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Separation, Deposition, and Characterization of Single Stranded DNA on Polymer Coated Surfaces

A Dissertation Presented

by

Eli Hoory

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Abstract of the Dissertation

Separation, Deposition and Characterization of Single-Stranded DNA on Polymer Coated Surfaces

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Analysis of DNA structure and behavior, up to and including full sequencing of a genome's bases, and of biological processes such as replication, transcription and translation, is essential for an understanding of genetic variation, heritable diseases and the effects of environmental factors. Recently, single-molecule techniques have been developed to study DNA properties in unprecedented detail. For a number of these techniques, controlled adsorption of linearly stretched DNA molecules on surfaces is necessary. In experiments where hybridization of adsorbed molecules to labeled probes is used to determine DNA structure, single-stranded DNA is needed. Conventionally, for long DNA's (up to Mbp), double-stranded DNA is deposited on a surface and denatured in-situ. While successful, this method has several disadvantages. This thesis reports efforts to directly adsorb long single-stranded DNA's out of solution as an alternative strategy. It consists of three parts:

1) Establishment of a simple method using Acridine Orange (AO) staining dye to determine whether DNA's are ss or ds on the surface. The method allows for the assessment of the degree of renaturation during deposition. Incubation of surface-adsorbed DNA in solutions of AO dye in the concentration range of 10-15uM were found to be effective for discriminating between ss DNA and ds DNA based on differences in the fluorescence emission spectra.
2) Deposition of ss DNA produced by heat denaturation on polymer-coated surfaces. Lambda DNA (48502bp) was adsorbed by drop evaporation or dipping/extraction of surface out of a buffered solution. The efficiency of deposition was optimized with respect to DNA concentration, buffer type and pH.
3) Separation of complementary single strands of Lambda, mono-cut digest and HindIII digest by gel electrophoresis. Using agarose gels in concentrations ranging from 0.4% to 1.4% (weight/volume), electric fields in the range 1-4V/cm in 1x Tris-Acetate-EDTA (TAE) buffer, good strand separation could be obtained. Both DC and pulsed electric fields were used and compared. Following separation, sense and anti-sense strands of lambda DNA were extracted from gels and deposited separately onto surfaces, and length distributions of the isolated molecules were measured by fluorescence microscopy.

Table of Contents

List of Tables

List of Figures

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Last but not least, I would like to thank my parents for their care and support throughout the years. I dedicate this dissertation to them.

Chapter 1. Introduction

1.1 Background and Summary

Analysis of DNA structure and behavior, up to and including full sequencing of a genome's bases, and of biological processes such as replication, transcription and translation, is essential for an understanding of genetic variation, heritable diseases and the effects of environmental factors.[1-7] Recently, single-molecule techniques have been developed to study DNA properties in unprecedented detail.[8-16] For a number of these techniques, controlled adsorption of linearly stretched DNA molecules on surfaces is necessary.[17-23] Various methods have been used to deposit DNA on surfaces, such as air-blowing ,spin-stretching and molecular combing (for a comparison, see reference 24). The most refined method is molecular combing, [17-19] where a receding meniscus of a DNA-containing solution on a surface provides the driving force for stretching the molecules left behind on the dry surfaces. The important parameters controlling the amount of DNA adsorbed and degree of stretching (including unwanted breakage) are 1) DNA concentration, 2) pH and the nature of the buffer solution used (including added ions such as Na^{+} and Mg^{+2}, 3) speed of the receding meniscus and 4) type of surface (hydrophobic, hydrophilic, etc.). In experiments where hybridization of adsorbed molecules to labeled probes is used to determine DNA structure, single-stranded DNA is needed. Conventionally, for long DNA's (up to Mbp), double-stranded DNA is deposited on a surface and denatured in-situ. While successful, this method has several disadvantages. These include desorption of both strands (reducing yield), the randomization of the strand left behind, incomplete denaturation, and possible renaturation. This thesis reports efforts to directly adsorb long single-stranded DNA's out of solution as an alternative strategy. It consists of three parts:

1) Establishment of a simple method using Acridine Orange (AO) staining dye to determine whether DNA's are ss or ds on the surface. The method allows for the assessment of the degree of renaturation during deposition. Incubation of surface-adsorbed DNA in solutions of AO dye in the concentration range of 10-15μM were found to be effective for discriminating between ss DNA and ds DNA based on differences in the fluorescence emission spectra.

2) Deposition of ss DNA produced by heat denaturation on polymer-coated surfaces. Lambda DNA (48502bp) was adsorbed by drop evaporation or dipping/extraction of surfaces out of a buffered solution. The efficiency of deposition was optimized with respect to DNA concentration, buffer type and pH.

3) Separation of complementary single strands of Lambda, mono-cut digest and Hind III digest DNA by gel electrophoresis. Using agarose gels in concentrations ranging from 0.4% to 1.4% (weight/volume), electric fields in the range 1-4V/cm in 1x Tris-Acetate-EDTA (TAE) buffer, good strand separation could be obtained. Both DC and pulsed electric fields were used and compared. Following separation, sense and anti-sense strands of lambda DNA were extracted from gels and deposited separately onto surfaces, and length distributions of the isolated molecules were measured by fluorescence microscopy.

Chapter 2: Sensitive differentiation of ds and ss DNA with a simple method using Acridine Orange

2.1 Introduction

A simple and sensitive method of determining if single-stranded DNA (ssDNA) is present on the surface, with the capability of discriminating between absorbed ssDNA and double-stranded DNA (dsDNA) is highly desirable. Most nucleic acid fluorescent dyes exhibit nearly identical emission spectra from ssDNA and dsDNA. The dye Acridine Orange (AO)[26-34] is special in this regard, having significantly different emission spectra for dsDNA and ssDNA. The interaction of AO with dsDNA occurs primarily by intercalation, while for ss DNA (and RNA), the interaction is believed to be due to electrostatic forces, and multiple AO molecules can stack onto the nucleic acid chains.[31] The emission maximum for dsDNA is 525 nm (green), while that for ssDNA occurs at 650 nm (red), conveniently distinguishable spectroscopically. Furthermore, the sensitivity is adequate to image single molecules on surfaces, though less sensitive than some other dyes − such as YOYO (Invitrogen) – which do not discriminate between dsDNA and ssDNA. The excitation maximum for AO occurs at 502 nm for dsDNA and 460 nm for ssDNA. The 488 nm line (blue) of an Ar laser may be used to probe both types simultaneously.

Although AO staining is useful for ssDNA/dsDNA discrimination, there is a drawback in that the level of discrimination is dependent on the amount of AO attached to the DNA and also to the AO present that is not attached to the DNA (either in solution or on surfaces).[29] In some circumstances, the discrimination may be lost altogether. For

example, at high attachment levels, both dsDNA and ssDNA fluoresce primarily in the red, while at very low levels, both types of DNA emit mostly in the green. To avoid this unwanted effect, the conditions for staining must be tuned to the given system, in our case, for DNA molecules stretched onto polymer-coated silicon substrates.

2.2 Experimental Section

Lambda phage DNA (500ug/ml) was purchased from New England Biolabs. The DNA solution was diluted to 5ug/ml a with a 6:50 NaOH: 2-(*N*-morpholino)ethanesulfonic acid (MES,fluka), composed of 6 parts (by volume) of 0.1M NaOH and 50 parts of 0.02M MES. This mixture was previously found to be effective for adsorption and stretching of DNA on polymer-coated surfaces. Acridine Orange (10mg/ml, or 33.13mM) was obtained from Invitrogen and 10X Tris Borate EDTA (TBE), 0.89M Tris-borate, 0.89M boric acid, and 0.02 M EDTA was purchased from Fisher Scientific. Polymethylmethacrylate (PMMA)(255Kg/mole, Pressure Chemical) diluted in toluene was used for spin casting films onto polished silicon substrates.

A Leica Confocal Microscope (TCS SP2) was used to image the DNA and to obtain fluorescence emission spectra. The microscope has three lasers (Ar, green HeNe and red HeNe) and a prism-based spectrometer for measuring emission spectra. Two photomultiplier tubes can be used simultaneously to record light intensity. A silicon-intensified target video camera (SIT 66, MTI) is also fitted for conventional (non-confocal) fluorescent imaging using emission filter cubes. The time to collect a typical emission spectrum is about five minutes. Magnifications of 10x, 40x and 150x were used and all samples were imaged in air on dried samples.

Polymer films were spun-cast using a Headway Research spinner and film thickness was determined by ellipsometry (Rudolf Auto El). A custom-built vacuum system with a turbo pump (Pfeiffer) and ion pump (Perkin Elmer), base pressure $5x10^{-7}$ torr, was used to vacuum anneal polymer –coated samples at 105 – 115^{o}C for approximately 12 hours prior to DNA adsorption experiments.

Silicon Substrated Cleaning Procedure. The modified Shiraki technique[35] for cleaning silicon was used. To remove organic contamination on the surface, Si substrates of area 1x1cm were placed in a beaker containing a 1:1:4 ratio of Hydrogen Peroxide, Ammonium Hydroxide, and de-ionized Water. The beaker was then placed on a hot plate set at 150^{o}C for ten minutes. The substrates were rinsed with water and then put in a second solution(1:1:3 ratio of hydrogen peroxide, ammonium hydroxide, and de-ionized Water) for ten minutes at room temperature to remove ionic/metallic impurities. Once the substrates were clean, a single one was placed on a spin caster and a 15mg/ml solution of PMMA diluted in toluene was used to cover the sample. The spin caster was set to spin at 2500rpm for 30 seconds. The resulting films were measured to be 600-700 Å thick by ellipsometry.

Preparation of Stock solutions. Ds-Lambda phage DNA was diluted with 0.1M NaOH and 0.02M MES in a 6:50 ratio. ss-Lambda phage DNA was prepared by heating 20ul of the ds DNA at 95^{o}C for ten minutes and snap cooling in ice water for 1.5 minutes. The acridine orange dye was prepared by diluting the stock solution (10mg/ml) using both the 6:50 0.1M-NaOH/0.02M-MES solution and 1X TBE. The following dilutions using 6:50 NaOH/MES were made with AO: 1.82 ug/ml, 2.00 ug/ml, 2.25 ug/ml, 2.50 ug/ml, 2.86 ug/ml, 3.3ug/ml, 4.00ug/ml, 5.00 ug/ml, and 6.67 ug/ml. The following

dilutions using 1X TBE were made with AO: 1.82 ug/ml , 2.50 ug/ml, 3.33 ug/ml, 4.00 ug/ml, 6.67 ug/ml.

Sample Preparation for Imaging. A home built apparatus was used to place a regular array of drops of volume 0.5- 2ul of DNA solution on the surfaces. Double and single stranded Lambda DNA drops of concentration 5ng/ul were deposited on the PMMA-coated silicon substrates. Several drying conditions were attempted. The most consistent protocol used for the study was as follows: droplets were exposed to air at room temperature for 4 minutes after being deposited, the substrate was then placed on a hot plate set at 60^{o}C for 4 minutes, the substrate was removed from the hot plate and allowed to cool for another 4 minutes at room temperature. The substrate was placed in a teflon holder containing the AO dye diluted in either 6:50 0.1M-NaOH/0.02M-MES or 1X TBE 0.89M Tris-borate, 0.89M boric acid, and 0.02 M EDTA for a 1 hour incubation. This time was picked for convenience and was left constant for all runs. The main parameter varied was the AO concentration. Upon completion of the incubation, the substrate was placed in either a 6:50 0.1M-NaOH/0.02M-MES or 1X TBE solution for 1.5 minutes to rinse any excess dye.

The dried samples were imaged with the confocal microscope. The following parameters were used for all imaging: excitation wavelength was set at 488nm, emission wavelengths were split into two intervals, one for the green region 505 – 575nm and one for the red region, 595-665nm, the confocal pinhole was set at 400um. When imaging single molecules, the photomultiplier voltage of each emission detector was set to 690 volts (high gain). The excitation laser power was set to 80% of the maximum. For DNA

rings, the excitation laser power was set to 20% of the maximum. An emission wavelength scan was taken from 505nm to 665nm in 25 steps via a 10nm wide emission detector window.

2.3 Results and Discussion

DNA diluted in 0.1M-NaOH/0.02M-MES solution has shown linearly stretched conformations on surfaces. Individual ds and ss lambda phage molecules were isolated and analyzed for intensity with Leica image analysis software. A region of interest (ROI) was selected and a ROI of the same area but displaced next to the DNA was used to obtain the background for subtraction. The ss/ds intensity ratios of single linearly stretched dna molecules were taken at various concentrations to optimize the discrimination between single and double stranded DNA. In the following, the emission spectra are characterized by the "intensity ratio ss/ds," defined as

$$R = \frac{I^{\text{background substrated}}_{595\text{nm}\rightarrow 665\text{nm}}}{I^{\text{background substrated}}_{505\text{nm}\rightarrow 575\text{nm}}} = I_{red}/I_{green}$$

For conditions showing good discrimination, we expect $R_{dsDNA} < 1$ and $R_{ssDNA} > 1$. In principle, for samples having a mixture of dsDNA and ssDNA. R may be used to estimate the amount of each present.

Figures 2.1 and 2.4 show the results for a series of dilutions of AO in 6:50 0.1M-NaOH/0.02M-MES solutions that range from 1.82μg/μℓ to 6.67μg/μℓ. The ratio R is calculated as the average over the isolated single molecules visible in the fluorescence images. Typical "red" and "green" images for ssDNA and dsDNA (Figure 2.2 and Figure 2.3, respectively), using 2.5μg/μℓ AO solutions in 6:50 0.1M-NaOH/0.02MMES. For the

range of concentrations of Figure 2.1, the ratio R varies from about 1.0 to 2.0, approximately linearly.

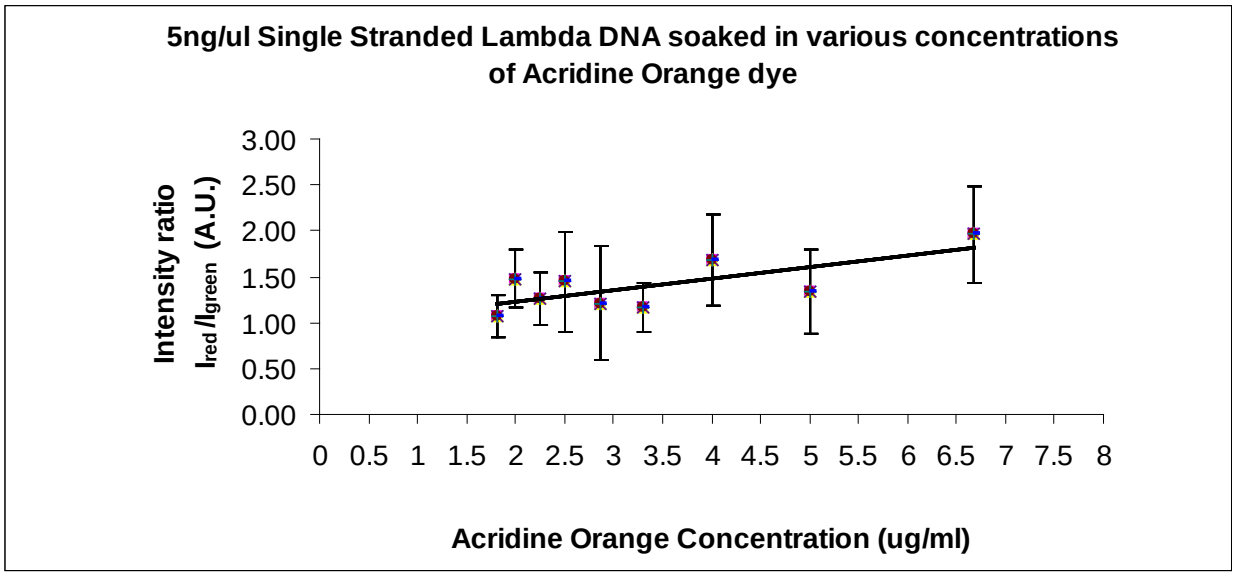


Figure 2.1 Analysis of Background subtracted ss/ds emission intensity ratios of ssDNA diluted in (6:50) 0.1M-NaOH/0.02MMES

The fluorescence is more red than green, but the green component is substantial, as is evident from Figure 2.2.

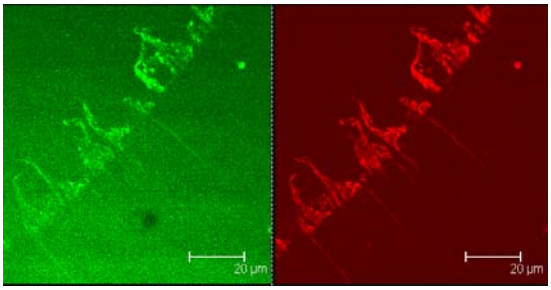


Figure 2.2: ss Lambda Phage DNA molecules (5ng/ul) on 900Angstrom coated PMMA silicon. DNA dyed in 2.5ug/ml Acridine Orange

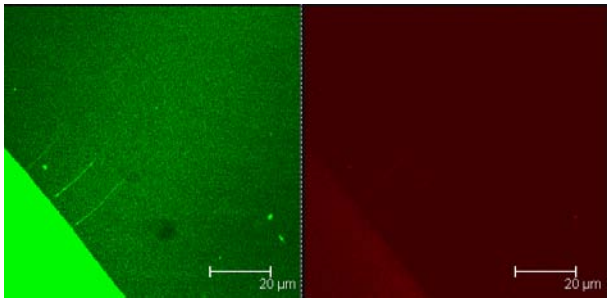


Figure 2.3: ds Lambda Phage DNA molecules (5ng/ul) on 900Angstrom coated PMMA silicon. DNA dyed in 2.5ug/ml Acridine Orange

Over the same range, the ratio varies from 0.3 to 1.8 for dsDNA single molecules, as seen in Figure 2.4

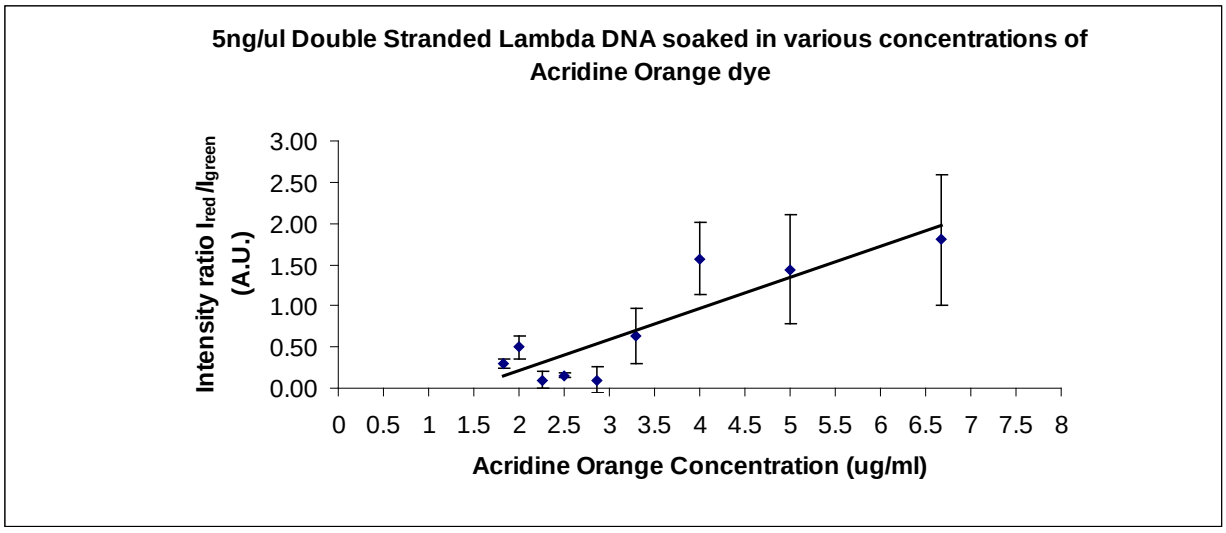


Figure 2.4 Analysis of Background subtracted ss/ds light emission intensity ratios of dsDNA diluted in 6:50 0.1M-NaOH/0.02M-MES

At the highest three concentrations, 4, 5 and 6.67µg/µℓ AO, the emission spectra are more reddish than greenish, having the ratios R similar to those ssDNA at the same concentrations, (hence no discrimination, within experimental error). In contrast, all the lower concentrations, 1.82 to 3.3µg/µℓ of AO, ssDNA spectra are more red than dsDNA, indicating good discrimination. The optimum concentrations are 2.25 to 2.86µg/µℓ,

where $R_{ss} / R_{ds} \cong 10$, indicating excellent contrast. The useful concentration is seen to be ±10% from the central value (2.5μg/μℓ), not unduly narrow.

When DNA molecules are deposited onto surfaces by evaporation of drops containing DNA, often the molecules end up concentrated in rings at the edge of the drop, due to preferential evaporation at the contact line written and the resultant convection. In many cases, a sample will have both isolated molecules and rings.

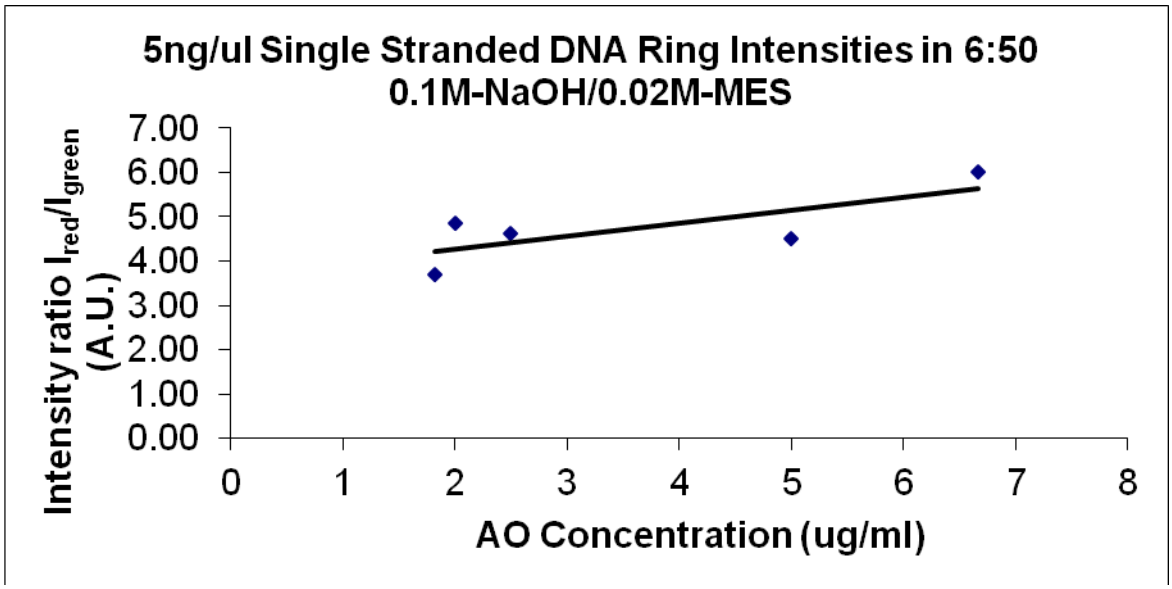


Figure 2.5 Analysis of Background subtracted Ired/Igreen light emission intensity ratios of ssDNA diluted in 6:50 0.1M-NaOH/0.02M-MES

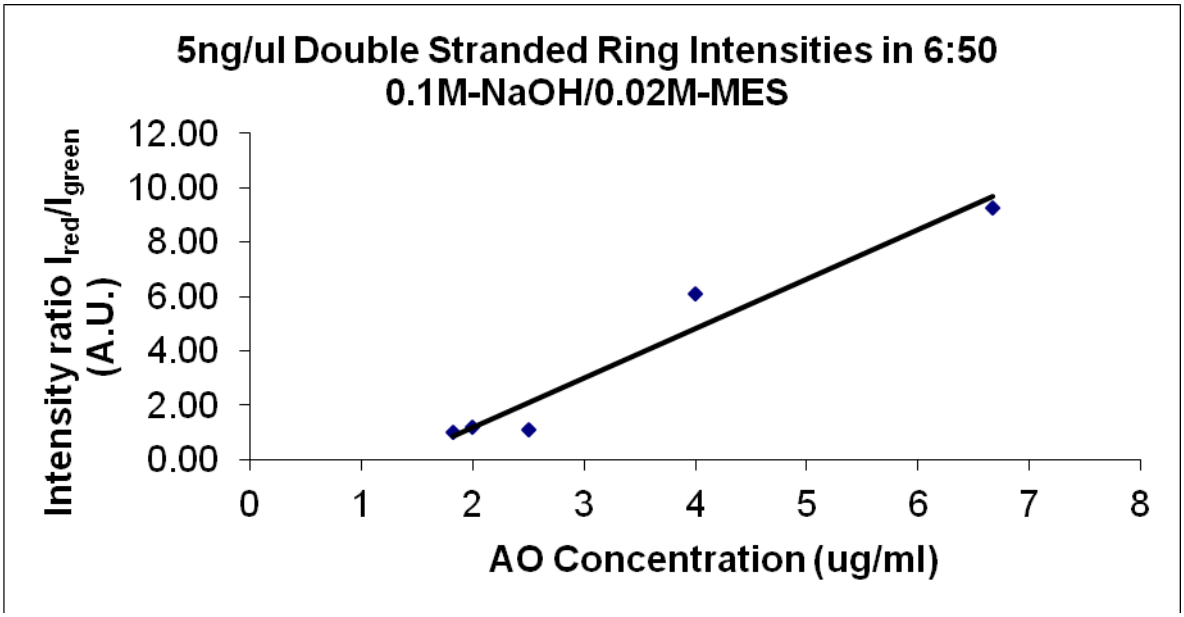


Figure 2.6 Analysis of Background subtracted Ired/Igreen light emission intensity ratios of dsDNA diluted in 6:50 0.1M-NaOH/0.02M-MES

In Figures 2.5 and 2.6 are shown the red-to- green intensity ratios for rings of ssDNA and dsDNA deposited on PMMA surfaces. As above, both the DNA solutions and AO staining solutions were made with 6:50 0.1M-NaOH/0.02M-MES. Qualitatively, the trend with increasing AO concentration is similar to the case of isolated molecules with an increased fraction of red intensity present in all samples. This may be due to trapped, unattached dye molecules present in the regions of highly concentrated DNA. There is still good discrimination between ssDNA and dsDNA over the AO concentration range 1.82μg/μℓ to 2.5μg/μℓ. However, at the highest concentration of AO, the contrast is actually reversed, with dsDNA emitting more red intensity to green than for ssDNA. Clearly, calibrating and controlling the staining conditions is important for obtaining reliable results.

The images of the rings with concentrated DNA have sufficient intensity such that a full emission spectrum is easily obtained. Figures 2.7 and 2.8 show rings and corresponding emission spectra of Figures 2.5 and 2.6 using the optimum AO concentration of 2.5μg/μℓ. As can be observed, the spectra are drastically different, and the level of differentiation is excellent.

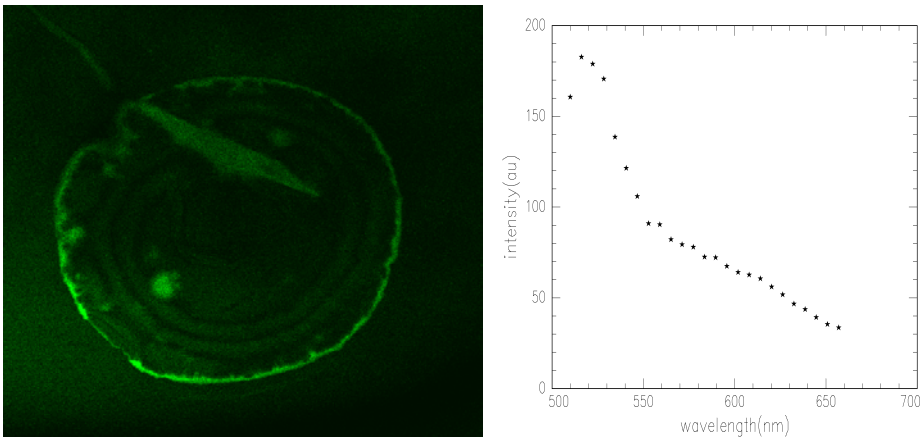


Figure 2.7: Left Image; Double stranded DNA ring on PMMA coated silicon. The ring were soaked in 2.5ug/ml Acridine Orange which was diluted in 6:50 0.1M-NaOH/0.02M-MES. Right
Image; Intensity Spectra

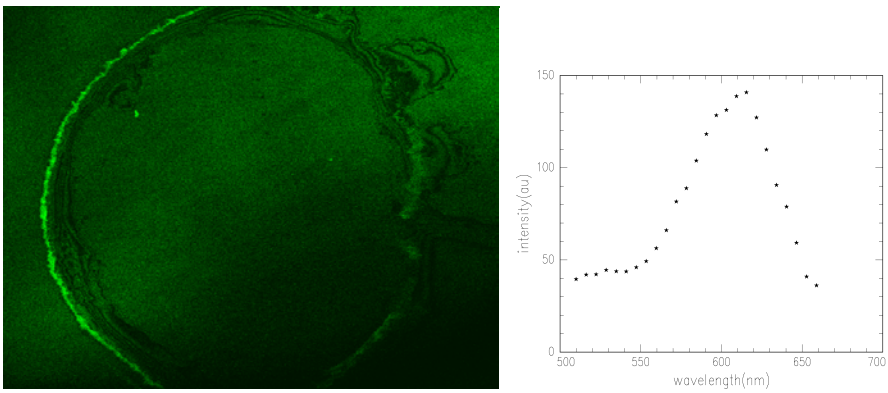


Figure 2.8: Left Image; Single stranded DNA ring on PMMA coated silicon. The ring was soaked in 2.5ug/ml Acridine Orange which was diluted in 6:50 0.1M-NaOH/0.02M-MES. Right Image; Intensity Spectra

Change of buffer staining. There are a number of factors which affect the degree of staining of the DNA molecules by AO. One significant factor which could influence the staining is the buffer used for the staining solution. A buffer commonly used for DNA gel electrophoresis is Tris-borate EDTA (TBE, 1X TBE is composed of 89mμ Tris base, 89mμ boric acid and 3mμ EDTA). A series of staining of staining experiment was conducted under conditions similar to those described in the last section but substituting a 1x TBE in place of 6:50 0.1M-NaOH/0.02M-MES. The buffer used for the DNA solutions for absorption was unchanged (6:50 0.1M-NaOH/0.02M-MES). The results, analyzing the emission from isolated single molecules, are shown in Figures 2.9 and 2.11 for ssDNA and dsDNA, respectively.

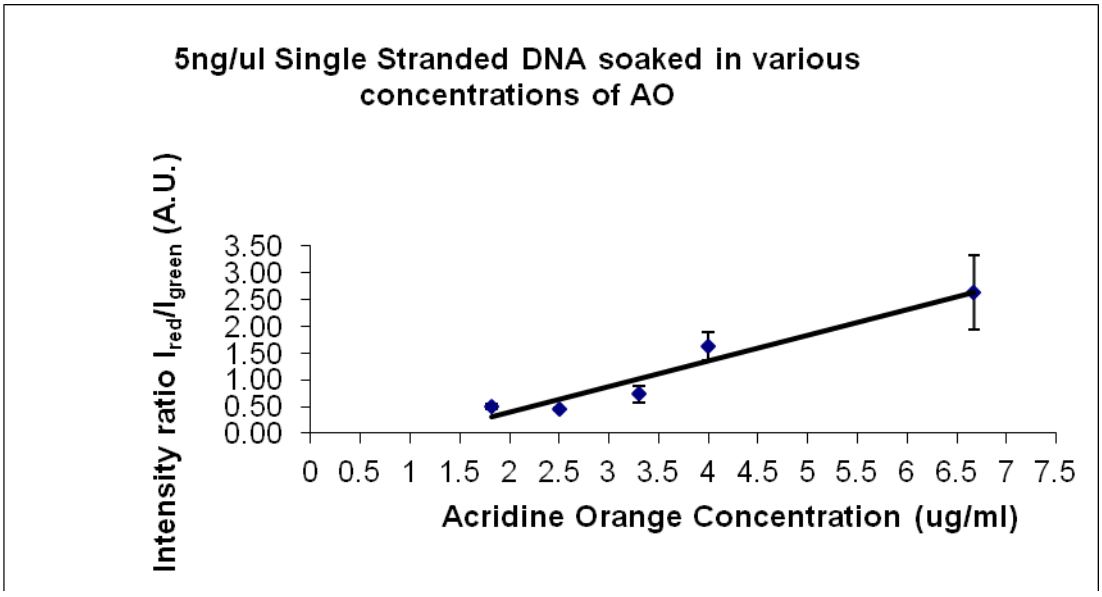


Figure 2.9 Analysis of Background subtracted light emission intensity ratios of ssDNA diluted in 1x TBE

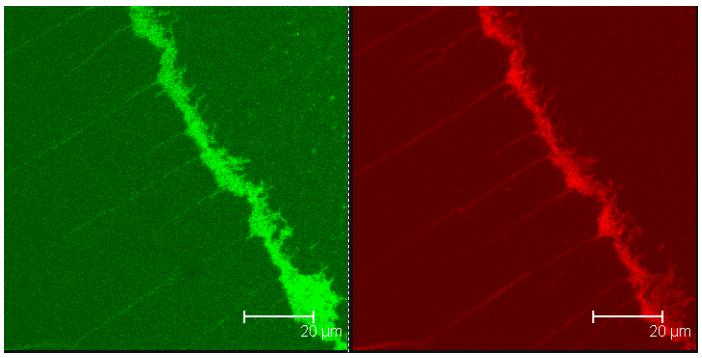


Figure 2.10 ssDNA strands soaked in 4ug/ml A.O that was diluted in 1X TBE

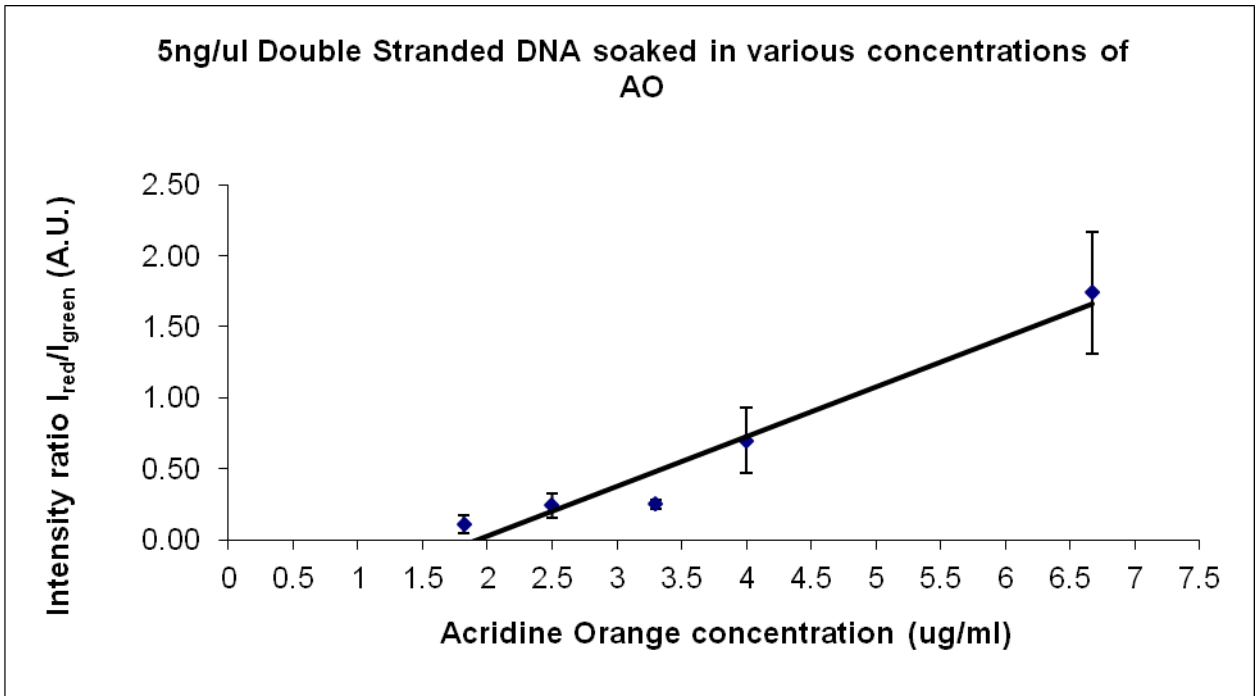


Figure 2.11 Analysis of Background subtracted Ired/Igreen light emission intensity ratios of dsDNA diluted in 1x TBE

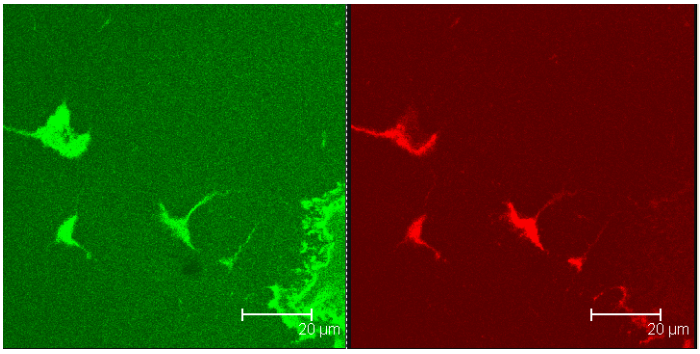


Figure 2.12 dsDNA strands soaked in 4ug/ml AO that was diluted in 1X TBE

Qualitatively, the results are similar to those using NaOH/MES staining buffer, with the best discrimination found for an AO concentration of 4µg/µℓ (where ssDNA emits strongly in the red, dsDNA in the green). However, at both extremes of concentration, 1.82µg/µℓ and 6.67µg/µℓ, strict color discrimination is lost – at the lower concentration both ssDNA and dsDNA emit more green than red, while at the higher concentration both emit more red than green. However, the ssDNA does have a higher red/green ratio than dsDNA for all concentrations. This means that they may still be differentiated but only when calibrated using both types of DNA.

Similarly, emission spectra and intensity ratios were determined for DNA in rings using 1 x TBE as the staining buffer (Figures 2.13 and 2.14).

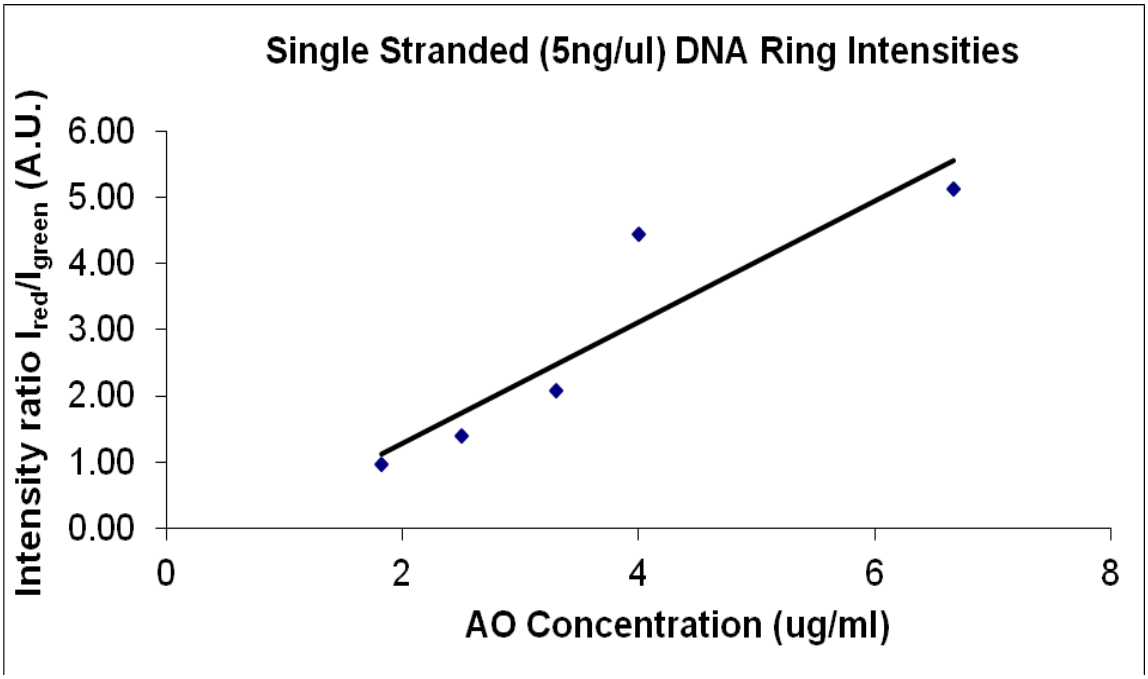


Figure 2.13 Analysis of Background subtracted Ired/Igreen light emission intensity ratios of ssDNA rings diluted in 1x TBE

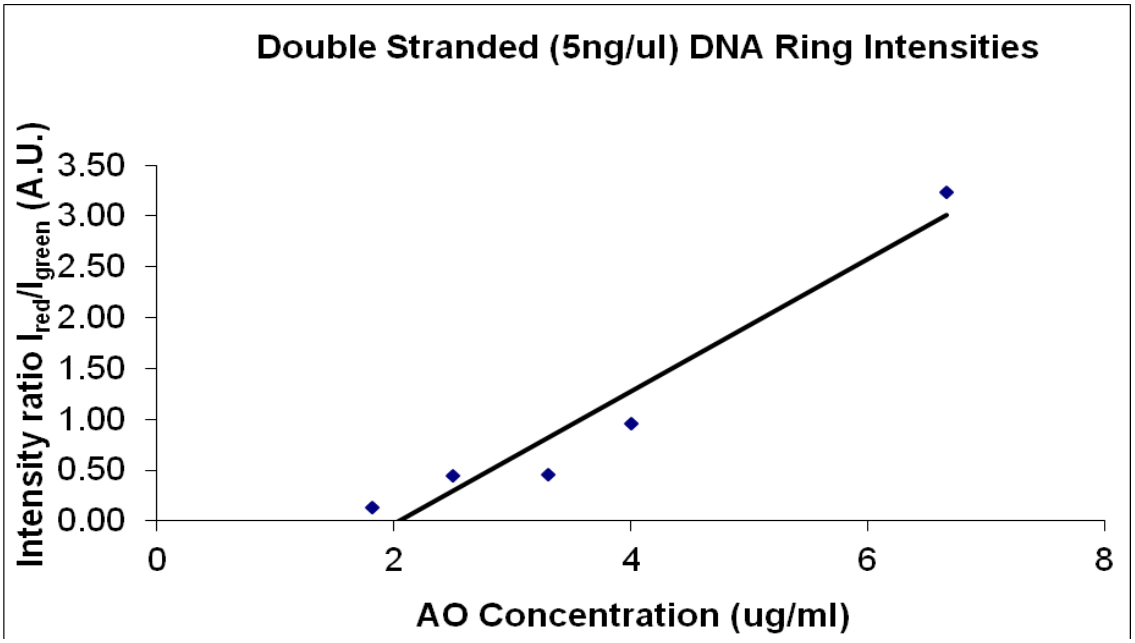


Figure 2.14 Analysis of Background subtracted Ired/Igreen light emission intensity ratios of ssDNA rings diluted in 1x TBE

The range of concentrations showing strict color discrimination is somewhat larger, from 2.5μg/μℓ to 4μg/μℓ. Again, even when both types of DNA have similar spectra, ssDNA has a higher red/green intensity ratio.

Effect of substrate surface. Another factor which may influence the staining is the nature of the surface. Different surfaces have a wide range of DNA-surface interactions which will influence the DNA conformations on the surface and affect the possible ways that dyes molecules absorb to the DNA and the substrate (which creates background emission). The polymer poly-ℓ-ℓysine (PLL) is widely used for strongly adhering DNA to a substrate, due to its becoming positively charged when in contact with aqueous buffers. Negatively charged DNA will be attracted electrostatically to PLL, while the positively charged AO molecules would not be expected to absorb well to a bare PLL surface. Therefore, PLL is an interesting surface to compare to the less polar PMMA surface. Figures 2.15 to 2.18 show emission spectra and intensity ratios for DNA rings on PLL-coated glass slides (LabScientific, Inc.).

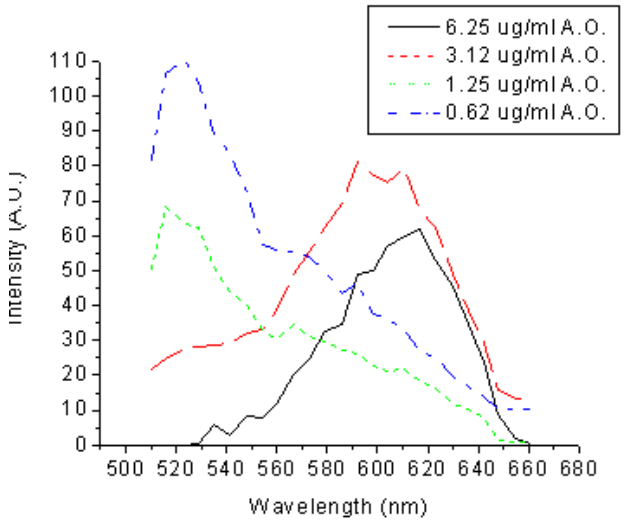


Figure 2.15 Analysis of Background subtracted light emission intensity of ssDNA rings diluted in 1x TBE at different concentrations

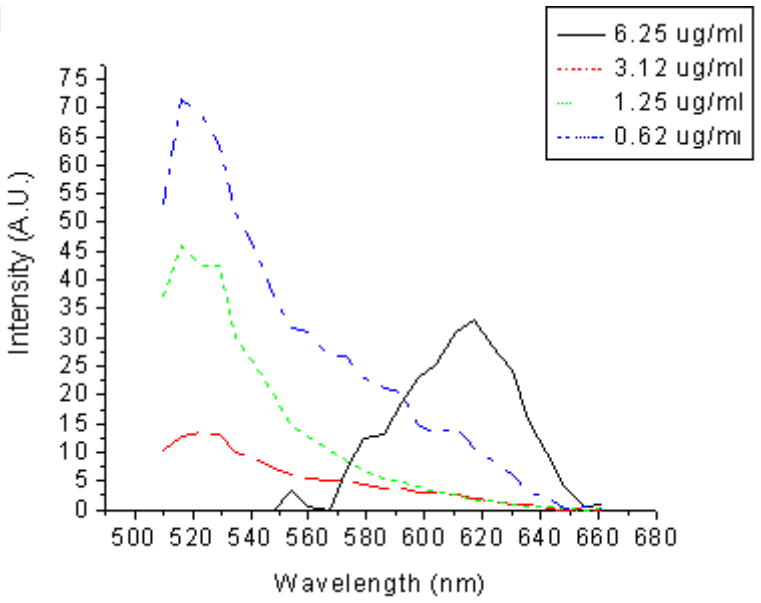


Figure 2.16 Analysis of Background subtracted light emission intensity of dsDNA rings diluted in 1x TBE at different concentrations

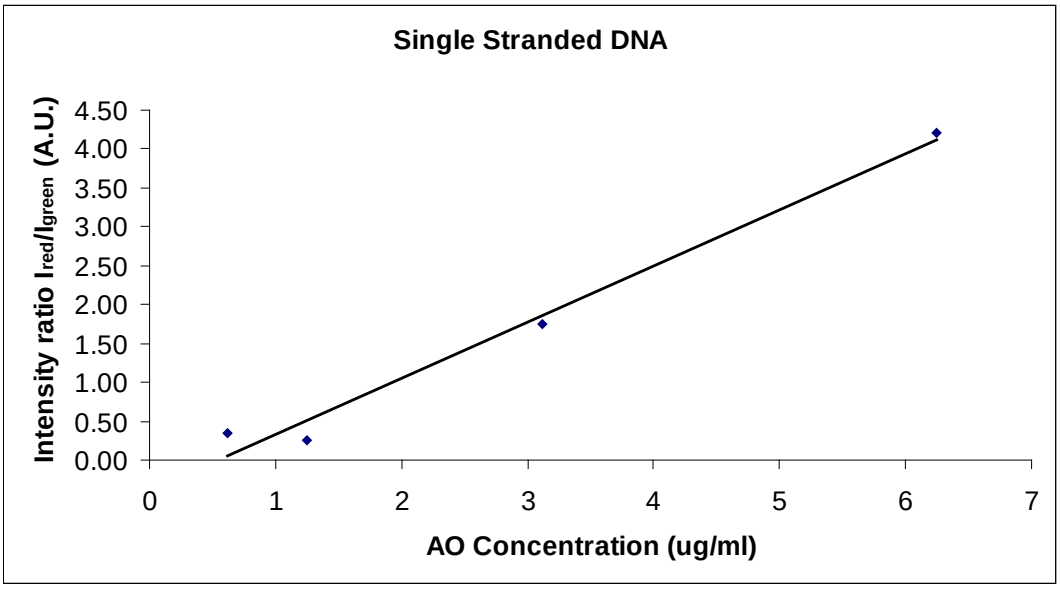


Figure 2.17 Analysis of Background subtracted Ired/Igreen light emission intensity ratios of ssDNA rings adsorbed to Poly-L-Lysine surface and diluted in 1x TBE

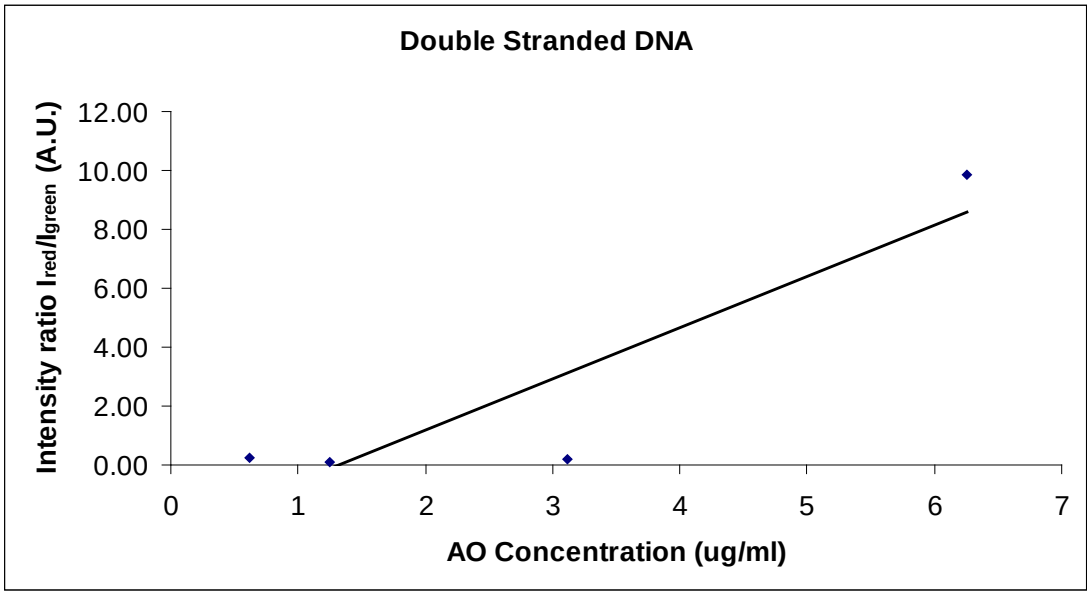


Figure 2.18 Analysis of Background subtracted Ired/Igreen light emission intensity ratios of dsDNA rings adsorbed to Poly-L-Lysine surface and diluted in 1x TBE

DNA solutions were made with 6:50 0.1M-NaOH/0.02M-MES buffer and AO staining solutions were made with 1 x TBE buffer. The AO concentration range used was from 0.62μg/μℓ to 6,25μg/μℓ. Figures 2.15 and 2.16 show the full emission spectra for all the concentrations, As seen in previous examples, the emission spectra show a transition from more green to more red with increasing concentration. The only concentration with strict color discrimination here is 3.12μg/μℓ. At the highest concentration, 6.25μg/μℓ, both are so strongly red that good discrimination is not possible. Overall, however, the results are similar to those using PMMA surfaces.

Finally in Figure 2.19, we show that a full emission spectrum may be generated for isolated molecules (though with considerable more noise than for DNA concentrated in rings). In this case, the DNA solution was made with 6:50 0.1M-NaOH/0.02M-MES buffer, and the 2.5μg/μℓ AO solution was made in 1X TBE. The shape of the emission spectra is close to that of the higher brightness ring data.

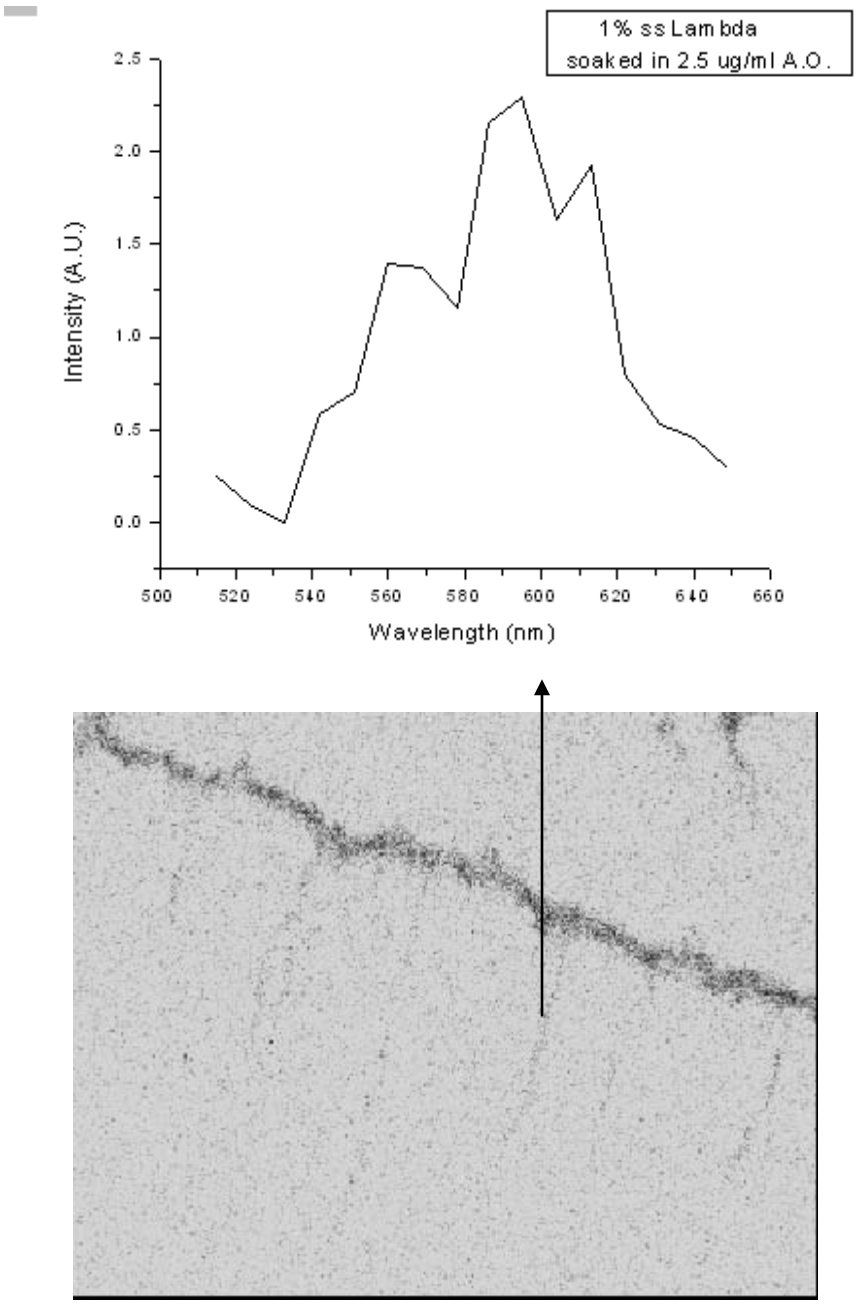


Figure 2.19 The graph represents an intensity analysis of various single molecules of lambda ssDNA on PMMA coated silicon. The DNA was soaked in 2.5 ug/ml AO which was diluted in 1X TBE. It can be seen that a strong red light emission confirms the single stranded DNA however, there is a small a presence of green light emission.

2.4 Conclusion

In summary, we have demonstrated that AO staining can be used effectively to discriminate between ssDNA and dsDNA absorbed on PMMA and PLL surfaces, either as isolated molecules or concentrated in densely packed rings, based on the emission spectra. Over a modest concentration range of AO staining solution, approximately $2 \rightarrow 2.5 \mu g/\mu \ell$ AO, strict color differentiation is still possible, based on the ratio of emitted red intensity to green intensity. In-situ calibration using both ssDNA and dsDNA is essential.

Chapter 3 Deposition of heat-denatured single-stranded DNA onto polymethylmethacrylate (PMMA)-coated substrates

3.1 Introduction

In this chapter, one of the simplest methods for depositing stretched single-stranded DNA (ssDNA) molecules on surfaces is described. The method is to deposit heat-denatured ssDNA onto a surface by dipping and retracting a polymer-coated substrate out of a DNA solution. The technique is simple yet versatile, since a number of experimental parameters may be varied independently to control the adsorption and stretching of the DNA molecules. Among others, these variables include: 1) DNA concentration in solution, 2) incubation (or soak) time of substrate in the DNA solution, 3) retraction rate of the substrate out of the solution (which results in the DNAs being stretched out on the surface), or multiple passes of retraction/insertion, 4) type of substrate surfaced used (glass, Si, polymer coatings of different types), type of buffer solution used, 5) pH of buffer solution (changed by adding bases such as NaOH or acids such as HCℓ, b) electric field-assisted adsorption, and 7) temperature. This chapter is mainly concerned with exploring the first three variables listed above. A separate issue, determining that the adsorbed molecules are indeed single-stranded (and not renatured in solution prior to adsorption, is taken up in the chapter on Acridine orange staining.

3.2 Experimental Section

DNA solutions were made with the Lambda DNA (48,502 base pairs, New England Biolabs, supplied in solution at a concentration of 500μg/mℓ) diluted in a 6:50 (by volume) solution of 0.1M NaOH : 0.02 M buffer. This buffer was used based on a

previous work with double-stranded DNA. The ratio of added NaOH was varied, 3:50 to 9:50 ratios produced good adsorption, and 6:50 was used throughout for the results shown here. The solution pH was measured to be 6.5. TBE buffer was also tried, but the results were poor. The range of DNA concentrations used was 0.5μg/mℓ to 12.5μg/mℓ. Lower concentrations produced unacceptably low densities of adsorbed DNAs, while higher concentrations resulted in overlap of DNA molecules, making analysis of individual chain lengths difficult. Solutions were gently vortexed to produce uniformity.

To visualize DNA molecules on the surfaces by fluorescence microscopy, the DNA was stained with YOYO-I (Molecular Probes, Invitrogen), a high-sensitivity dye enabling isolated single molecules to be images. The dye : bp ratio was designed to be approximately 1:20 to reduce the increase in denaturation temperature observed at high dye : bp ratios[1]. Typically, 7.5μℓ of 0.1 mM YOYO-1 solution was added to 20μℓ of the DNA stock solution (500μg/mℓ) and then diluted further to the desired working concentrations. Prior to use, the DNA/dye solutions were heated at 45°C for $0.5 \rightarrow 1$ hour to promote dye binding.

Polished silicon wafers, used for the substrates, were cut to size using a homemade scribe-and-break setup. The sample size was 10-15 mm square. To clean the silicon, prior to polymer spin-casting, the following procedure was used: 1) sonication in ethanol for 10 minutes, 2) boiling in 1:1:4 solution of ammonium hydroxide : hydrogen peroxide : deionized water for 10 minutes, followed by rinsing in deionized water, 3) immersion for 1 minute in 1:1:3 solution of sulfuric acid : hydrogen peroxide : deionized water, 4) deionized water rinse and 10 minute soak in deionized water. Following cleaning, a thin polymer layer (500-800Å thick) was spun-cast onto the silicon substrates.

The polymer used was Polymethylmethacrylate (PMMA), which is one of several polymer castings which have been successfully used for deposition of double stranded DNA[2]. PMMA of molecular weight 255kg/mole was dissolved in toluene at a concentration of 15mg/mℓ and spun for 1 minute at 2000 → 2500 rpm onto the silicon samples. Thickness was measured by ellipsometry. (Rudolf Auto El). Before DNA deposition, the surfaces were annealed at 105°C for at least 1 hour in an ion-pumped vacuum system with a base pressure of 1×10^{-7} torr.

The sample dipping apparatus is shown in Figure 3.1. A computer-controlled stepper motor system (Arrick MD-2) was connected to a linear stage which raises and lowers the sample-holding tweezers. The DNA solution 100 → 200μℓ in volume, is contained in a teflon reservoir with a sample well measuring 2mm x 12mm x 6mm (width x length x depth). The useful range of speeds of retraction is 5μm/s up to 8mm/s. A Basic-language program is used to control the stepper motor, determining the soak time, retraction speed and distance traveled. DNA samples to be deposited as single-stranded were denatured by heating to 95°C for 10 minutes and then immediately cooled in an ice-water bath. The DNA solution was then quickly pipetted into the teflon well, and the dipping experiment was run.

Imaging of DNA molecules on the surfaces was done using a Leica Laser Scanning Confocal microscope (TCS SP2), equipped with an Ar ion laser and two HeNe lasers (red and green), as well as an Hg lamp source for conventional (non-confocal) fluorescence microscopy using filter cubes. The images shown below were taken using the Hg lamp and a filter cube with a blue excitation filter. A cooled CCD camera (Leica DC350F) was used, and typical exposure times were 30-60s. Image analysis, including

measurement of areal density of DNAs on the surface and their length distributions, was done with ImageJ software (NIH). All images in the figures were taken at 10X magnification and the image area is 866um x 686um.

3.3 Results and Discussion

A series of experiments was run focusing on the variables of DNA concentration, soak time of the substrate in the DNA solution prior to retraction and the retraction rate of the substrate out of the solution.

DNA concentration. As expected, increasing the DNA concentration leads to increased density of adsorbed molecules on the surface. For concentrations of DNA below 0.5μg/mℓ, the density was unacceptably sparse, typically only a few molecules per image view. At concentrations greater than 25μg/mℓ, the overlap of DNA molecules became high enough to make individual size measurements difficult or impossible, and also the rate of unwanted renaturation during disposition will increase. Therefore, the experiments were conducted at Lambda DNA concentrations ranging from 0.5 to 12.5μg/mℓ (0.1% to 2.5% of the stock solution density). Figure 3.2 shows a plot of the area ℓ density of adsorbed DNAs versus concentration with the retraction speed fixed at 1mm/s and the soak time of 30s. Amongst the plot are shown the corresponding fluorescence images, all taken at 10x magnification. Excellent molecular "combing[17-20,37]" can be seen, with the DNAs stretched parallel to the literature value[38] 16 – 20μm for lambda DNA (see below for analysis).

Thus, there appears not to be excessive breakage of the single-stranded DNA (ssDNA), even though ssDNA is considerably more fragile than double-stranded DNA (persistence lengths of ssDNA in solution are typically 2-5nm, while those of dsDNA are about 50nm). The density variation is weaker than linear, a 25-fold increase in concentration producing a 5-fold increase in density.

The same general trend may be observed in Figure 3.3-3.6 for combinations of different soak times (30s, 120s and 300s) and different retraction rates (1mm/s versus 5mm/s). Good combing of DNA is obtained for the entire range of densities varying by a factor of 10.

Retraction speed. In another series of experiments, the substrate retraction speed was varied over a large range at fixed DNA concentration and soak time. Here, the interest is not only to determine the variation of adsorbed molecular density but also to test if high retraction rates show an increased tendency to break the DNA molecules during deposition (due to the large forces). Figure 3.7 shows the results for speeds ranging from 0.005mm/s to 8mm/s, a factor of 1600x, with DNA concentration fixed at 1ug/ml and soak time set at 30s. There is no regular trend observed with change of retraction speed, though the density does decrease somewhat at the highest rate of 8mm/s. Interestingly, as shown in Figure 3.8, the average length of the stretched molecules is approximately constant at 19um, within the normal range of contour lengths for unbroken chains. The histograms to the right of the images in Figure 3.7 indicate the number of molecules of various lengths in the micrographs (in the histograms, 15 stands for the range 10-15um, etc.). The distribution is seen to be fairly narrow, with standard deviations of 5um.

Soak time. The final parameter which was varied systematically was the soak (or incubation) time, the time the sample was left in the DNA solution before retraction. The range of soak times studied was from 30s to 300s (longer times were avoided due to possible renaturation during the soak). Figure 3.9 shows a series of images taken using different soak times with the DNA concentration fixed at 12.5ug/ml and retraction speed set at 5mm/s. The plot in the figure shows the corresponding molecular densities vs. soak time and it may be seen that the density is, within experimental error, constant. Hence, it is preferable to use the shorter times when working with ssDNA.

3.4 Conclusion

It has been demonstrated that single-stranded Lambda DNA (48502 bases) can be stretched onto polymer-coated surfaces by retraction from solution. A useful range of retraction speeds, soak time and DNA concentrations has been mapped out for obtaining isolated molecules at moderate densities where the majority of adsorbed molecules are of lengths close to the natural contour length. The molecules in these configurations are well suited for further applications such as hybridization experiments[39].

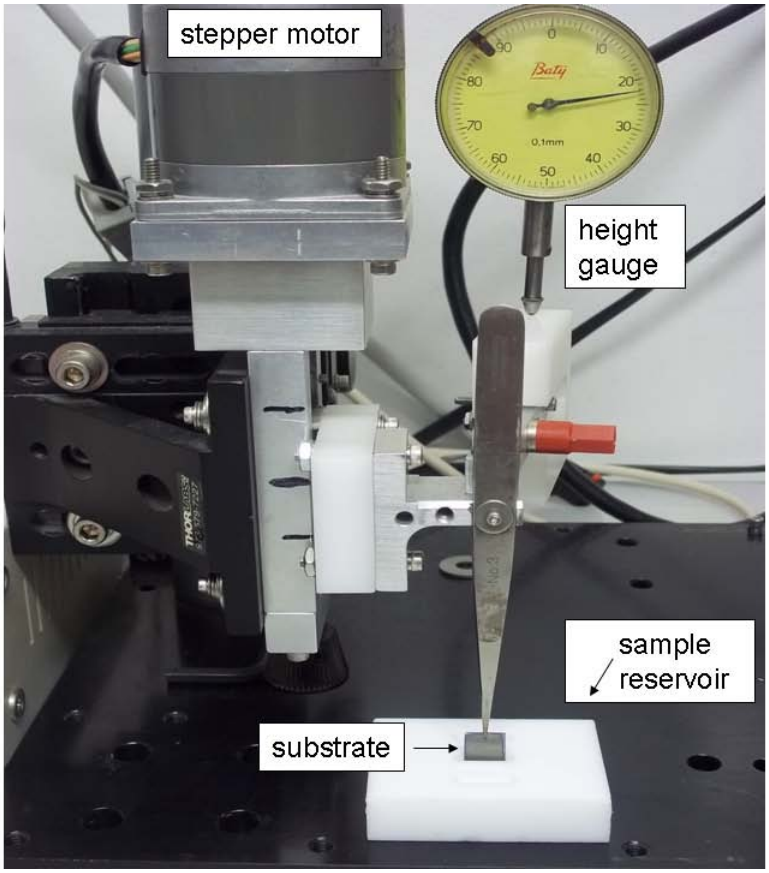


Figure 3.1: Substrate dipping apparatus

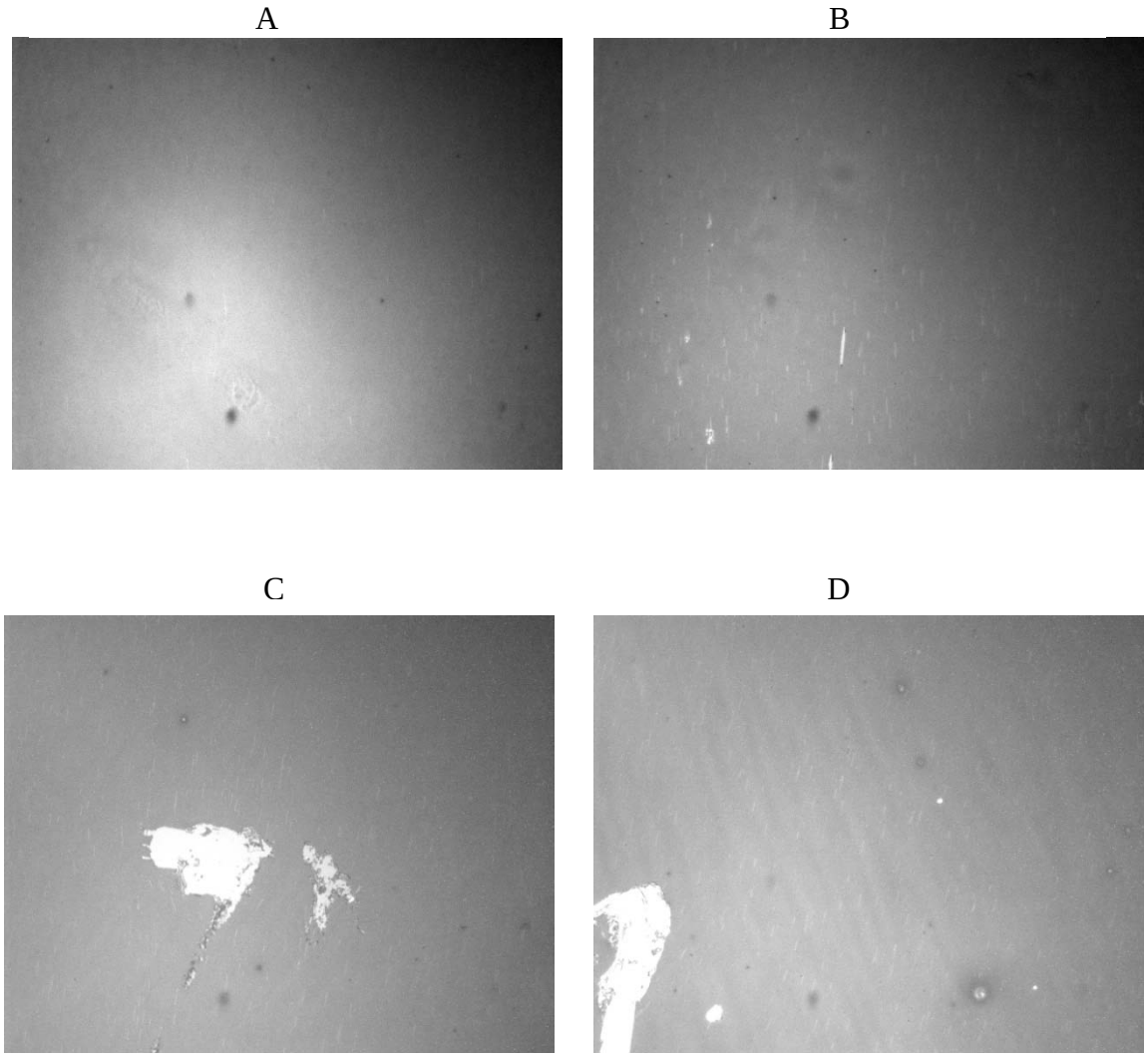
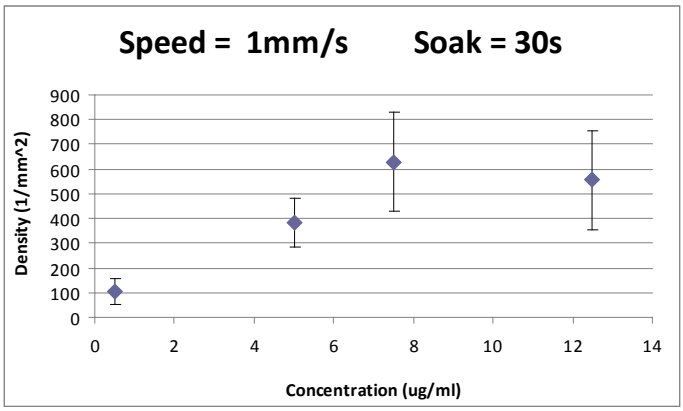


Figure 3.2. Variation of adsorbed molecular density with ssDNA concentration. Substrate retraction rate fixed at 1mm/s and soak time fixed at 30s.
(A)0.5µg/ml, (B) 5µg/ml, (C) 7.5 µg/ml, (D)12.5 µg/ml, (image size: 868µm x 686µm)

E) Graph of Molecular Density vs. ssDNA concentration



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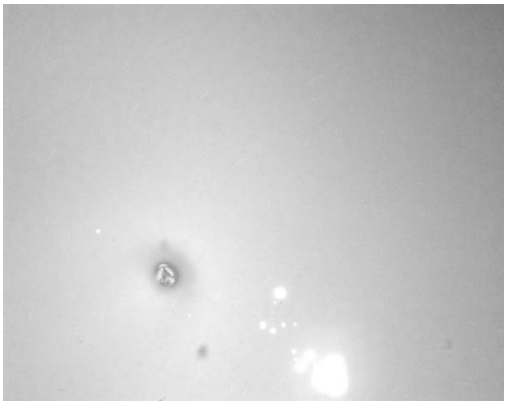

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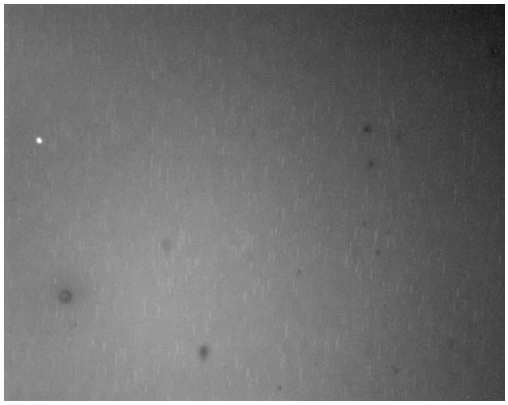

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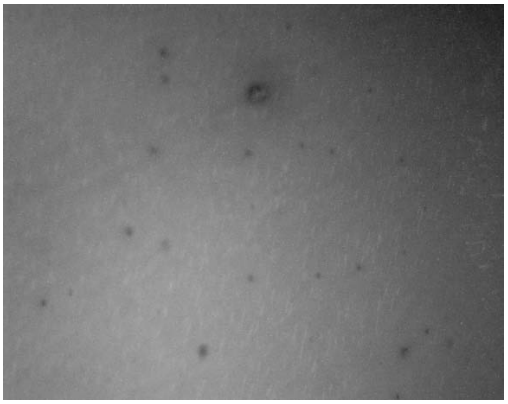

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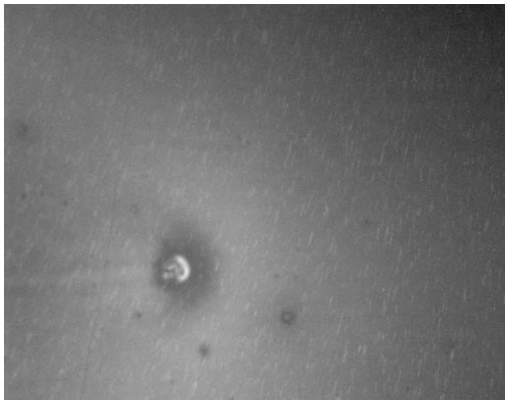
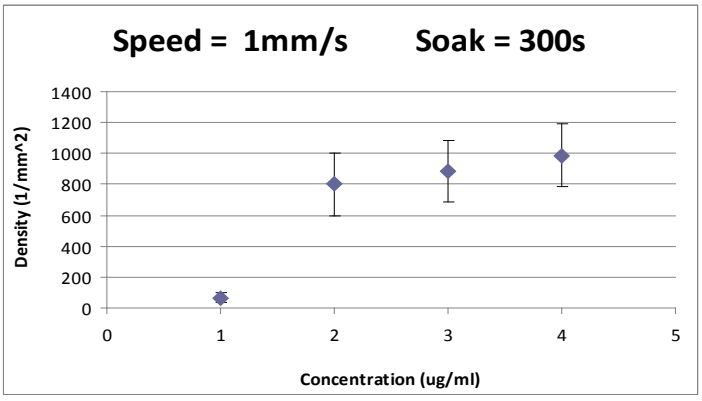
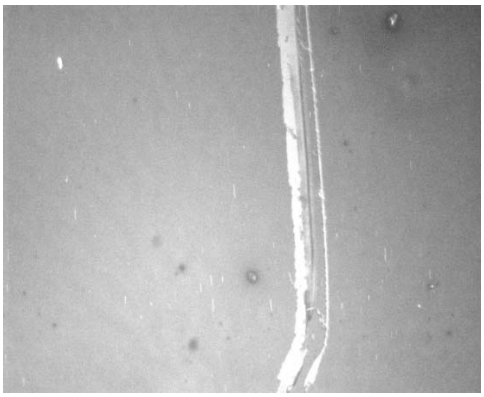


Figure 3.3 Variation of adsorbed molecular density with ssDNA concentration. Substrate retraction rate fixed at 1mm/s and soak time fixed at 300s.
(A) 0.5µg/ml, (B) 5µg/ml, (C) 7.5 µg/ml, (D)12.5 µg/ml, (image size: 868µm x 686µm)

E) Graph of Molecular Density vs. ssDNA concentration





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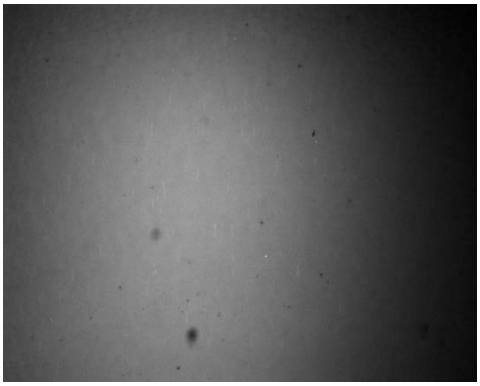

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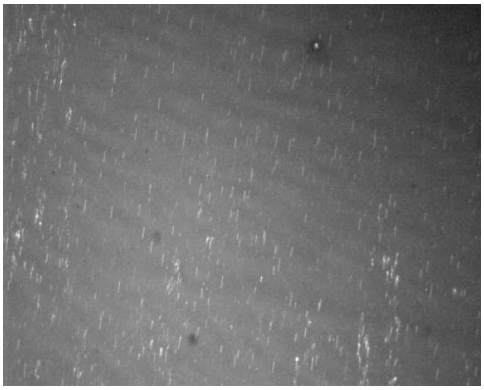

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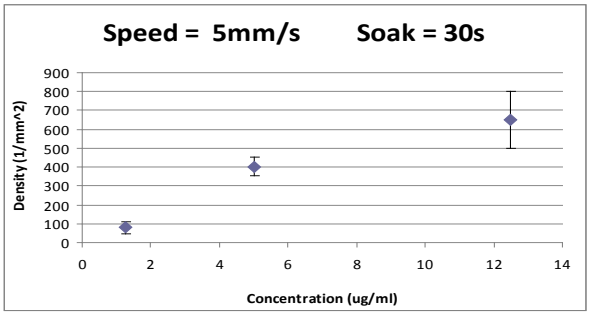
Figure 3.4 Variation of adsorbed molecular density with ssDNA concentration. Substrate retraction rate fixed at 5mm/s and soak time fixed at 30s.
(A)1.25µg/ml, (B) 5µg/ml, (C) 12.5 µg/ml, (image size: 868µm x 686µm
(D) Graph of molecular Density vs. ssDNA concentration



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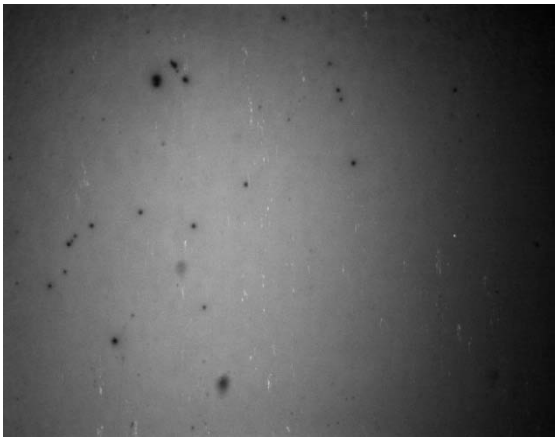

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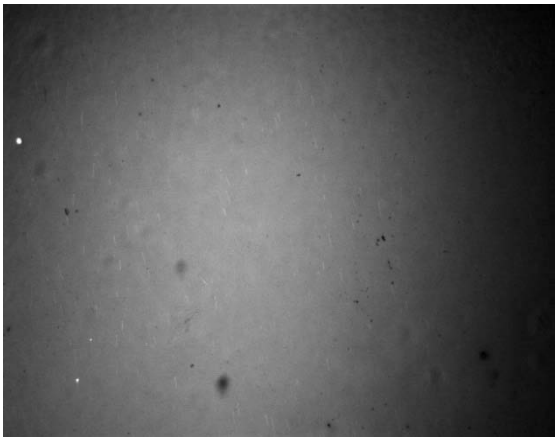

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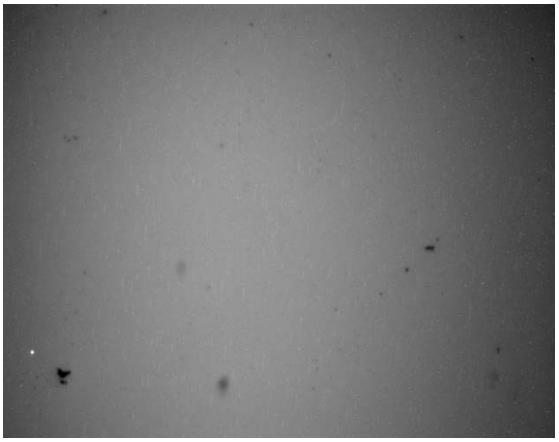

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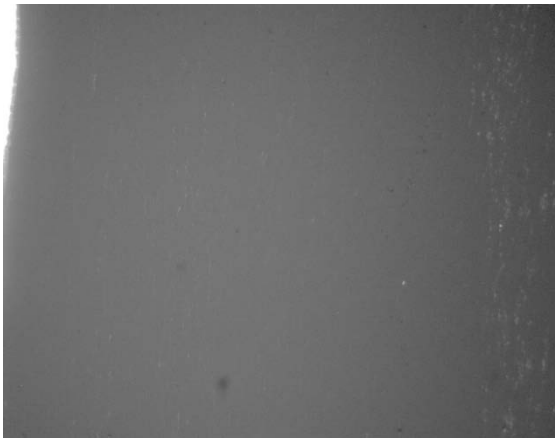
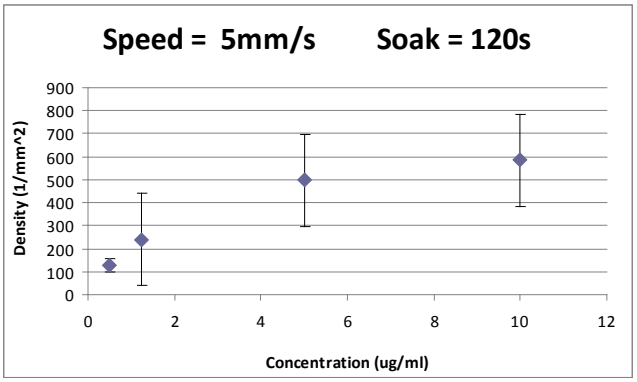


Figure 3.5 Variation of adsorbed molecular density with ssDNA concentration. Substrate retraction rate fixed at 5mm/s and soak time fixed at 120s.
(A)0.5µg/ml, (B) 1.25µg/ml, (C) 5 µg/ml, (D)10 µg/ml, (image size: 868µm x 686µm)

(E) Graph of Molecular Density vs. ssDNA concentration



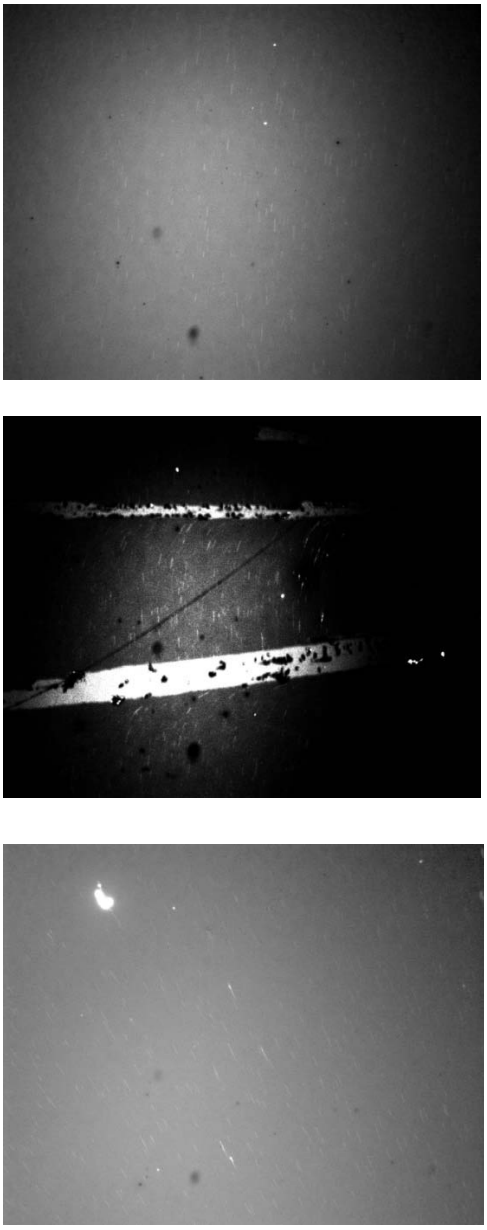
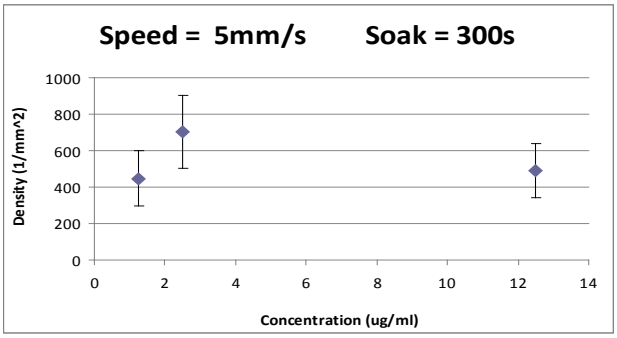


Figure 3.6 Variation of adsorbed molecular density with ssDNA concentration. Substrate retraction rate fixed at 5mm/s and soak time fixed at 300s.
(A)1.25µg/ml, (B) 2.5µg/ml, (C) 12.5 µg/ml, (image size: 868µm x 686µm)
(D) Graph of Molecular Density vs. ssDNA concentration



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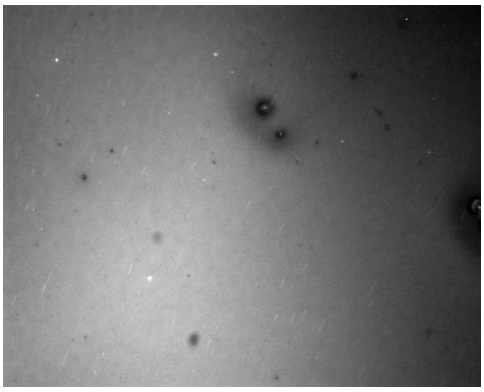

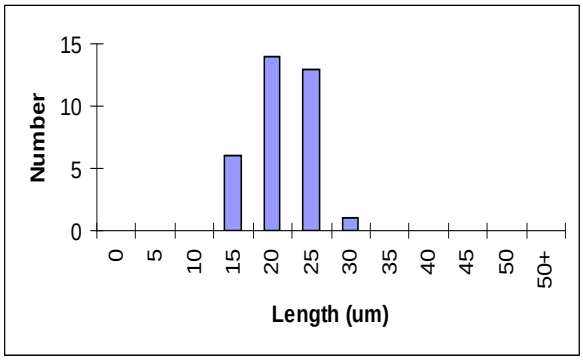

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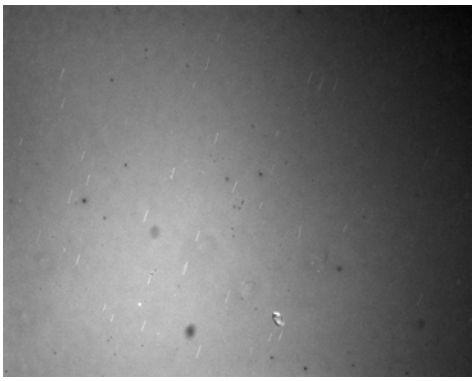

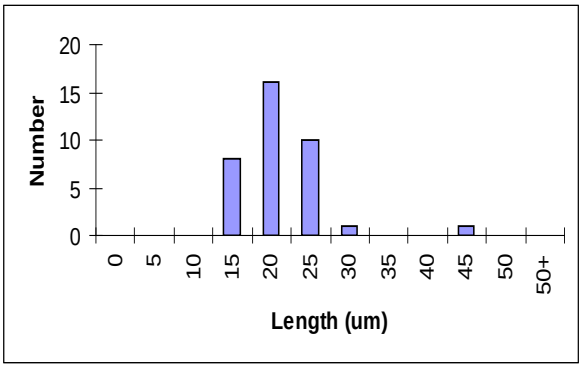

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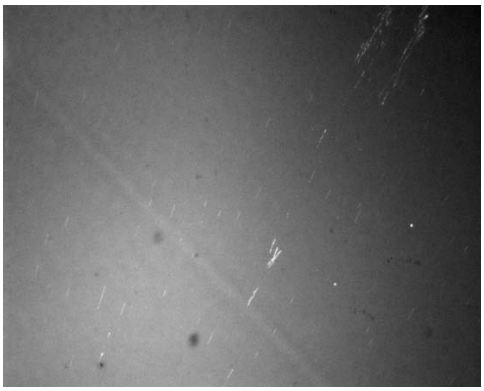


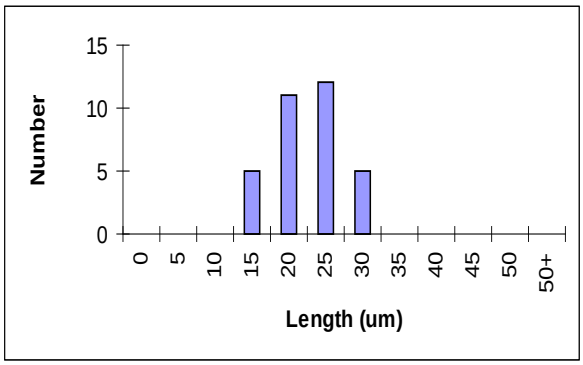
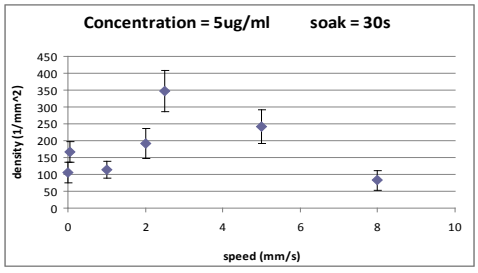


Figure 3.7 Variation of adsorbed molecular density with substrate retraction rate. ssDNA concentration fixed at 5µg/ml and soak time fixed at 30s. (image size: 868µm x 686µm) (A)0.005mm/s, (B) 0.05mm/s, (C) 1mm/s, with histograms of molecular length (to right) (D) Graph of Molecular Density vs. speed



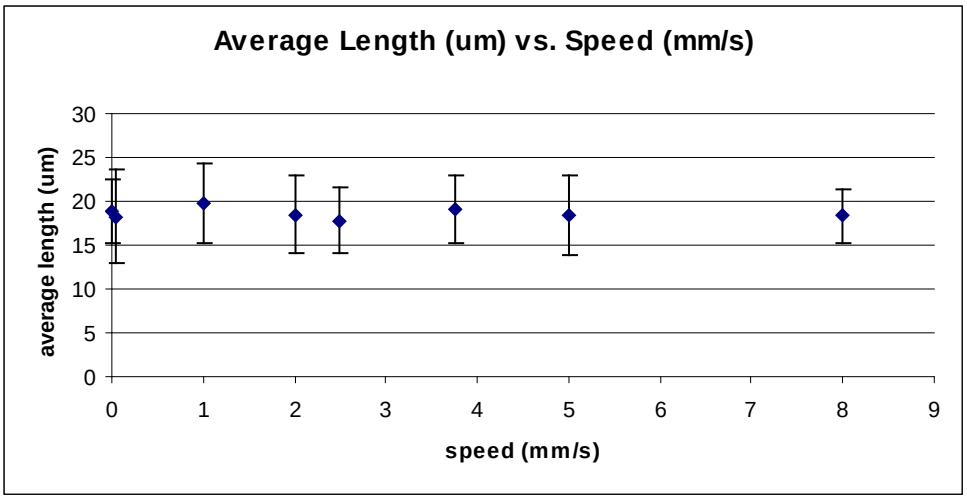
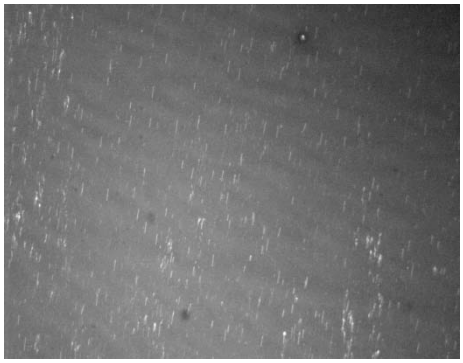
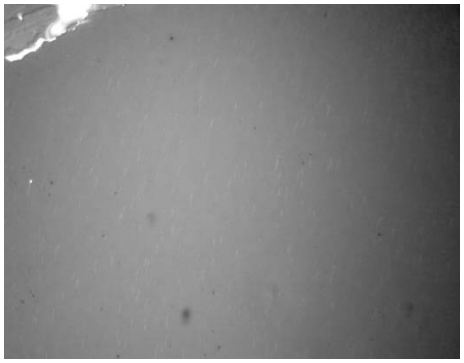


Figure 3.8 Variation of average length of ssDNA strand with substrate retraction rate.

A B





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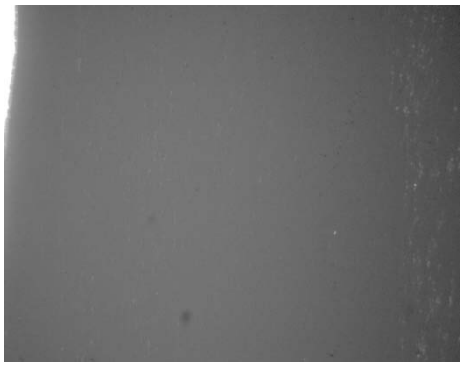


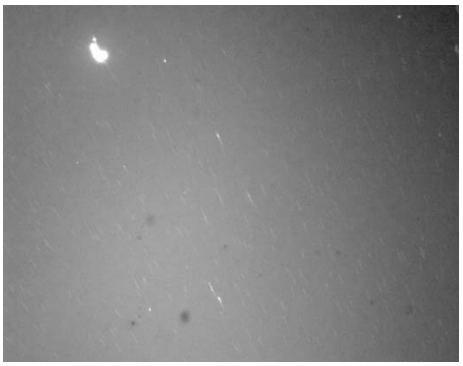
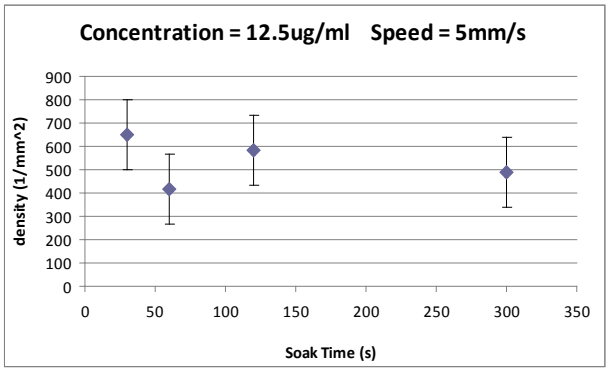


Figure 3.9 Variation of adsorbed molecular density with soak time. ssDNA concentration fixed at 12.5µg/ml and retraction rate fixed at 5mm/s (A) 30s, (B) 60s, (C) 120s, (D) 300s, (image size: 868µm x 686µm)

E) Graph of Molecular Density vs. Soak Time



Chapter 4: Separation of complementary strands of DNA by Field Inversion Gel Electrophoresis and Deposition of single sided DNA on polymer coated surfaces

4.1 Introduction

Many analytical techniques for the study of DNA structure require adsorption of single-stranded DNA (ssDNA) molecules to surfaces. In DNA microarray technology, short ssDNA segments, usually less than 100 nucleotides long, are attached to surfaces and their hybridization to DNA molecules is used to characterize the DNA. Other methods use a long DNA molecule stretched on a surface as a probe for short ssDNA segments (of known sequence) to hybridize to as a means of locating particular sequences along the DNA chain. Generally, the DNA is adsorbed to the surface as a double-stranded molecule (dsDNA), followed by in-situ denaturation at elevated temperatures. In this chapter, an alternative method, using gel electrophoresis to separate and purify complementary single strands for subsequent deposition and analysis on surfaces, is described.

The use of pulsed-field gel electrophoresis is an effective technique that can be used to separate complementary DNA strands[40] and ensure they stay that way for convenient extraction and deposition. Gel electrophoresis is a technique commonly used to separate proteins and DNA of different sizes. In some cases, DNA fragments of varying lengths can co-migrate and end up in the same position in a gel.[41] To combat this issue, a pulsed-field is often utilized in electrophoresis to help separate DNA. A specific type of pulsed field commonly used is Field-Inversion Gel Electrophoresis (FIGE) which is a simple method that switches periodically the polarity of the electrodes. A forward to back ratio of 3 to 1 is widely used.[41] It is also known that one of the most

important factors when dealing with pulsed-field gels in separating different size ranges of molecules is the switch time intervals.[41]

Single stranded nucleic acid molecules in gels form coiled structures of various sizes. The size parameters are influenced by base composition and sequence size range of up to 2-300bp length. [49-51] Here we demonstrate that we are able to efficiently separate 48.5kb ss lambda DNA in urea based gels and deposit them onto a surface. We optimized pulsed-field parameters for our applications in combination with buffer concentration, field strength, and experiment duration by analyzing ssDNA bands to obtain the best resolution and separation. We also calculated ssDNA mobility as the main measure to describe separation.[41,44-48]

4.2 Experimental Section

Electrophoresis and pulsing equipments. We used a Hoefer Scientific Pulsed Controller, Model PC500, with a switch time set to vary from 1.5 to 0.5 seconds during the run and a forward to reverse ratio of 3 to 1, unless otherwise stated. The basic setup consisted of a DC power supply (HP 6241) routed through the pulse controller the output of which was connected to a USA Scientific gel box. The gel box was kept in a refrigerator with the temperature set to approximately 9^{o}C. This measure was used to prevent the system from overheating and to reduce the effect of buffer evaporation. Experiment times ranged from 24 hours to 72 hours.

DNA type and preparation for agarose gel electrophoresis. Lambda DNA (48502bp) and Monocut DNA purchased from New England Biolabs (NEB) was used. T4 DNA (166kbp) was purchased from Wako. DNA concentrations of 12.5, 20, and

25ng/ul were prepared. For example a 20ng/ul lambda DNA solution consisted of 20ul stock of Lambda DNA (500ug/ml), 340ul of 8M Urea made in 1x TAE(40mM Tris-acetate, 1mM EDTA, pH 8) and 40ul of 6X tracking dye (NEB). The tracking dye made up 10% of the final volume of the solution. Samples were denatured by heat at 95^{o}C for ten minutes.

Preparing Gels for Agarose Gel Electrophoresis. The types of agarose used were low-endoosmotic (LE) powder from acros organics #400410250, certified mega base agarose and certified low melting agarose from Biorad, #1613108 and 1% premade gels purchased from Biorad, #1613015. A 1X TAE Electrophoresis Running Buffer was used by adding 15g of Urea to 250ml of 1XTAE to make a final Urea concentration of 1M. The solution was mixed with magnetic stirring rod until the solution was clear. In experiments where urea was not needed, 250ml of 1X TAE solution was used as a running buffer.

DNA preparation for agarose gel electrophoresis. A 25ng DNA solution was made for the study using 20ul stock of Lambda DNA purchased from NEB, N30115 500ng/ul DNA concentration. A 20ul volume of Lambda DNA was added to 340ul of 8M Urea made in 1x TAE(40mM Tris-acetate, 1mM EDTA, pH 8). We added 12 g of Urea powder into 25ml of 1X TAE to make an 8M Urea solution and mixed with a vortex until solution was clear. We added 40ul of 6x tracking dye (NEB). The tracking dye made up 10% of the final volume of the solution. The ssDNA was loaded following heat denaturation at 95^{o}C for ten minutes and snap cooling in ice water. dsDNA samples were loaded without the heating step.

Table 4.1

Gel Concentration (%) (weight/volume)	**0.4**	**1.0**	**1.4**
Agarose Weight *(g)	0.1	0.25	0.35
1X TAE Volume (ml)	25	25	25
8M Urea Weight(g)	1.5	1.5	1.5

*Selected type of desired agarose for application

The following components from table 4.1 were added to a beaker for the desired gel concentration (note: the addition of 8M urea was removed based on application)
The solution contained in the beaker was heated until boiling. Aluminum foil with holes in it on the top of the beaker was placed above. The solution was removed after boiling for 1 minute and cooled for 1-2 minutes. The solution was poured in the casting tray of the electrophoresis gel casting tray with the comb positioned in the slots. The rubber seals on the sides of the casting tray were affixed firmly to the sides of the electrophoresis tray. The tray was covered with aluminum foil and allowed to cool at room temperature for at least 30 minutes. The lower concentration gels needed longer cooling times and were cooled for at least 1 hour. For more rapid cooling of the gels, the cell was placed in the refrigerator. This is only necessary for low melting agarose powders at 0.4% concentrations. The comb was removed from the gel and repositioned in the casting tray 90 degrees so that the wells were close to the designated negative electrode. The electrophoresis buffer was poured over the gel until completely submerged. DNA samples from 2-10ul, were inserted into the wells. ssDNA contained at least 6.8M Urea before heat denaturation at 95^{o}C. The DC Power

Supply was connected to the pulser and the pulser was connected to the electrophoresis tank. (Hoeffer model # PC 500 Switchback) Desired fields/Pulse times were run. The best conditions for separation were found to be 48 hours at 20V over a distance of 14.5cm with a pulse field ratio of 3 to 1 and a switch back time of 1.5 seconds to 0.5 seconds. Once the run was completed, the gel was removed and stained. Urea based gels were cleaned by rinsing in 1X TAE(40mM Tris-acetate, 1mM EDTA, pH 8) for 45 minutes with gentle rocking back and forth. If urea was not used in the gel, the gel was rinsed with pure 1X TAE for 5 minutes with gentle rocking back and forth. The gels were then stained in 10,000x diluted SyBr Gold, (Cat. # S-11494 from Invitrogen). 5ul of SyBr Gold staining dye was added to 50ml of 1X TAE solution and this solution was shaken for 30 minutes in a plastic container. (CSH protocol). Excitation wavelengths for SyBr Gold are 300nm in uv range and 495nm in the visible range. The emission wavelength is 537nm.

Once completed, the gel was ready for analysis by placing in UV Imaging system or on top of UV box for extraction. A Zymo clean gel DNA recovery kit (cat# D4001) was used to extract pure DNA. DNA was not extracted from 1% premade gels from Biorad since higher melting temperatures of 95^{o}C seemed to compromise the integrity of the DNA extraction. A plastic gel extractor from USA scientific, #5454-2500 was used to cut out DNA bands. A gel slice was cut out and placed into a 1.5ml microcentrifuge tube. ADB buffer was added in the ratio of 3 volumes to 1 of the gel slice by weight and converted to microliters. For example if 100mg was measured 300ul of ADB Buffer was added. Three gel slices at most were added to the filtration tube when the solution is transferred. The solution was placed in a 55^{o}C bath for 5-10 minutes until completely dissolved. Once dissolved, the solution (up to 750ul) was quickly placed in a filter tube provided by the kit and centrifuged down at least 14,000rpm

for 1 minute. The DNA was then attached to the filter membrane and the remaining buffer can was discarded. The solution was then cleaned for 2 cycles through the same filter tube by addition of 200ul of cleaning solution and centrifuging down at least 14,000rpm for 1 minute. The DNA was then eluted into a new microcentrifuge tube by the addition of 20ul of 60^{o}C heated DI water to the same filter tube used for the cleaning steps allowing 2 minutes for the filter to prime with the water and centrifuging down at 14,000rpm for 1 minute.

Imaging. The gels were viewed using a Biorad UV Gel doc (302nm) and printing box. A Leica TCS SP2 confocal microscope with a SIT 66 Video camera was used to view the dna on surfaces.

Polymer thin film solutions and silicon cleaning. PMMA of molecular weight (255Kg/mole) and Polystyrene were diluted in toluene and spun-cast on cleaned silicon wafers at 3000rpm for 30 seconds. The silicon wafers were cut to be 8mm x 8mm and were cleaned to remove organic debris by RCA solution. The RCA procedure was as follows:

The silicon pieces were placed in methanol and shaken to remove residual dust by sonication for ten minutes. The methanol was removed and de-ionized water was added to rinse the pieces. To remove organic contaminants, the silicon pieces were added to a solution of de-ionized water, hydrogen peroxide, and ammonium hydroxide in a ratio of 4:1:1 by volume. The solution was heated until boiling and left for ten minutes. The pieces were then thoroughly rinsed with de-ionized water. To remove ionic/metallic impurities, the silicon pieces were added to a mixture of DI water, hydrogen peroxide,

and sulfuric acid in the ratio of 3:1:1 by volume and left to soak for ten minutes. The silicon pieces were thoroughly rinsed with de-ionized water and ready for use.

Pump Equipment. A Pfieffer Balzers (TCP-121) turbo pump and Digital 5000 ion pump were used to anneal the samples for 60min. at 105°C prior to deposition.

4.3 Results and Discussion

We conducted a concentration study using ds lambda DNA (48502bp) to identify the upper and lower limits of DNA resolution and band sharpness. The experiment was run for 1 hour in a 1% (Biorad #1613015) premade gel at 4V/cm. Based on Figure 1, lane 4 containing 0.5ng DNA by mass is barely visible. Most of our studies were conducted with DNA concentrations of less than 50ul/mg and mass values falling in the range of 50 -500ng for both ds and ss DNA. These DNA concentrations are within the range found to be independent of mobility.[59]

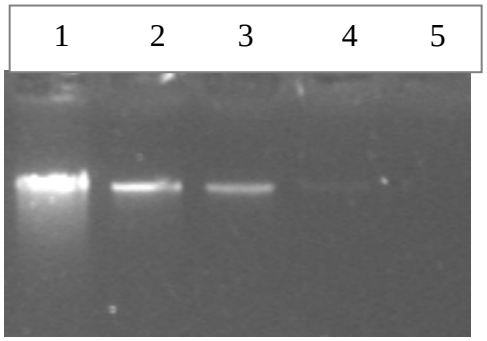


Figure 4.1: Gel electrophoresis run with different loadings of ds lambda DNA in a 1% premade agarose gel from Biorad. E-Field: 4V/cm in 1x TAE for 1 hour.
Lane 1; 500ng ds lambda DNA
Lane 2; 50ng ds lambda DNA
Lane 3; 5 ng ds lambda DNA
Lane 4; 0.5ng ds lambda DNA
Lane 5; 0.05ng ds lambda DNA

The ability to keep ss DNA denatured upon heat treatment of 95^{o}C in the presence of urea, was extremely important since we needed to get ssDNA on a surface before it renatured. In capillary electrophoresis experiments, ss DNA samples containing 4M urea

were sufficient to keep complementary strands from reannealing.[53] However in pulsed-field gel electrophoresis experiments we conducted, it was critical to add a range from 6.8 to 8M urea to prevent ssDNA complementary bands from renaturing. As shown in Figure 4.2, a ss Hind III DNA sample diluted in a 4M urea solution renaturing to form double stranded DNA during the electrophoresis. As indicated by the arrows, we line up the bands in lanes 3 and 4 with the dsDNA bands in lanes 1 and 2 to confirm they are dsDNA.

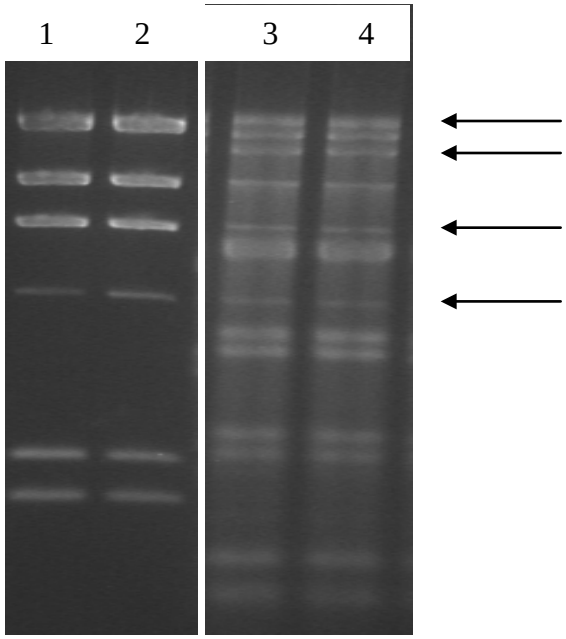
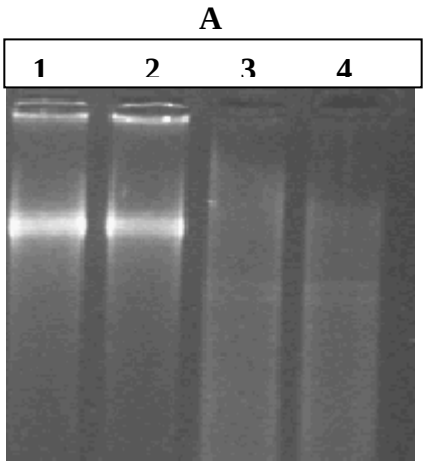


Figure 4.2: Lanes 1-2 ds Hind III, Lanes 3-4 Heat denatured ss Hind III DNA in a 1.5% acros LE agarose with renaturation occurring indicated by arrows. E-Field: 1.9V/cm for 24 hours.

Possible incomplete denaturation before electrophoresis and DNA renaturation during experiments as evidence by the increased background in lanes 3 and 4 runs demonstrate that ss DNA solutions containing 4M Urea are inadequate to keep complementary bands separate. Although many gel electrophoresis protocols include urea in the preparation of gels and electrophoresis buffers,[52,54-55] the primary factor to keep DNA single stranded was that urea need only be in the DNA samples[40]. To confirm urea was indeed necessary for keeping complimentary DNA strands apart, we compared DNA that was

denatured at 95°C in the presence of 6.8M Urea to DNA denatured at 95°C void of Urea via PFGE (Figure 4.3). The picture of the gel with DNA in lanes 3-4 containing 6.8M Urea upon heat denaturation illustrates separation and mobility differences between ds and ss DNA(figure 3a). The gel containing DNA void of urea in lanes 3-4 of Figure 3b shows a high background indicating that the complementary DNA strands are re-hybridizing.



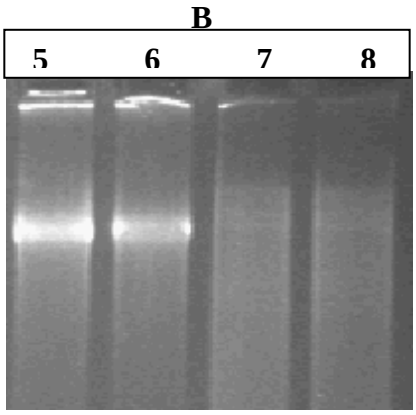


Figure 4.3: E-Field; 1.38V/cm, 48 hour run for both gels. **A)** 1.2% acros LE agarose gel with 8M Urea in DNA samples. Lanes 1-2, ds Lambda DNA, Lanes 3-4, ss Lambda DNA **B:** 1.2% acros LE agarose gel without Urea in DNA samples. Lanes 5-6, ds Lambda DNA, Lanes 7-8, ss Lambda DNA.

Upon establishment of separation of complimentary bands in agarose gels first shown by Hayward and more recently by Materna et al. Later Hegedus et. al. demonstrated separation of complementary strands in urea based gels and we investigated the possibilities of separating even longer DNA's such as Lambda Phage DNA (NEB #N3011s), Monocut digest (NEB N3019) , and T4 DNA (Wako). The purpose is to separate them to study their conformations by depositing them on different polymer-coated surfaces. To do this we conducted different experiments with varying conditions including gel concentration, pulse times, electric fields. We analyzed the gels through Image J software and calculated DNA mobilities ($V= \mu \bullet E$), separation distances, and FWHM. After a picture was taken, ImageJ was used to create a light-intensity graph of a desired lane in the gel. All gels analyzed were calibrated for distance before any data was tabulated. The distance was taken in inches for visibility purposes and immediately converted to centimeters for calculations, and the time was converted from hours to seconds. FWHM was calculated by finding the midpoint on the peak between the background (base) and the tip. The horizontal difference between the midpoint and the tip was doubled to find the FWHM. The normalized resolution of a pair of ss DNA strands was determined by subtracting the travel distance of the second strand from the first and dividing the difference by the travel distance of the first band.

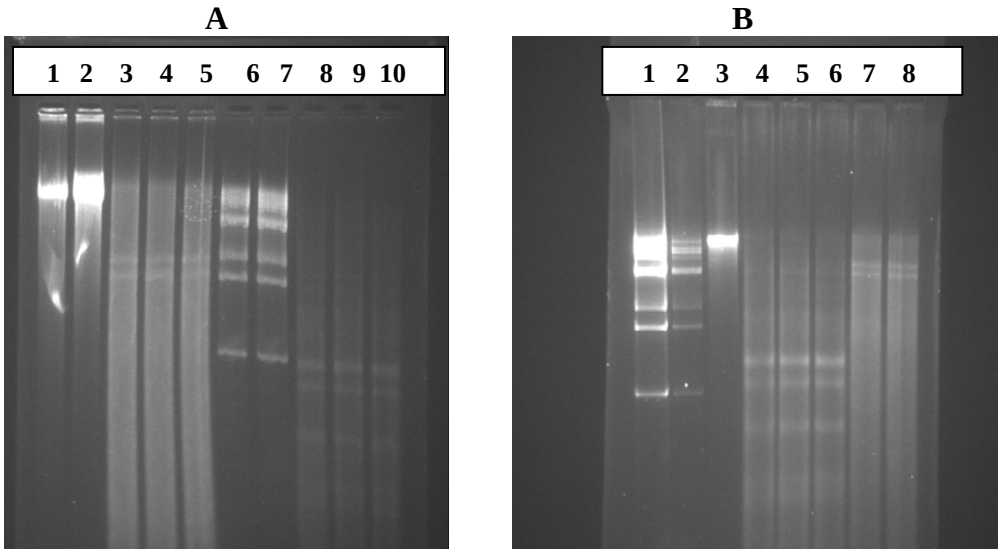


Figure 4.4: (A) 0.8% acros gel in 1X TAE pulsed 3 to 1 ratio with switch back time 1.5s to 0.5s. E-Field was 1.38V/cm, run time was 68 hours. (B) 1.0% premade Biorad gel in 1X TAE pulsed 3 to 1 ratio with switch back time 1.5s to 0.5s. E-Field was 2V/cm, run time was 44 hours.

In Figure 4.4, we demonstrate the ability to separate complimentary bands of lambda DNA (48502bp). Lanes 1-2 in figure 4A are ds lambda DNA. Lanes 3-5 are ss lamba DNA. Lanes 6-7 are ds Monocut DNA and 8-10, ss monocut . Lanes 4-6 in figure 4B are ss monocut DNA bands. Lanes 7-8 in Figure 4B are ss lambda DNA. All ss DNA samples in each gel were produced by heat denaturation at 95^{o}C in the presence of 8M urea. The ss lambda bands show greater separation in the 0.8% gel but were also electrophoresed for a longer period. The ss lambda DNA bands in the 1.0% gel are far more resolved but less separated. The ability to separate ds lambda DNA gave initiative to find the best conditions for both separation and resolution. We were also able to successfully separate the first few bands in the ds monocut DNA ladder. As shown in Figure 4B, the first ss band in monocut DNA (lane 6), aligns exactly with that of ss lambda DNA(lane 7).

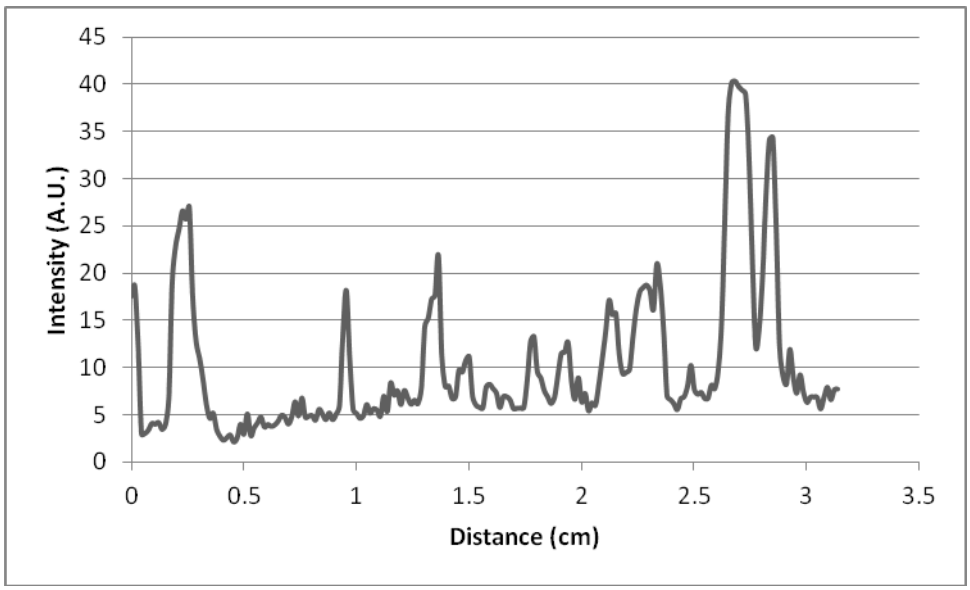


Figure 4.5: Travel distance of ss monocot complimentary bands in a 1.0% agarose gel. E-Field 1.38V, 48 hour experiment

Figure 4.5 illustrates an electropherogram of the first four complementary bands of monocut ssDNA (from largest to smaller). The ability to separate complimentary strands of the first few bands in the monocot ladder is feasible based on previous work done by Hegedus[40].

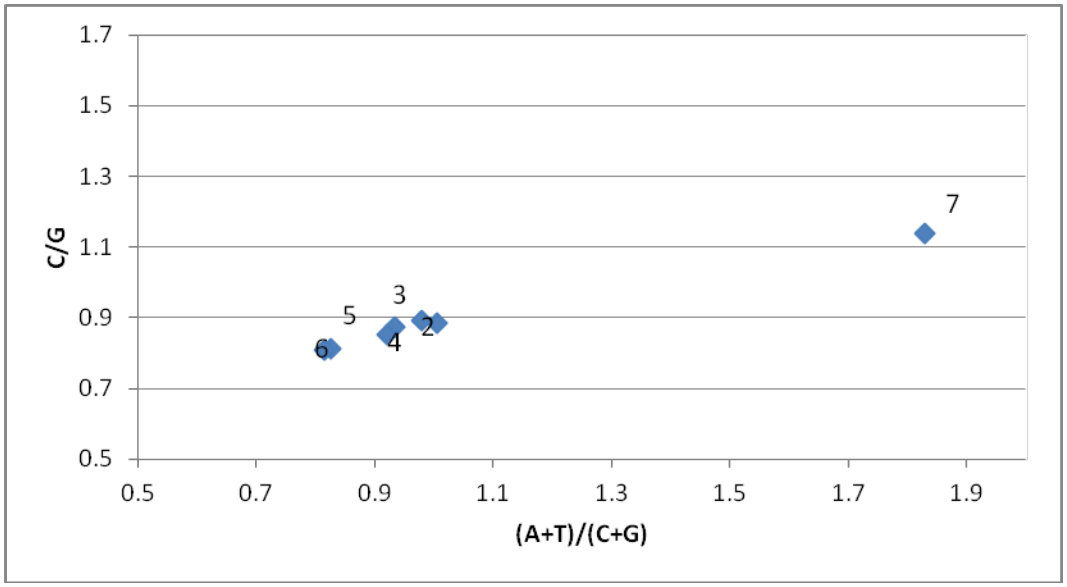


Figure 4.6: Separation of complementary strands with respect to base content.

. Figure 4.6 indicates the ability to separate single strands of DNA by base content. Number 1 represents the ss sense strand of lambda. Numbers 2 through 6 indicate the ss sense strands of the 2nd through 6th bands from the top of the Monocut ladder. Number 7 represents the sense strand of T4 DNA. Numbers 1-6 fell in a region of good separation. However, T4 DNA did not separate. The data corresponds with the results of Hegedus et. al. who demonstrated that DNA base content in the region where numbers 1-6 fall showed good separation with the antisense band being retarded and T4 being in a region showing no separation.

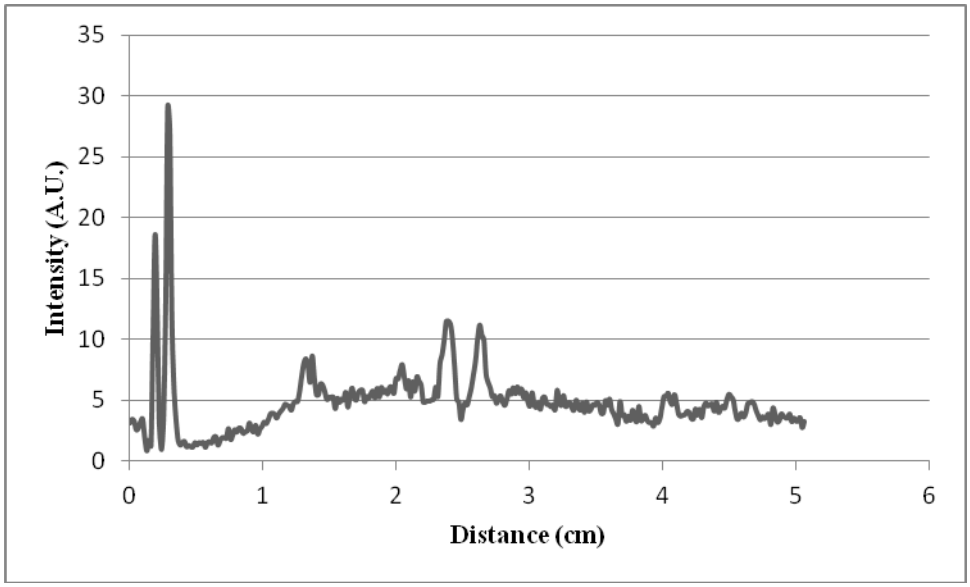


Figure 4.7: Travel distance of ss lambda DNA bands in a 0.8% agarose gel; E-field is 1.38V/cm, 68 hour experiment.

Figure 4.7 illustrates a background subtracted electropherogram of complementary Lambda DNA (48502b). Although identification of sense and antisense bands is best done with labeling probes, separation with respect to base content correlates to data indicating the first band in Figure 4.7 is the antisense strand.[40]

Gel conc.(%)	1st peak (cm^2/Vs)	2nd peak(cm^2/Vs)
0.4	1.64E-05	1.77E-05
0.6	1.47E-05	1.54E-05
0.8	8.28E-06	8.90E-06
1	6.5E-06	6.88E-06
1.2	5.1E-06	5.43E-06
1.4	3.69E-06	4.12E-06

Table 4.2: Mobilities of ss lambda DNA in various gel concentrations; E-Field for all runs 2.0 V/cm, 48 hour experiments

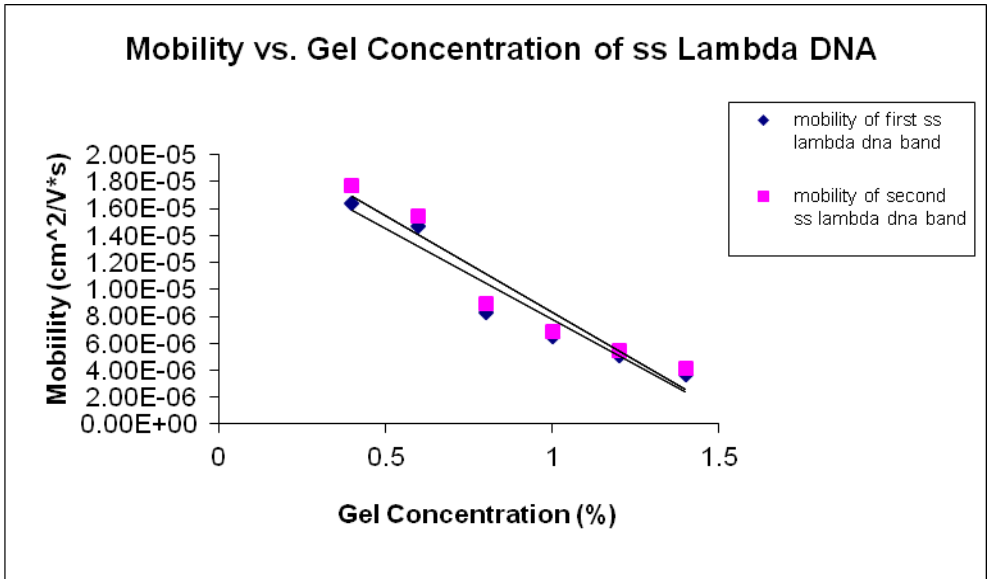


Figure 4.8: Mobility vs. Gel Concentration of 48.5kb ss lambda DNA; E-Field for all runs 2.0 V/cm, 48 hour experiments

As gel concentration increased, DNA mobilites for sense and antisense bands decreased as expected. We see a linear trend for both sense and antisense lambda DNA bands with constant pulsing parameters and E-fields, suggesting that gel concentration is the primary factor in influencing ss lambda DNA mobility.

Normalized relative separation was calculated as the difference in distance between peaks of the sense and antisense strands of lambda DNA divided by the distance of travel of the first peak. Using an E-Field of 2.07 V/cm (Figure 4.9), DNA separation is fairly

constant within error across the gel concentrations indicating gel concentration does not affect band separation. An E-Field of 0.83V/cm was used for 0.4% gel (weight/volume) to prevent the DNA bands from running off the gel.

Gel concentration(%)	Normalized Relative Separation (A.U.)
0.4	0.07
0.6	0.05
0.8	0.06
1.0	0.06
1.2	0.07
1.4	0.07

Table 4.3: Relative separation of 48.5kb ss lambda DNA normalized for distance; E-Field for runs 2.0V/cm,48 hour experiments.

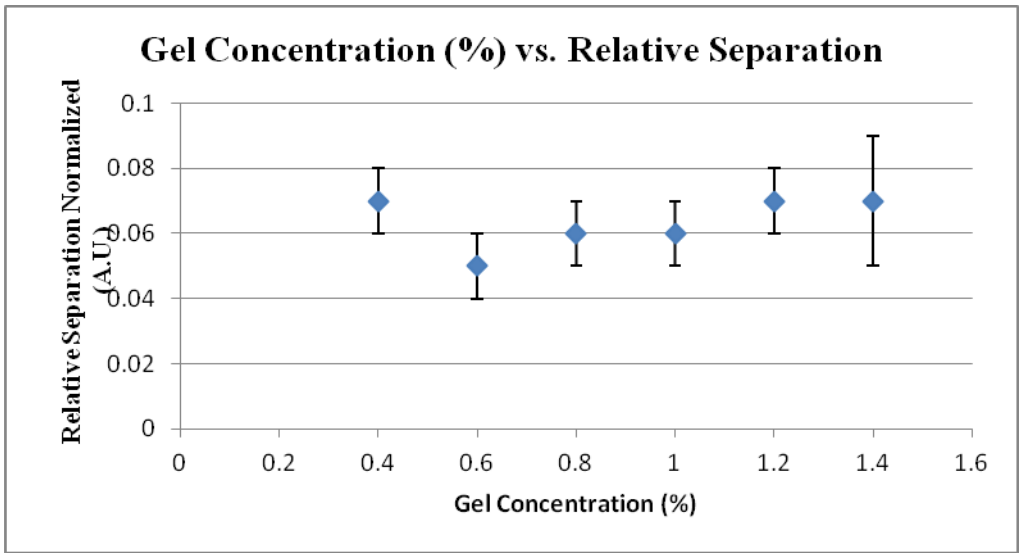


Figure 4.9: Normalized relative separation of ss lambda DNA bands vs. Gel Concentration (%); E-Field 0.6% gels and higher were 2.0V/cm,48 hour experiments. The E-Field for the 0.4 gel was 0.83V/cm. The width of a well is 0.1cm.

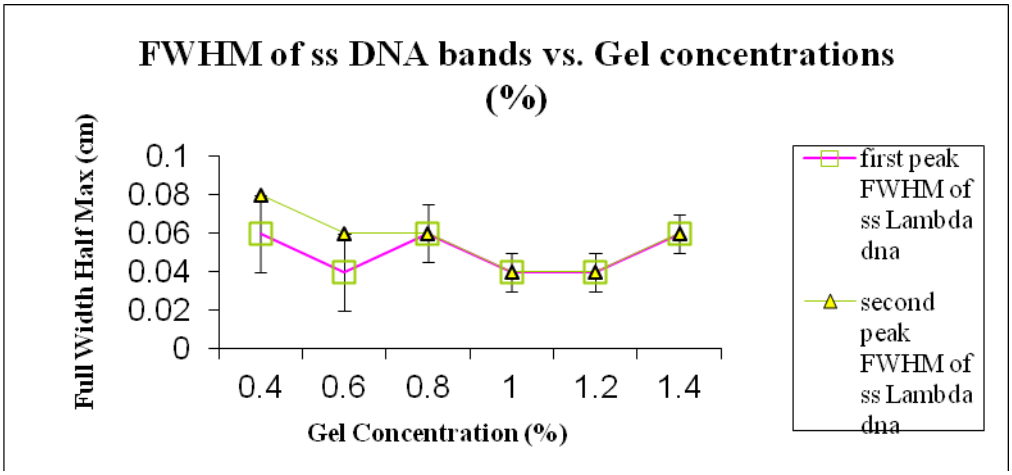


Figure 4.10: Full-Width Half-Max of ss lambda DNA bands vs. Gel Concentration (%); E-Field for all runs 0.6% and higher were 2.0V/cm,48 hour experiments. E-Field for 0.4% gel was 0.83V/cm. The width of a well is 0.1cm

Gel conc. (%)	1st peak	2nd peak
0.04	0.06	0.08
0.06	0.04	0.06
0.8	0.08	0.06
1	0.04	0.04
1.2	0.04	0.04
1.4	0.06	0.06

Table 4.4: Full-Width Half-Max of ss lambda dna in various gel concentrations E-Field for all runs 0.6% and higher was 2.0 V/cm, 48 hour experiments. E-Field for 0.4% gel was 0.83V/cm.

Figure 4.10 shows the FWHM values for the bands remain relatively constant within error across gel concentration. The width of the well was measured to be roughly 0.1cm wide. In all cases, the FWHM for both sense and antisense bands were measured to be less than the well width. This indicates DNA dispersion did not occur to great effect.

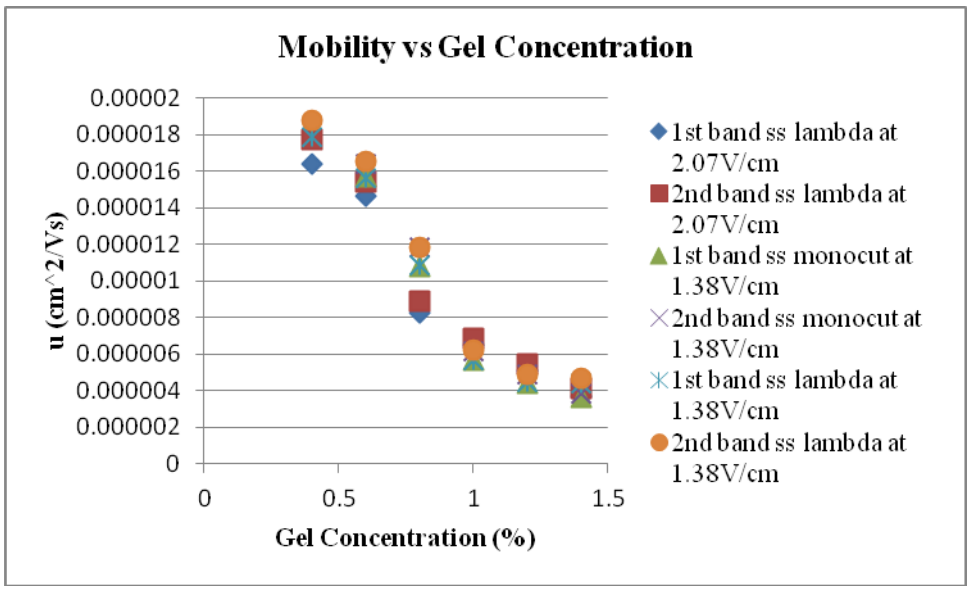


Figure 4.11: Comparison of ss lambda DNA and the 1st pair of ss monocut bands with respect to mobility vs. gel concentration. E-Field for ss lambda was 2.0V/cm and 1.38V/cm, E-Field for 1st band of ssmonocut was 1.38V/cm, all runs were 48 hours. Note: E-field was 0.83V/cm for ss lambda in the 0.4% concentrated gels.

Figure 4.11 illustrates the mobilities of lambda DNA at different voltages. A decreasing linear trend is observed increasing gel concentration. Figure 4.12 compares the mobility of ds lambda DNA and the average mobility of the complementary ss lambda DNA bands. A slower avg mobility for ss lambda DNA compared to ds lambda DNA was noted at 1.4% and above for both E-fields. This may attribute to the inability to accurately resolve the DNA bands at those gel concentrations. Data for separation could not be obtained for gels below 0.4% since they are too weak to handle.

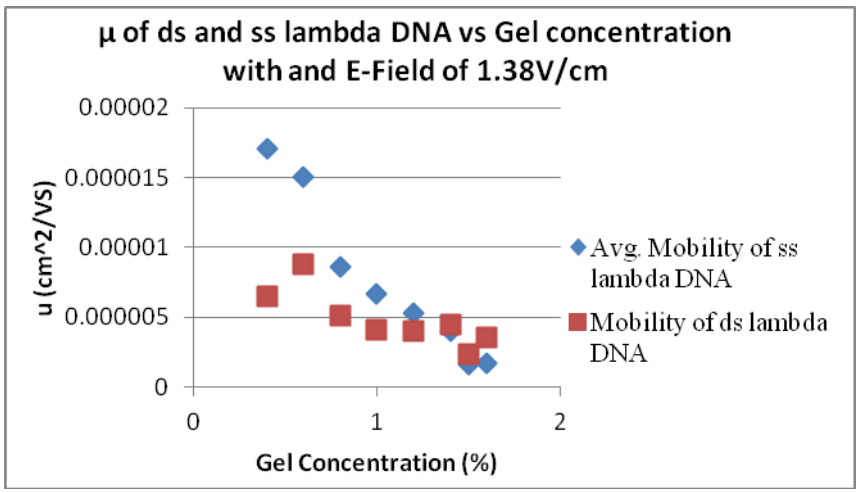


Figure 4.12: Comparison of the avg. ss lambda bands and ds lambda mobility with respect to gel concentration. E-Field for lambda dna was taken at 1.38V/cm

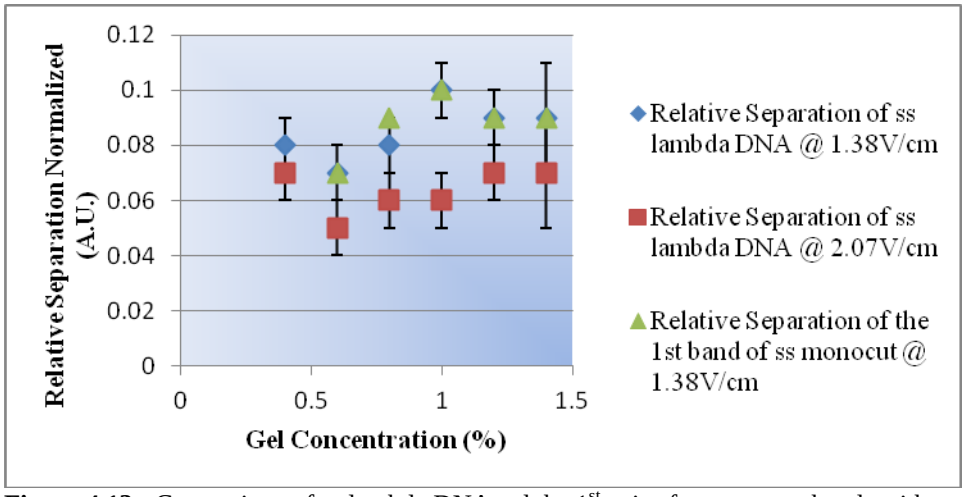


Figure 4.13: Comparison of ss lambda DNAand the 1st pair of ss monocut bands with respect to normalized relative separation vs. gel concentration. E-Field for ss lambda was taken at 2.0V/cm and 1.38V/cm, E-Field for 1st band of ss monocut was 1.38V/cm, all runs were 48 hours. Note: E-field was 0.83V/cm for ss lambda in the 0.4% concentrated gels.

The ss lambda DNA bands show a general improvement in separation vs gel concentration under the lower electric field of 1.38V/cm (Figure 4.13). In theory, this may be due to the ability of a DNA strand to wiggle through the pores more easily at a lower E-field.[43]

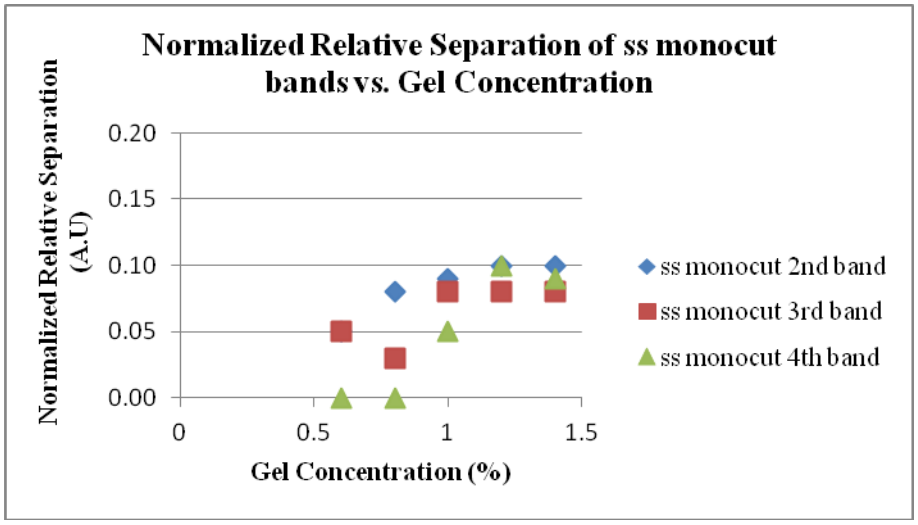


Figure 4.14: Comparison of relative separation of ss monocut with respect to gel concentration. E-Field for ss lambda was 2.0V/cm, E-Field for 1st band of ss monocut was 1.38V/cm, all runs were 48 hours.

Figure 4.14 illustrates the relative separation of the pairs of monocot ss bands 2 through 4. An increasing trend in separation can be seen for all bands vs gel concentration. This data illustrates the greater separation occurred as the DNA molecules decreased in size.

We also studied the effects of Urea on ss lambda DNA mobilities and separation by comparing three conditions. The first condition was to analyze ss lambda DNA mobility and separation by experimenting with concentrations of Urea of 8M, 1M, 1M, in the DNA, gel and buffer respectively. The second condition was to analyze ss lambda with Urea of concentrations 8M and 1M in the DNA and buffer respectively. The third was to analyze the mobility and separation with just 8M Urea in the DNA. A pH measurement was taken for running buffers with and without 1M Urea pre and post experiments as well as 8M Urea solutions prior to experimentation. The measurements were steady with a pH range from 8.2-8.3.

Condition	Mobility 1 (u)	Mobility 2 (u)	rel. separation ± S.D.
D8, GM1, RB1	6.98E-06	7.36E-06	0.050 ± 0.01
D8, RB1	7.05E-06	7.44E-06	0.050± 0.01
D8	7.10E-06	7.39E-06	0.050± 0.01

Table 4.5: Mobilitiy and Separation of ss lambda complimentary DNA bands in the presence of Urea in a 1% gel. E- field; 2V/cm, 48 hour experimental run. D8: ss dna denatured at 95°C in the presence of 8M urea, GM1: Gel Mix consisting of 1M urea, B1: Running Buffer consisting of 1M urea.

After analysis, we do not see a major effect of Urea on mobility or relative separation of ss lambda dna based on the conditions tested, hence to save time, we conducted most experiments thereafter with condition D8 only.

Various pulse times and forward to reverse ratios were also tested to determine optimal separations conditions for lambda dna. Table 4.6 lists the relative separation for these various conditions.

pulse time (s)	Forward to Reverse Ratio	Voltage (V/cm)	rel. separation (cm)
0.3 - 30	3 to 1	2	0.06
0.5 - 1.5	3 to 1	2.48	0.06
1.0 - 50	3 to 1	2.0	0.04
1.0 - 99	3 to 1	2	0
6.0 - 45	3 to 1	2.48	0.04
1.5 - 0.5	3 to 1	2	0.06
15 - 5.0	3 to 1	2	0
30 - 10	3 to 1	2	0
1.5 - 0.5	4 to 1	2	0.04
1.5 - 0.5	2 to 1	2	0.05

Table 4.6: Pulse times, forward-reverse ratios and resulting separation of ss lambda complimentary DNA bands in the presence of 6.8MUrea in a 1% gel. E-field; 2V/cm, 48 hour experimental run. D8: ssDNA denatured at 95°C in the presence of 8M urea, GM1:

The pulse time of 1.5 – 0.5 seconds was chosen since it was one interval that produced the greatest separation amongst the others as well as yielded the sharpest bands.

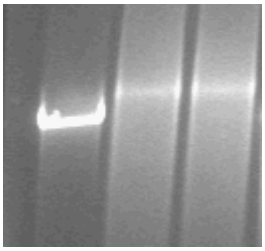


Figure 4.15: Lane 1) 60ng ds T4 dna, Lane 2-3) 200ng ss T4 DNA heat denatured at 95C with 8M Urea; pulsed field at 1.38V/cm in a 1% gel for 48 hours. Pulse field was 3 to 1 ratio with switch back time of 1.5s to 0.5s. Buffer was 1XTAE.

We attempted to separate T4 DNA which has a length of roughly 168Kb pairs shown in Figure 4.15. Various pulsed field parameters were used to separate ss T4 DNA. However, this proved to be difficult as Hegedus et. al found that DNA having G-C base content around 35% was difficult to separate. T4 dna has 34.5% G-C base content[41].

We also attempted to separate ss lambda DNA with 1x TBE buffers with and without pulsing however resolution and separation quality was very poor (data not shown). It is known that TAE has a lowering buffer capacity and provided better resolution for long DNA especially over longer experimental runs.

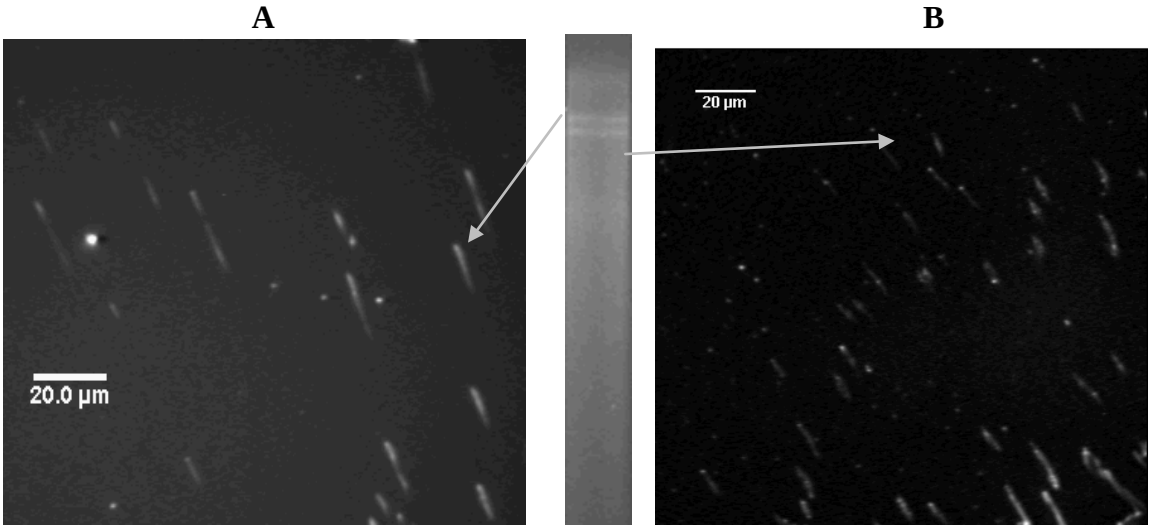


Figure 4.16: Sense and antisense lambda ss DNA bands taken with 40x lens. (**A**) 48.5kb single stranded lambda DNA extracted from a 0.4% low melt agarose and deposited on a 500Ă PS thin film coated substrate. (**B)** 48.5kb single sided lambda DNA extracted from a 0.4% low melt agarose and deposited on a 700 Ă PMMA thin film coated substrate.

After successfully separating complementary bands of lambda DNA, we looked to extract them to study their conformations on surfaces. Lambda DNA drops of volumes 0.5 to 2ul were put on PS and PMMA coated surfaces that were positioned on hot plates set to 60^{o}C. The droplets were allowed to dry and viewed under the fluorescence microscope using a Silicon-Intensified Target (SIT, dage model 66) camera. The DNA was stained in the gel in a 1:10,000x SYBR Gold solution which was prepared in 1X TAE for 30 minutes.[52,55] Figure 4.16 shows extracted sense and antisense DNA strands from a 0.4% agarose gel and the subsequent deposition of fully extended single sided lambda chains on both surfaces. We found optimal film thickness to be between 500 – 1000Ă for PMMA and PS. However, surface cleaning and polymer thin film preparation proved to be critical for consistency in the thin films.

Exposure of DNA to ultraviolet (uv) light can cause it to cleave and/or crosslink. Since the DNA was exposed to uv light, we wanted to make sure that the it was not affecting the DNA by breaking or cleaving it. We examined both extracted uv exposed and non-uv exposed ss DNA by placing them on the same polymer surface. The non-uv exposed DNA was generated by running agarose gels with multiple lanes of heat denatured ss lambda DNA in the presence of 8M Urea via PFGE. The gel was cut in two halves, one half exposed to uv light with the subsequent extraction of ss bands, the other half not exposed to uv light. The two gel-halves were put back together and aligned with a ruler to extract the non-uv exposed ss bands. The non-uv exposed half of the gel was then placed on the uv box to confirm that the ss bands were extracted properly. Figure 4.17 illustrates the approximate full-contour length of ss lambda DNA in specific regions chosen for both uv and non-uv exposed images on both PMMA and PS surfaces.

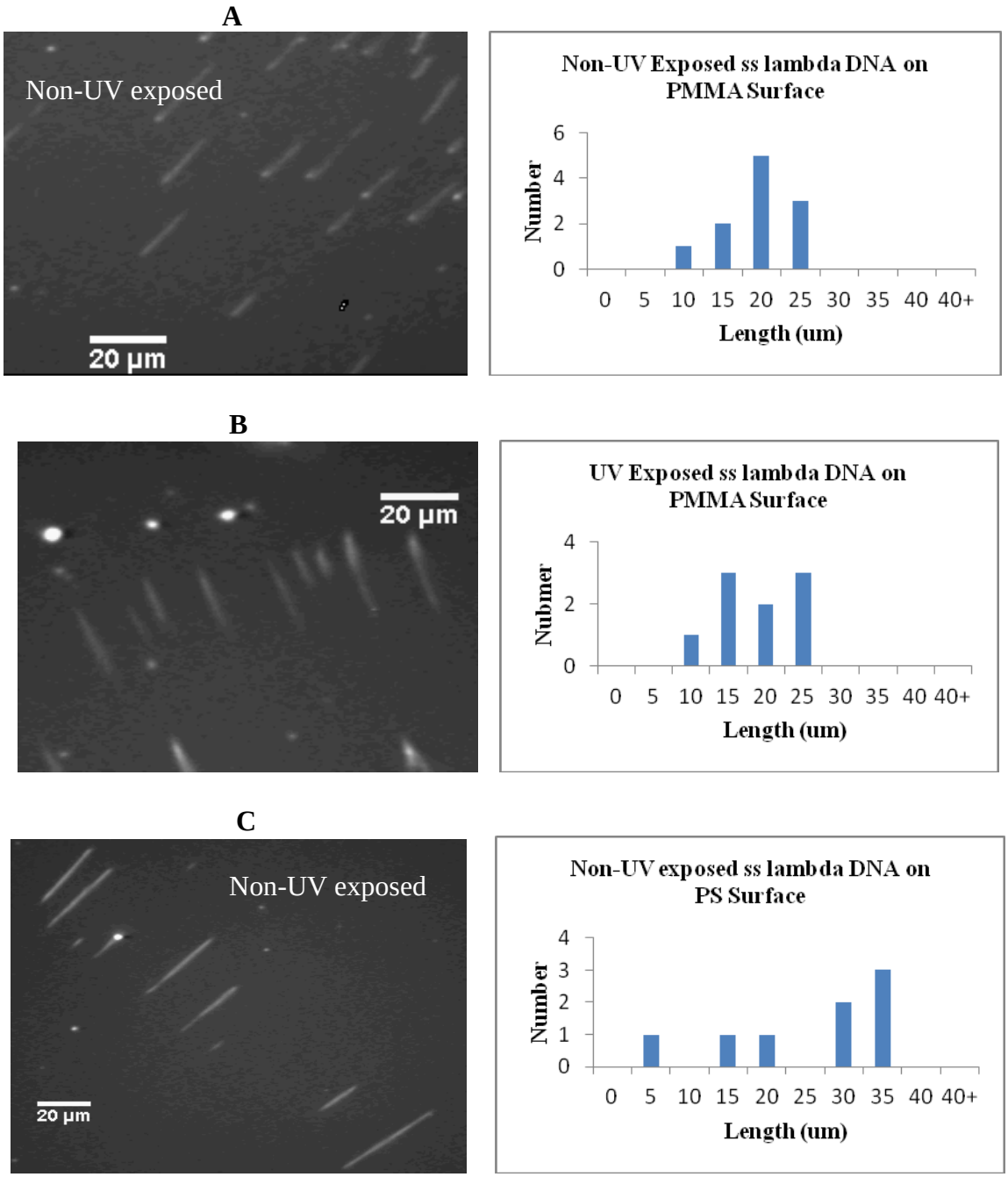


Figure 4.17: 48.5kb ss lambda DNA deposited on a polymer coated surfaces with histograms, images taken with a 40x lens; DNA was stained in the gel for 30 minutes with a 1:10,000x SYBR gold solution prepared in 1X TAE and then extracted from a 1% acros LE gel; (A) non-uv exposed ss lamba DNA on pmma ~ 700Ă thick; (B) uv-exposed ss lamba on pmma ~ 700Ă thick, (C) non-uv exposed ss lambda DNA on ps ~ 500Ă thick;

D

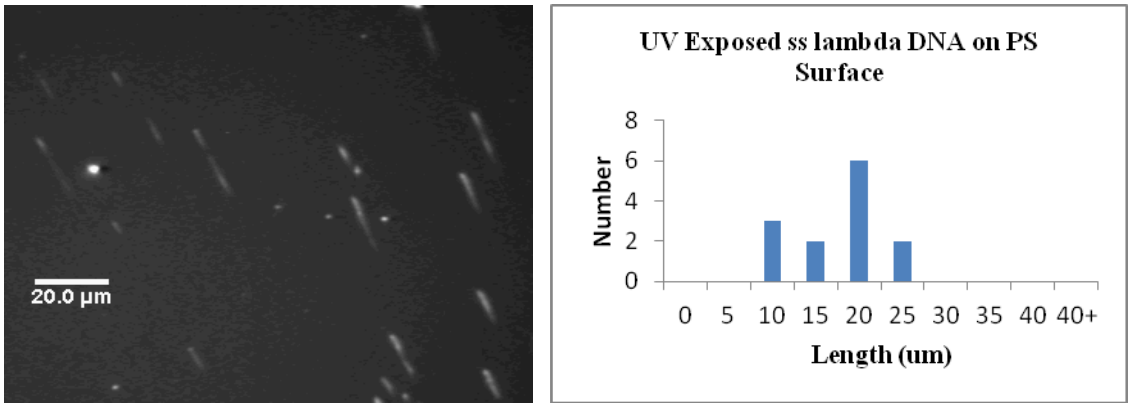


Figure 4.17: 48.5kb ss lambda DNA deposited on a polymer coated surfaces with histograms, images taken with a 40x lens; DNA was stained in the gel for 30 minutes with a 1:10,000x SYBR gold solution prepared in 1X TAE and then extracted from a 1% acros LE gel (D) uv exposed ss lambda DNA on ps ~ 500Ă thick

We analyzed the lengths of a population of ss DNA strands exposed and unexposed to uv light on PMMA and PS surfaces. Histograms were generated to statistically compare the lengths of the DNA. In most cases (Fig. 4.17-4.19), the majority of DNA lengths analyzed fell within the 10-25um range. The full length of lambda DNA measures to be approximately 20um. However, very few lengths of above 30um were also found for the ssDNA unexposed to uv light and ssDNA strands exposed to uv light. Although, the DNA was not exposed for very long periods to uv light, the data cannot suggest that uv does affect the length of ssDNA.

Figure 4.18:

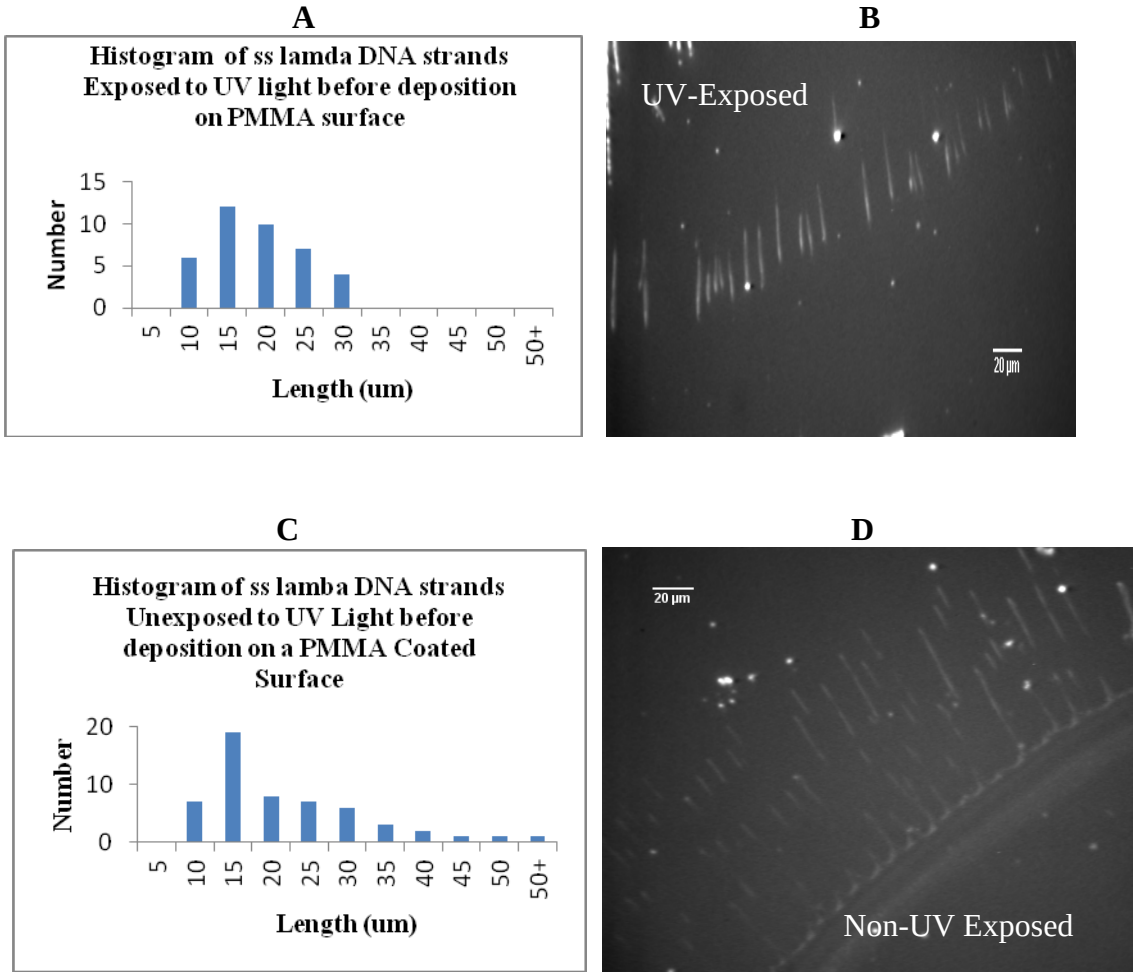


Figure 4.1 8: A) Histogram of ssDNA strands exposed to uv light analyzed on a ~700A coated PMMA surface in image B (40x lens). C) Histogram of ss DNA strands unexposed to uv light on a ~700A coated PMMA surface in image D (40x lens).

Figure 4.19:

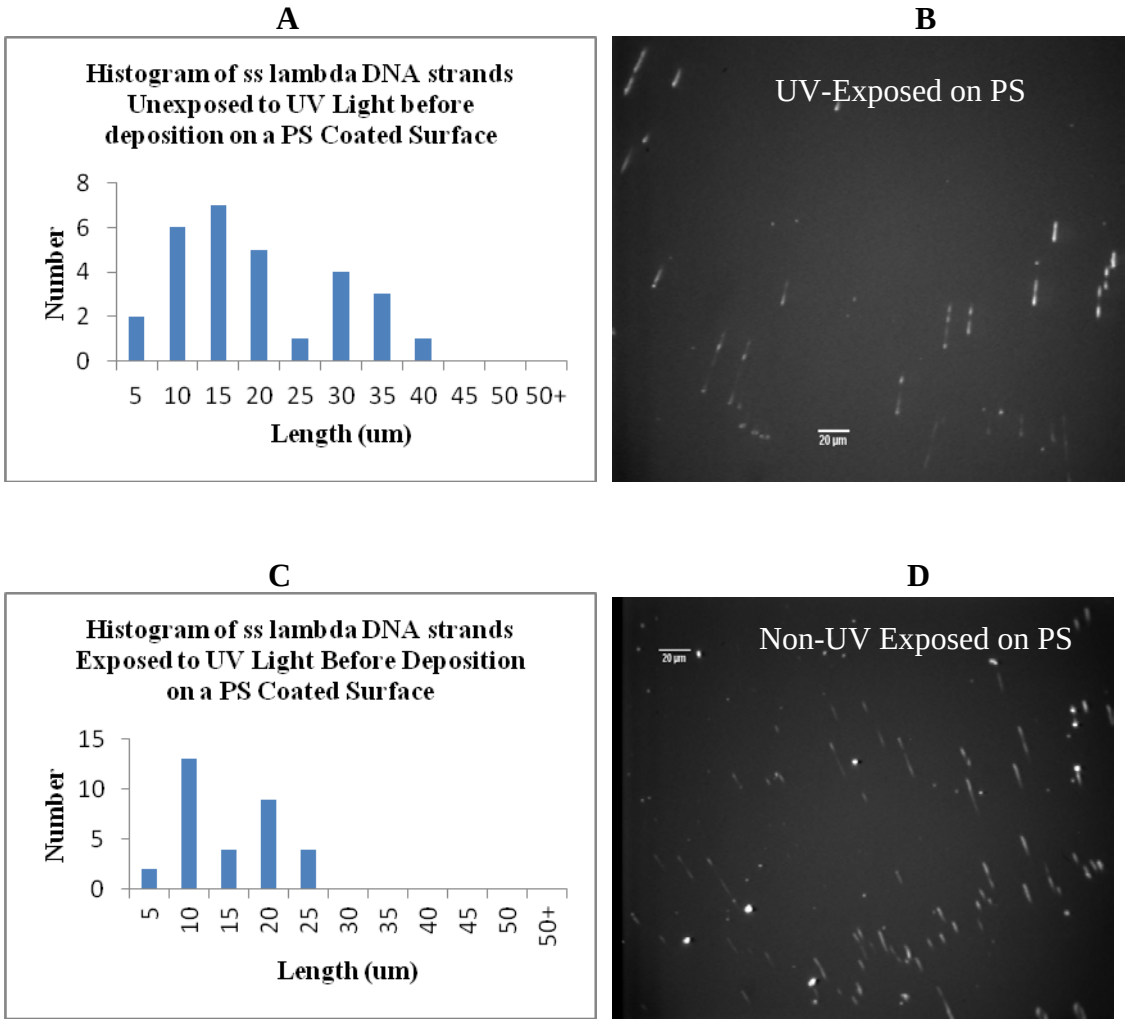


Figure 4.19: (A) Histogram of ssDNA strands exposed to uv light analyzed on a ~500A coated PS surface in image (B) (40x lens). (C) Histogram of ssDNA strands unexposed to uv light on a ~500A coated PS surface image (D) (40x lens).

We also observed areas where ss DNA conformations formed loops or were curved. This may be attributed to various conditions such as the drying process, surface uniformity as well as the exposure to uv light. Loops must occur when center segments absorb and anchor molecules or two chains join at ends (may be indicated by dots at joining points). However, not significantly, these types of DNA conformation seemed to be present in both ss bands of DNA as well as those exposed and unexposed to uv light.

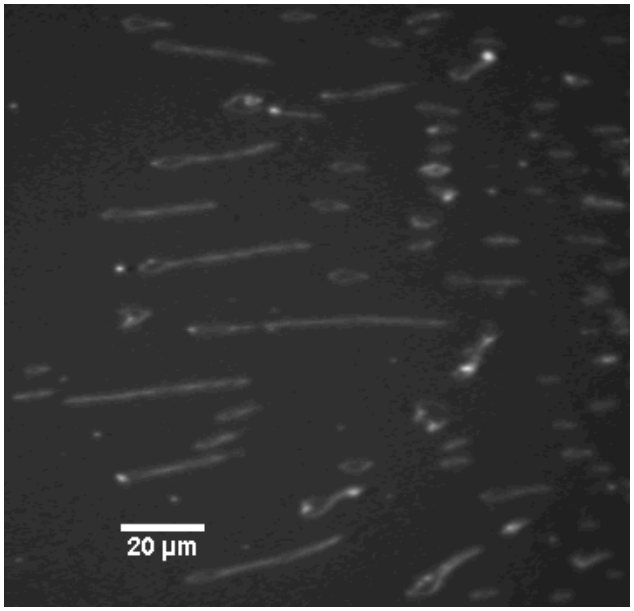


Figure 4.20: ss lambda DNA of various lengths and conformations deposited on a polymer coated surfaces; uv exposed ss lamba DNA exposed on pmma ~ 700Ă thick; image taken with a 40x lens.

4.4 Conclusions

The ability to separate complementary strands of up to 48.5kb ss lambda DNA in urea based gels and its subsequent extraction and deposition on surfaces has been demonstrated. PFGE is a technique that can separate ssDNA strands and ensure they stay that way for convenient extraction and deposition. We found by optimizing pulsing parameters, electric fields, and concentration of agarose gels, we can successfully separate longer ss DNA molecules and extract them to analyze on surfaces. The methods are easy to follow and can potentially be used in diagnostic applications such as sequencing.

We cannot definitively say whether UV exposure is affecting the DNA to great extent when comparing the two conditions. In some cases, we also see different

conformations of ss DNA forming such as loops and coiling on surfaces which may occur due to varying surface conditions.

4.5 Future Work

The success of separating sense and anti-sense strands of 48.5kb lambda by pulse-field gel electrophoresis and depositing them on a surface in-tact shows this to be a feasible method for subsequent analysis of DNA structure. The next measures could employ other pulsing mechanisms such as contour clamped homogeneous electric field (CHEF) or transverse alternating field electrophoresis (TAFE) to separate even longer strands in combination with different pulse schemes and longer gels. Introducing controlled single-strand breaks by enzyme digestion or cutting of one strand followed by separation may be another feasible method. DNA labeling of one or both strands to modify mobility may also be investigated as a possible separation method.

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