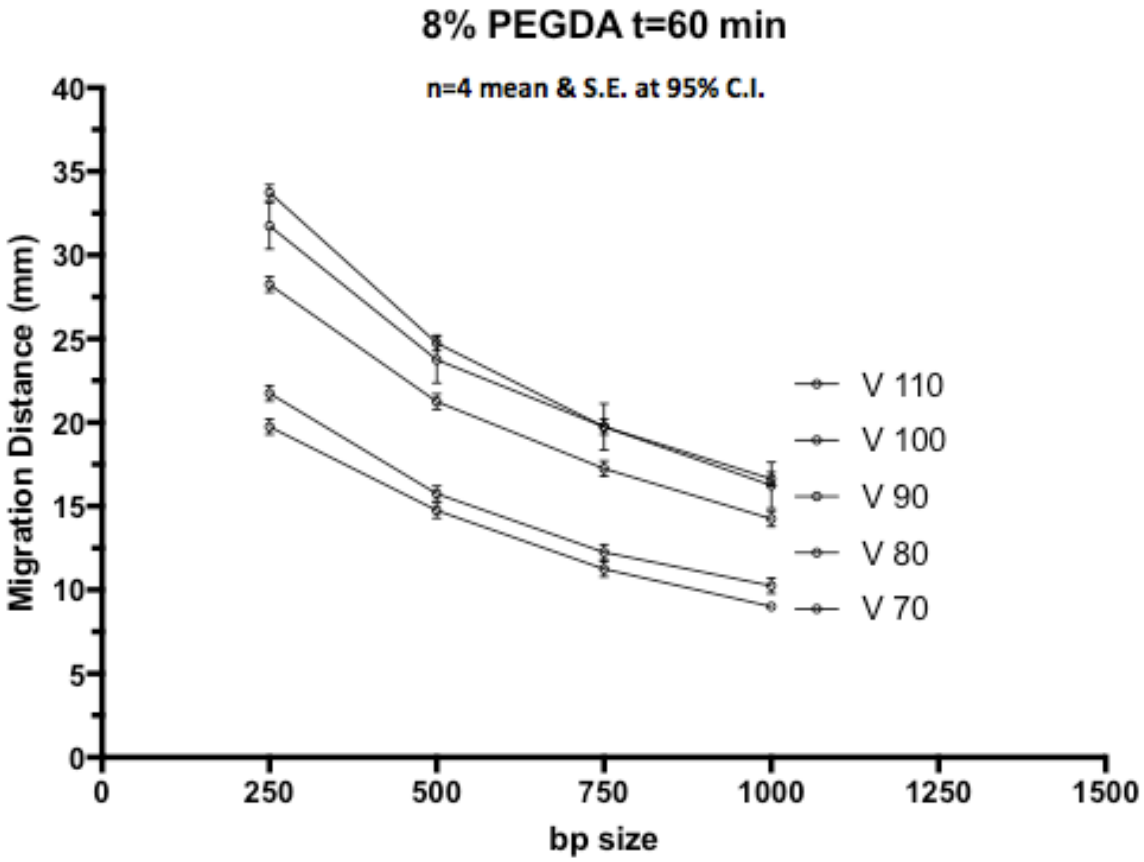

Figure 8. Average DNA Band Migration of 250-1000 bp DNA fragments at varying running voltages across four separate runs.

We can see from our data that for the DNA fragment sizes of interest (ranging 250-1000 bp) there are similar average migration distances when using running voltages between 70-80V. These voltages are sufficient for separating these DNA fragments reliably in our 8% PEGDA hydrogel.

From the data we obtained running our 8% hydrogels, as shown in figures 5 and 6, our system was able to reliably separate the 1kb DNA ladder. The optimized PEGDA hydrogel performed best at separating the smaller bp end of the range of DNA fragments and had trouble distinctly separating DNA fragments greater than 3000 bp in size. In our summary and conclusions we address why we think this is due to the pore size of our hydrogel, which we believe we can

control by varying the molecular weight of the PEGDA. In Figure 7 we are comparing the DNA separation and migration of the standard 1% agarose to our optimized 8% PEGDA hydrogel. We were able to run both of these hydrogels at the same voltage and running time to compare the performance in separating a 1 kb DNA ladder. In Figure 8 we focused on the smaller bp end of the range of the 1kb DNA standard ladder to characterize our systems performance for separating these DNA fragments across a range of voltages.

CHAPTER 4: SUMMARY AND CONCLUSIONS

In our study, we focused on optimizing the parameters surrounding the fabrication methods of our novel PEGDA hydrogel system and characterized its performance in gel electrophoresis applications. We created four distinct molds with various design parameters such as well geometry, thickness, length and width, and used these to fabricate PEGDA hydrogels with varying thicknesses to determine which combinations supported the best gel clarity and ease of fabrication. We altered the concentration of our PEGDA hydrogels and tested how the resulting hydrogels could be cross-linked and removed from our molds. We found that a PEGDA concentration of 8% resulted in the most robust hydrogels that could be handled easily during the fabrication process and the running of the gel electrophoresis procedure. This concentration also allowed our system to separate the 1 kb DNA ladder more reliably than other concentrations tested.

Across our four custom molds, we established the optimum UV exposure times to ensure cross-linking and stability of the PEGDA hydrogels. Over cross-linking resulted in gels that did not separate bands effectively, while under cross-linking resulted in gels that were not easily removed from their molds or easily handled. We also determined which of our mold designs resulted in the best hydrogel fabrication. The design we used for molds A and B proved to be the most reliable for consistently forming the loading wells and for removing the hydrogel from the mold. We also showed that decreasing the thickness of our hydrogels allowed for better imaging of the DNA fragments. For our mold A, of

thickness 0.5 mm, we found a UV exposure time of 360 seconds to be the best parameter for hydrogel formation. UV exposure times as low as 300 seconds had been tested, but in these cases well formation was not was reliable, and removing the hydrogel from the mold was not as consistent. Our 0.5 mm thick 8% PEGDA hydrogels showed to have the most clarity due to minimizing the thickness of the gel. For these hydrogels, we found that hand cut loading wells could be made more reliably than photo-mask fabricated loading wells. This was due to the tendency for the photo-mask loading wells to over cross-link, or even under cross-link, and form incorrectly.

The fidelity of the loading well geometry proved to be essential for lining up the DNA fragments and enabling the fragments to migrate in straight lines. It was also crucial to ensure the geometry of the loading wells supported the correct amount of DNA sample loading volume. When the loading well volume was too small, not enough sample would be concentrated into the running lanes once the voltage was applied. Additionally, if the loading well volume was too large then the sample would diffuse out of the loading well. We designed our molds to enable efficient loading of the DNA samples by increasing the thickness of the hydrogel only at the loading site, and maintaining a lower thickness for the running lanes of the hydrogel. We did not encounter any problems with the DNA samples failing to migrate from the bottom of the wells into the thinner portion of the hydrogel when the voltage was applied. This may have been because the glass cover slide at the bottom of the hydrogel ensures the DNA fragments migrate at the correct depth.

Loading the 8% 0.5 mm thick PEGDA hydrogels with DNA samples less than 5 microliters did not result in easily or reliably detectable DNA bands. We found that 5 microliters per well was the optimal volume of sample to be loaded, and that doubling the sample load to 10 microliters did not increase band resolution or brightness. Furthermore, we found that an optimal bathing time in S.S. dye to be no more than 30 minutes at 40 rpm following the running of the gel. Increasing bathing time or rpm of the shaker any more than this would not improve the band resolution or brightness. Initially we had tested our bathing time for as long as 3 hours to ensure the DNA stain was able to diffuse into our hydrogel and attach to the DNA fragments. The S.S. stain was effective at binding to the DNA fragments in all thicknesses of our hydrogels in as little as 3o minutes. Additionally, bathing the hydrogels in S.S. overnight did not improve band brightness on the imager.

While optimizing running parameters including time and voltage, we found that for 8% PEGDA hydrogels a run time of 60 minutes at 80 voltage gave us the most consistent band migration and separation. Furthermore, our data supports that our optimized 8% PEGDA hydrogel is able to effectively separate DNA fragments ranging from 250-1000 bp comparable to standard 1% agarose gels. Our system used PEGDA-750, and became fine tuned for best detecting DNA fragments in this range. We suspect this to be due to the pore size of the PEGDA-750 hydrogel, and that by altering the molecular weight of the PEGDA hydrogel, we could customize our system to detect specific target ranges of DNA fragments.

Our PEGDA platform combined two distinct technologies not used in standard agarose gels. First, we attach our PEGDA hydrogel to a cover glass providing stability for thinner, less than millimeter thickness scale hydrogels compared to the thicker agarose. This cover glass attachment also enables easier handling of the hydrogel during the fabrication and gel electrophoresis running process. Secondly, our PEGDA hydrogels are fabricated using UV exposure, rather than the boiling and cooling method for standard agarose gels. UV exposure cross-linking allows for the fine-tuning of the hydrogel and may be a distinction in the fabrication process that we can continue to build upon as we remain focused on optimizing our system.

Our results suggest that our platform based on PEGDA hydrogels can be used as an alternative approach for DNA gel electrophoresis, especially when targeting specific ranges of small DNA fragments. By optimizing various parameters surrounding the fabrication and running of the PEGDA hydrogel in the gel electrophoresis apparatus, we can change the characteristics of our gel and improve DNA band separation in distinct ranges. The steps taken in these initial experiments provide a detailed picture of the optimization that has gone into our system, and opens possibilities for continuing to improve our system.

CHAPTER 5: FUTURE STUDIES

The goal for our PEGDA hydrogel is to continue developing a robust, micro scale platform for detecting small nucleic acid molecules of interest. Based on the results from our study, we have systematic evidence of the performance of our hydrogel in regards to thickness of the hydrogel, PEGDA concentration, UV exposure time, running time, and running voltage. We also fine-tuned a number of fabrication parameters involving the design of our system. The aims of our future studies include characterizing PEGDA hydrogels of differing molecular weights, and refinement of our platform for ranges of DNA band separation across various base pair sizes.

We believe that by altering the molecular weight of the PEGDA, we can fabricate hydrogels with distinct ranges in pore sizes that will allow us to create custom hydrogels for detecting specific DNA fragment lengths of interest. We have established a protocol for creating PEGDA hydrogels, and also have noted how the hydrogel system reacts to changes in specific parameters. These results will allow us to fast track the process for optimizing PEGDA hydrogels of other molecular weights. We believe that increasing the molecular weight of the PEGDA could allow us to fabricate larger pores for optimally separating larger DNA fragments, while decreasing the molecular weight could allow us to create smaller pore sizes for the separation of smaller bp fragments.

A direction that we would like to take our platform is to continue to modify the cover glass in new and interesting ways to support the optimization and customization of our hydrogel. One way that we have begun to do this is shown

in Figure 9, where we used a laser cutter to remove a portion of the cover glass creating a window beneath our hydrogel in effort to improve imaging resolution and brightness.

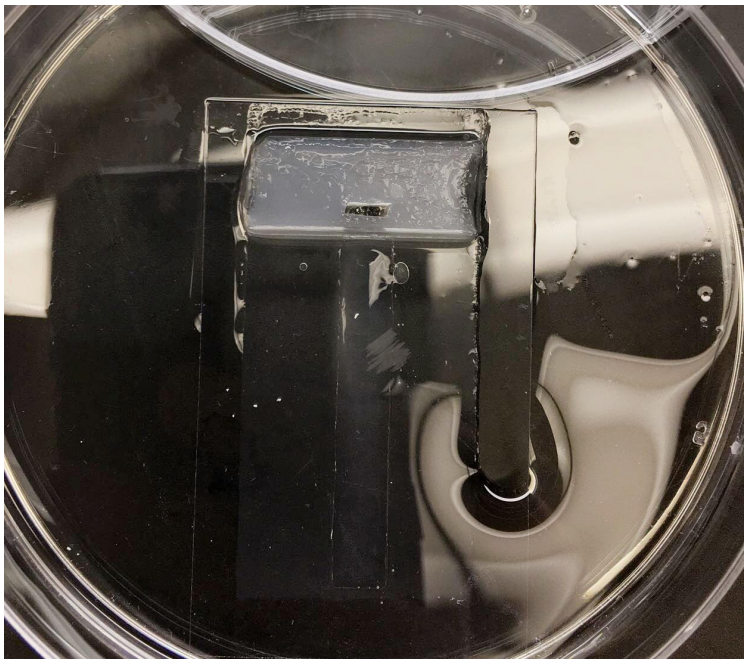

Figure 9. Fabrication of an 8% PEGDA hydrogel attached to a laser cut modified cover glass with a running window.

In Figure 9, you can see our 8% PEGDA hydrogel on a cover glass where the area of the cover glass where the DNA sample will run has been removed by a laser cutter prior to hydrogel attachment. This is possible because we are able to modify and store our cover glass slides before we use them to fabricate our hydrogels. Preliminary results are promising for increased band brightness, although many challenges are left to overcome regarding the UV exposure needed to produce this design as well as providing support for the DNA fragments to migrate properly in this windowed region.

As we continue to develop our platform, there are many possibilities to combine more technologies into our system. We would also like to test how our system performs in laboratory procedures such as the Southern blot and develop our platform in ways to support this. The advantages of our hydrogel platform include millimeter size thickness, UV exposure fabrication methods, and the potential to build onto and modify our cover glass support.

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