SINGLE MOLECULE STUDIES OF FLEXIBLE POLYMER SYSTEMS

BY

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DISSERTATION

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Abstract

Polymeric materials play a profound role in our daily lives. There have been many key advances in polymer processing over the last several decades, but much of the underlying molecular behavior and physics of polymer solutions is not fully understood. Single molecule studies of polymer solutions provide an avenue for studying polymer dynamics and can aid in developing new molecular models of dynamic behavior. For nearly two decades, fluorescently-labeled double stranded DNA (dsDNA) has been the model system for studying single molecule polymer dynamics in non-equilibrium conditions; however, dsDNA is a semiflexible polymer with markedly different local molecular properties compared to flexible polymer chains, such as synthetic organic polymers.

This thesis presents a new methodology for studying truly flexible polymers at the single molecule level. We have demonstrated the ability to synthesize long strands of fluorescently-labeled ssDNA, and we directly imaged single ssDNA polymers stretching in fluid flows in microfluidic devices (Chapter 2). In addition, we have developed an automated flow-based method to isolate individual polymer chains for long periods of time in planar extensional flow (Chapter 3). By combining the tools we developed we were able to study the longest polymer relaxation time dynamics of flexible polymers (Chapter 4), and utilizing both Brownian dynamics simulations and single molecule experiments, we were able to study the relaxation dynamics of flexible chains. In addition, we were able to use to automated hydrodynamic trap to study dynamics of polymers in precisely controlled flow conditions that have not been studied previously (Chapter 6), as well as the dynamics of a different class of materials: ring polymers.
Acknowledgements

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Chapter 1: Introduction

1.1 Overview

Polymer molecules exhibit complex dynamic behavior in non-equilibrium hydrodynamic flow conditions. Polymer chains stretch and orient in solution-based fluid flows, which gives rise to flow-dependent material properties such as bulk stress and viscosity. Ultimately, the macroscopic response of a polymeric material is determined by the underlying microstructure and molecular properties of deformed polymer chains. Polymer dynamics has been studied using a wide array of experimental and theoretical tools [1, 2]. Single molecule techniques allow for the direct observation of chain dynamics at the molecular level, thereby enabling characterization of polymer backbone motion and chain stretching dynamics. An increased understanding of flexible chain dynamics at the single molecule level can be utilized in conjunction with bulk rheological measurements to enhance our knowledge of how to improve polymer processing and aid in applications such as polymer solution based turbulent drag reduction.

1.2 Bulk Rheological Measurements

Over the last 50 years, polymer solution dynamics has been an active area of research. There are many techniques for measuring both linear and non-linear rheology of polymer solutions including rheometry, light scattering, polarimetry, and dichroism. Two important processes relevant to the proposed research that have been characterized in bulk measurements are polymer relaxation and polymer deformation in extensional flow. Relaxation of double stranded DNA has been studied using birefringence relaxation [3], flow dichroism [4, 5], creep recovery [6], and light scattering [7]. These experiments have provided useful information that has been used to
test classic theories of polymer dynamics, such as the Zimm prediction of relaxation time. In addition, light scattering [8] and birefringence [9-12] have been used to infer extent of polymer deformation in extensional flow. This work led to important results such as critical strain rates for stretching and saturation of the molecular extension in flow. While these results provide useful information about the macroscopic fluid response, it is difficult, if not impossible, to extract molecular behavior of the polymer chains in solution because these “bulk” level techniques rely on average measurements of many molecules. Single molecule experiments overcome these challenges by enabling direct observation of polymer chain behavior.

1.3 Single Molecule Studies of Double Stranded DNA

Over the last 15 years, double stranded DNA (dsDNA) has served as the model system to study single molecule polymer dynamics using fluorescence microscopy [13]. In particular, single molecule experiments have almost exclusively relied on lambda (λ-) phage DNA as a model system. Lambda DNA exhibits several advantages as “model” polymer, including facile sample preparation and fluorescent labeling. Moreover, monodisperse samples of λ-DNA are readily available from commercial vendors. In addition, the dynamic time scales for λ-DNA are convenient for studying at the single molecule level. A myriad of single molecule experiments have been performed using λ-DNA including chain relaxation [14], coil diffusion [15], stretching in uniform flow [16], dynamics in extensional [17-19], shear [20] and mixed flow [21], and polymer conformation hysteresis [22]. Recently, dsDNA has been used to directly observe polymer dynamics in confined geometries [23-31]. A typical single molecule setup is depicted in Figure 1.1.

1.4 Molecular Properties of Polymers
1.4.1 Local Chain Flexibility

Although λ-DNA has provided useful information in single molecule studies, ds-DNA is a semiflexible polymer with markedly different molecular properties compared to flexible polymer chains. In hydrocarbon-based polymer chains, chain flexibility is determined by bond rotations about torsion angles, while the bond angle between carbon atoms remains relatively constant.

Chain conformational energy depends on the torsion angle with three energy minima corresponding to the trans (0°) and gauche (±120°) states. A polymer chain is considered *statically flexible* if the energy difference between the trans and gauche states, $\Delta \varepsilon$, is small, i.e. $\Delta \varepsilon \approx kT$. Under these conditions, the trans and gauche states would be equally preferred at thermal equilibrium. A chain is considered *dynamically flexible* if the energy barrier between trans and gauche states, $\Delta E$, is small, i.e. $\Delta E \approx kT$. When a chain is dynamically flexible, it can rapidly explore many conformations without being trapped in a narrow subset of conformation space.

The flexibility mechanisms are fundamentally different for a semiflexible polymer such as dsDNA due to the double helix structure. Double stranded DNA exhibits uniform flexibility along the entire length of the polymer backbone. The flexibility arises due to contour fluctuations [32] as opposed to torsion bond angles in flexible chains. The elasticity of semiflexible polymers is described by the worm-like chain model, and chain stiffness is determined by a parameter called the persistence length, which is a measure of local flexibility and represents the length over which a preferred backbone orientation persists along the chain. Double stranded DNA is relatively stiff with a persistence length of \(~53\) nm [33, 34], whereas polyethylene would be considered flexible with persistence length \(0.57\) nm [35]. This disparity in persistence lengths is further enhanced when considering labeled dsDNA, which has a persistence length of \(~66\) nm when stained with YOYO-1 [15, 16, 36]. The molecular structures of dsDNA and ssDNA, a
flexible polymer, are displayed in Figure 1.2, which suggests that local flexibility can alter global conformation.

1.4.2 Global Flexibility

Based on previous work, λ-DNA has been shown to follow dynamical scaling laws for flexible polymers, mainly due to the relative length scales associated with the polymer chain. In the limit of a small persistence length relative to contour length, the chain will appear flexible at intermediate length scales longer than the persistence length. Global flexibility can be defined as the number of persistence lengths in a polymer chain:

\[
\frac{L}{l_p} \sim N
\]  

(1.1)

where \(L\) is the contour length, \(l_p\) is the persistence length, and \(N\) is the number of statistical steps. Generally speaking, polymer chains with \(N>100\) are considered globally flexible; therefore, λ-DNA is globally flexible because it contains \(N\approx310\) persistence lengths (\(L\approx16.3 \mu m\)). Although λ-DNA is considered flexible under these circumstances, the possibility arises for different dynamics for flexible polymers due to excluded volume effects or intrachain hydrodynamic effects, which arise due to local chemistry.

1.4.3 Monomer Aspect Ratio

The monomer aspect ratio describes the local asymmetry of the polymer chain and plays a role in equilibrium and dynamic chain properties. The aspect ratio, \(a\), is defined as the ratio of the Kuhn step size, \(b=2l_p\), to the chain diameter, \(d\):

\[
a = \frac{b}{d}
\]  

(1.2)

The monomer aspect ratios for fluorescently-labeled double stranded DNA, polystyrene, and single stranded DNA are listed in Table 1.
Monomer aspect ratio plays an important role in both excluded volume and intrachain hydrodynamic interactions. For asymmetric monomers, the ratio of the excluded volume to the occupied volume is equal to the aspect ratio [37]. If a chain has a large monomer aspect ratio, monomer-monomer interactions along the backbone are suppressed, whereas chains with small aspect ratios show dominant monomer-monomer interactions thereby resulting in non-linearities in polymer chain behavior, e.g., a non-linear low force elasticity. In addition, as the monomer aspect ratio increases, the number of intrachain contacts decreases, which results in increasingly free-draining coil behavior when compared to nearly symmetric monomers. Free-draining coils suppress intrachain hydrodynamic interactions (HI) in the coiled state, which suggests that HI is not important for λ-DNA. Indeed, this was confirmed in both Brownian dynamics simulations [17, 38] and in experimental measurements of conformation hysteresis in extensional flow [22], where a 1.3 mm piece of λ-DNA was required before hysteresis in chain conformation in extensional flow was observed. Conformation hysteresis is a spectacular display of non-linear behavior that is generally suppressed in polymer chains with high aspect ratios.

1.4.4 Chain Elasticity and Force Extension Measurements

In the past, the chain elasticity of synthetic polymers has generally been modeled using either the inverse Langevin (ILC) or the Warner force-extension relation [2]. The elasticity of λ-DNA and other semiflexible biopolymers has been well described by the Marko-Siggia force-extension relation [34]. An underlying assumption for all three of these models is the absence of monomer-monomer interactions. For a force-extension relation with no monomer interactions, there is typically a linear Hookean response at low extensions:

\[ x \sim f \]  

(1.3)
where \( x \) is dimensionless extension. For polymer chains in which long range, monomer-
monomer interactions are relevant, e.g. chains with small monomer aspect ratios, chain elasticity
is non-linear at low extensions such that:

\[
\chi \sim f^{2/3}
\]

(1.4)

This low force non-linear response was originally predicted by Pincus in 1976 [39], but was only
recently seen experimentally using ssDNA and polyethylene glycol in a magnetic tweezer assay
[40, 41]. These results further suggest that \( \lambda \)-DNA may not serve as a suitable “model” polymer
chain for flexible polymers.

1.5 Project Overview

Despite the recent exciting progress in our understanding of polymer dynamics via single
molecule studies, there is a strong need to study a new model system of truly flexible polymers
that can adequately model the behavior of typical synthetic flexible polymers. This thesis lays
out a framework for our approach to extending single molecule studies to flexible polymer
systems. In Chapter 2 we describe the synthesis and characterization of our new model system:
fluorescently labeled single stranded DNA. In Chapter 3, we present a new methodology for
studying dynamics of polymeric systems at the molecular level using automated hydrodynamic
trapping and manipulation of single polymer chains. By combining the approaches laid out in
Chapters 2 and 3, we describe our efforts at a first experimental dynamic measurement of
flexible polymers at the single molecule level with our study of relaxation dynamics in Chapter
4. In addition to the study of our new model system, the hydrodynamic trap has also enabled a
host of other controlled single molecule dynamic experiments which are discussed in Chapter 5
and include controlled strain pathways, large amplitude oscillatory extension, and dynamics of ring polymers.
1.6 List of Figures and Tables

![Diagram of a common single molecule setup for studying fluorescently labeled polymer chains.](image)

**Figure 1.1** Schematic depicting a common single molecule setup for studying fluorescently labeled polymer chains. Experiments require an excitation source (typically a laser or mercury lamp), beam optics, a high numerical aperture objective, high quantum yield fluorescent dyes, and a sensitive detector (typically EMCCD camera).
Figure 1.2 Polymer chain structures are shown for (a) double stranded DNA, a semiflexible polymer with a double helix backbone and (b) single stranded DNA, a flexible chain consisting of 5-carbon sugars linked by phosphodiester bonds. For ssDNA, a modified base with primary amine (aminoallyl-uracil) is shown, and ‘B’ represents a natural nucleobase.
Table 1.1 Molecular properties for common model polymer chains.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>dsDNA</th>
<th>Polystyrene</th>
<th>ssDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspect ratio, $a$</td>
<td>66</td>
<td>2</td>
<td>1.2</td>
</tr>
<tr>
<td>Kuhn step size, $b$ (nm)</td>
<td>132</td>
<td>1.5</td>
<td>1.2</td>
</tr>
<tr>
<td>Molecular diameter, $d$ (nm)</td>
<td>2</td>
<td>0.8</td>
<td>1</td>
</tr>
</tbody>
</table>
1.7 References


Chapter 2: Biochemical Synthesis and Characterization of Fluorescently-Labeled Single-Stranded DNA*

2.1 Introduction

In this work, we require high molecular weight, flexible polymers with control over composition and labeling chemistry. Rolling circle replication (RCR) is a method to rapidly synthesize long single stranded DNA containing many copies of tandem repeated sequences of DNA or RNA. RCR is an ideal technique for creating periodic nanoassemblies, biosensors, and repeated DNA aptamers[1]. Rolling circle replication has also been performed in a microfluidic flow system to synthesize surface-tethered DNA molecules[2]. RCR typically begins with a linear single stranded template that is hybridized to a complementary primer strand, thereby creating a circular template structure. Next, the template is ligated to form covalently closed circular ssDNA[3-5]. DNA polymerase is added and bonds to the free 3’ hydroxyl group and initiates replication of the circular template. In rolling circle synthesis, DNA polymerase replicates around and traverses circular templates for hundreds to thousands of cycles, thereby generating long strands of ssDNA.

In prior studies, different experimental aspects of RCR were optimized including template size and ideal DNA polymerase[5]. Templates ranging in size from 26 to 74 nucleotides in size were all efficiently replicated using a variety of DNA polymerases. In this work, phi29 DNA polymerase was used for its exceptional processivity and strand displacement activity[6, 7]. We observed phi29 to generate ssDNA products in excess of 65 kilonucleotides in length. RCR reactions were supplemented with aminoallyl dUTP (aa-dUTP), which is a modified nucleotide containing a primary amine as a reactive chemical moiety to facilitate subsequent

dye-labeling with succinimidyl ester dyes. The RCR reaction scheme used here is shown in Figure 2.1.

The labeling scheme implemented in this work has several advantages over base intercalation labeling methods for double stranded DNA. In the past, \( \lambda \)-DNA has been labeled with intercalating dyes such as YOYO-1 and the Sytox family of dyes. Although these dyes allow for single molecule imaging of dsDNA, they alter the backbone structure of the molecules and rigidify the polymer chain. In most work, the backbone is saturated with dye molecules at a rate of one dye molecule per four base pairs. Using covalent incorporation of dye molecules and via careful adjustment of the number of dye labeling sites, we believe ssDNA is labeled without alterations to backbone structure and at easily tunable dye incorporation ratios.

2.2 Materials and Methods

2.2.1 Rolling Circle Replication Reactions

Oligonucleotide templates and primers for rolling circle replication (RCR) were designed using VectorNTI software and synthesized by Integrated DNA Technologies (Coralville, IA). Table 2.1 shows the sequences of template oligonucleotides used in these experiments. Template oligonucleotides were designed to generate ssDNA products rich in either purine or pyrimidine nucleotides. In some cases, product ssDNA was designed to be nearly homopolymeric. All template oligos contained a 5’ phosphorylated terminus to enable formation of the minicircle template by ligation.

Linear templates were circularized by a ligation reaction using T4 DNA ligase (New England Biolabs). First, a reaction mixture (500 \( \mu \)L) consisting of 200 nM template and primer oligonucleotides in T4 DNA ligase buffer (10 mM Tris/Tris-HCl, 200 \( \mu \)M EDTA, 2 mM NaCl)
was prepared. Template and primer oligonucleotides were hybridized by heating at 70°C for 2.5 minutes, followed by slow cooling to room temperature (20°C). Next, 600 units of T4 DNA ligase was added, and the ligation reaction proceeded for 5 hours at 16°C.

RCR reactions consisted of 25 nM ligated/primed minicircle, 200 μM dTTP/aa-dUTP, 1 mM custom-mix dNTPs (containing only nucleotides in the synthesized product sequence) and 200 μg/mL BSA in phi29 DNA polymerase reaction buffer (50 mM Tris-HCl, 10 mM (NH₄)₂SO₄, 10 mM MgCl₂, 4 mM dithiothreitol, pH 7.5) in a total reaction volume of 50 μL. The polymerization reaction was initiated by addition of 5 units of phi29 polymerase, and the reaction proceeded for 30 minutes at room temperature, unless otherwise noted. In negative control (NC) reactions, no phi29 polymerase was added. Reactions were terminated by addition of EDTA to a final concentration of 20 mM, and the products were stored at 4°C.

2.2.2 Gel Electrophoresis Characterization

Gel electrophoresis was used to characterize ssDNA products from RCR reactions: agarose gels (0.6%) were run in TAE buffer using 1 kb DNA ladder (NEB) supplemented with 50 ng of λ-DNA as a standard marker. Agarose gels were run for 35 minutes at 100 V and pre-stained with SYBR Gold added to the loading buffer (1X). All gels were imaged using a Gel Doc 2000 (Biorad), and fluorescent gel images are inverted for ease of display. All enzymes, including T4 DNA ligase, phi29 DNA polymerase, Exonuclease I, and DNA markers for electrophoresis and dNTP stocks were purchased from New England Biolabs. Aminoallyl dUTP (aa-dUTP) and SYBR gold were purchased from Invitrogen. All other chemicals were obtained from Sigma-Aldrich or Fischer Scientific and were molecular biology grade purity.

2.2.3 Fluorescent Dye-Labeling and Quantification
Single polymer imaging experiments were performed using Sequence 1 (Table 2). RCR reaction times were increased to 90 minutes to generate long ssDNA products appropriate for single molecule fluorescence microscopy. Prior to fluorescent labeling, ssDNA from RCR reactions was purified using a HiTrap desalting column and fast protein liquid chromatography (AKTA FPLC, GE Biosciences), thereby removing excess oligonucleotides and dNTPs. This process also acted as a buffer exchange step for labeling. Approximately 800 µL of the RCR reaction mixture was loaded onto the FPLC and eluted into diluted Alexa Fluor 532 labeling buffer (25 mM sodium bicarbonate, pH 8.0)[8]. Purification and ssDNA concentration prior to labeling was performed using Vivacon2 spin columns, 100 kDa MWCO (Sartorius). Approximately 2 mL of eluted ssDNA sample was loaded onto the spin columns and centrifuged at 2,500xg for 45 minutes, which generally yielded a 25-30X increase in DNA concentration, as determined by measuring absorbance at 260 nm using a Nanodrop 1000 (Thermoscientific). Typically, sample is washed through the Vivacon 2 columns twice to further improve buffer exchange and to remove phi29 polymerase (MW: 65 kDa) that was eluted from the FPLC step. Purified ssDNA (5 µg) at a volume of approximately 32 uL was concentrated further using a speedvac (Genevac SP Scientific) to bring the final sodium bicarbonate concentration to 100 mM. The resulting ssDNA solution was labeled with 2 uL of 30 µg/µL NHS-ester Alexa Fluor 532 (Invitrogen). Solutions were mixed thoroughly by pipet and the reaction proceeded for 1-2 hours at room temperature in the dark. In all labeling reactions, DNA and dye concentrations were maintained constant to standardize reaction conditions. Labeling reactions were quenched by addition of 1 M Tris/Tris-HCl (pH 8.0) to a final volume of ~15 µL.

Following the dye labeling reaction, ssDNA products were purified from unreacted fluorescent dye and remaining template structures to enable single molecule imaging and for
quantification of dye labeling ratios. Immediately following quenching of the ssDNA labeling reactions, reaction mixtures were supplemented with an appropriate denaturing agent (6 M urea, 8M guanidine hydrochloride, or 80% formamide) to a final volume of ~75 μL. These mixtures were purified using Biospin 6 columns (Biorad), which also served as a buffer exchange to elute fluorescently-labeled ssDNA products in 10 mM Tris/Tris-HCl buffer (pH 8.0). Dye labeling ratios were determined using absorbance measurements. Fluorescently-labeled ssDNA for single molecule experiments was supplemented with EDTA (10 mM) and diluted with 50% (v/v) glycerol for storage at -20°C.

Bulk fluorescence measurements of enzymatically-digested fluorescently-labeled ssDNA were used to quantify the extent of putative dye interactions (if any) along the polymer backbone. Here, 1.5 μg of labeled ssDNA sample was split into two, equal concentration reactions. One reaction treated with an excess of DNase I for 30 minutes at 37 °C, and the second reaction was untreated. Bulk fluorescence measurements were performed by illuminating samples at excitation wavelengths between 400-540 nm, while monitoring emission at 555 nm using a Cary Eclipse Fluorescence Spectrophotometer (Varian).

2.2.4 Fluorescence Microscopy

Direct visualization of single fluorescently-labeled ssDNA molecules was performed using epifluorescence microscopy. Single stranded DNA was imaged using an Olympus IX71 inverted microscope equipped with a 100x oil immersion objective lens (Olympus UPlanSApo) and an Andor Ixon EMCCD camera. A solid-state laser (CrystaLaser) was used as an illumination source at a wavelength of 532 nm. Polymers were imaged in viewing solution containing 50 mM Tris/Tris-HCl (pH 8.0), 1 mM EDTA, 5 mg/mL glucose, 20 mM NaCl, and ~95% glycerol by weight. Prior to microscopy, solutions were purified with a 0.45 μm membrane filter, followed
by addition of β-mercaptoethanol (140 mM), glucose oxidase (~65 U/mL) and catalase (1.1 kU/mL) as oxygen scavenging reagents to minimize photobleaching of the Alexa Fluor dye. For viewing, ~50 ng of fluorescently-labeled ssDNA was added to 1 mL of viewing solution (yielding ~1-10 pM ssDNA). Individual ssDNA molecules were visualized both in quiescent conditions on a microscope slide and in planar extensional flow generated in a PDMS-based microfluidic device, which effectively stretches ssDNA molecules for backbone visualization. Images were processed using ImageJ software. All images were corrected for background, false color was applied, and in some cases, levels were adjusted to filter approximately 15-20% of the lowest intensity pixels for noise reduction.

### 2.2.5 Product Separation

In an attempt to reduce product polydispersity, gel filtration-based separation techniques were utilized. Sephacryl S-1000 resin was packed into (GE Life Sciences) following the manufacturer’s protocol. Control experiments were performed using 25 μg of the HindIII digest of λ-DNA and DNA ladders. To test the ability of this resin to separate ssDNA, several RCR reactions with reaction times varying from 1-15 minutes were pooled together. Samples were pumped through the column at a flow rate of 0.1 mL/min and collected in 500 uL fractions. To characterize separation, pulsed field gel electrophoresis was utilized. Pulsed field gel electrophoresis increases the size resolution of DNA fragments as compared to native gel electrophoresis by switching the polarity of the electric field between the electrodes. In this manner, DNA moves in both forward and reverse directions in the gel, thereby resulting in enhanced separation of larger samples [9]. Ultimately, this approach was successful for separating dsDNA fragments, but was unsuccessful for ssDNA.
2.3 Results and Discussion

2.3.1 Template Design

Table 2.1 contains a summary of oligonucleotide template sequences and RCR reaction results. Template ssDNA sequences were designed to allow for dye labeling and to prevent intramolecular base pairing. To facilitate labeling, adenine monomers were designed to occur in template strands at semi-random locations, thereby yielding amine-modified thymine or uracil nucleotides in ssDNA product strands at tunable intervals. In addition, we designed purine-rich or pyrimidine-rich templates consisting of only 2 nucleotides (A/G or C/T), thereby preventing intrachain base pairing. In some cases, a third nucleotide (A) was present at low ratios to facilitate dye-labeling by incorporation of amine-modified nucleotides (aa-dUTP). Using this method, a wide-range of template sequences was successfully replicated by RCR, yielding long ssDNA products (>65 kb). In some cases, we observed limitations on template sequence design, as evidenced by low reactions yields or short products. In general, RCR reactions were inhibited for templates containing consecutive stretches of a single nucleotide, particularly cytosine (C) or guanine (G). Indeed, previous research has demonstrated that some polymerases are inhibited by homopolymeric sequences due to enzymatic ‘slippage’ on the template, and guanine-rich sequences can form quaternary structures, which may prevent ligation[7]. For some sequences, purine-rich templates failed to replicate entirely, and many of the unsuccessful reactions contained templates with larger amounts of guanine compared to the successful reactions. Overall, RCR success or failure appeared to be independent of sequence length for circular DNA templates in the range of 28-66 bases, as observed for Sequences 1-3 and Pyr 4-5.
2.3.2 Reaction Time Results

The effect of RCR reaction time on ssDNA product length distributions was characterized using Sequence 1. Figure 2.2 shows an image of an agarose gel containing ssDNA products from timed RCR reactions with durations ranging from 0.5 to 180 minutes. As expected, ssDNA product length increases with increasing reaction time. In addition, product bands appear to broaden and intensify for long reaction times, indicating increases in both product yield and polydispersity. Typical yields for a single RCR reaction were on the order of 1-2 µg depending on reaction time. To increase yield for dye labeling, multiple reactions were carried out in parallel.

2.3.3 Aminoallyl-dUTP Incorporation Results

The ratio of natural to modified nucleotide (dTTP:aa-dUTP) in the reaction mixture was varied to determine the effect of unnatural bases on product lengths (Figure 2.3). Upon increasing the amount of aa-dUTP, product length, yield, and polydispersity remained nearly constant, which suggests that the base modification is non-perturbative for replication by phi29 polymerase. Figure 2.4 shows alkaline agarose gel electrophoresis for ssDNA samples containing variable ratios of aa-dUTP:dTTP. Alkaline agarose gel electrophoresis was performed using standard protocols in molecular biology, and the denaturing conditions allow for accurate determination of molecular weight. The results shown in Figure 2.4 are consistent with those in Figure 2.3 in that ssDNA samples appear relatively monodisperse within the resolution of the gel, with the general absence of broad molecular weight distributions; however, as the fraction of aa-dUTP is increased, some amount of ssDNA product exhibits inhibited mobility in the denaturing gel, presumably due to the modified aa-dUTP nucleotide and the chemical nature of the additional primary amine. Single molecule visualization directly reveals that after dye labeling, ssDNA
molecules from these samples (Sequence 1) stretch to reveal clean, linear and pristine ssDNA polymer backbones.

2.3.4 Pyrimidine-Rich ssDNA Product Results

As shown in Table 2.1, RCR reactions were performed to yield products rich in either pyrimidines or purines. In general, higher molecular weight products were obtained for ssDNA rich in purines (A/G) compared to pyrimidine rich products. For most ssDNA sequences, products migrated with similar mobility on both native and denaturing gels, suggesting the absence of base pairing in ssDNA products. Interestingly, ssDNA products rich in pyrimidine bases (generated by Sequences 4-6 and Pyr 6, 8-10) showed inhibited migration through native agarose as demonstrated in Figure 2.5a. For these sequences, denaturing gels were used to quantify product length distributions, in addition to single molecule visualization. Alkaline conditions (Figure 2.5b) enabled product migration but demonstrated large smears over a wide range of product lengths, indicative of a large increase in polydispersity compared with purine-rich ssDNA products.

2.3.5 Base Pairing and Base Stacking Interactions

In this work, ssDNA sequences are designed to prevent intrachain base pairing and base stacking interactions. In nature, base stacking is an important phenomenon that aids in stabilization of the double helix in dsDNA. In ssDNA, base stacking can give rise to helical domains. Stacking interactions in ssDNA are dominant for poly(dA) and poly(dC) homopolymeric sequences, as evidenced by plateaus in AFM force-extension elasticity data for homopolymeric ssDNA molecules[10-13]. Poly(dT) shows minimal or no evidence of base stacking and exhibits force-extension curves similar to random ssDNA sequences (denatured λ-DNA)[14].

23
For the majority of the designer sequences (including Sequence 1), base stacking is not expected. Stacking is primarily relevant for homopolymers with long poly(dA) domains[10], which do not occur in any of the designer sequences except Sequences 8 and 10, which were not used in labeling experiments. Furthermore, base stacking interactions are weak between chemically distinct bases[10] and mainly dominate in homopolymeric sequences, which has been previously verified in molecular beacon experiments where a single base defect in a poly(dA) loop significantly impacted the enthalpic barrier to hairpin closing[15]. Base stacking involves only interactions between neighboring nucleobases and is either non-cooperative or weakly cooperative[10], which implies that short stretches of single base connected by a differing nucleotide are not impacted by base stacking.

Finally, precautions were taken to minimize or completely eliminate intramolecular base pairing in ssDNA products. By design, hairpins are avoided in all sequences used in this work. Templates for RCR were designed to be either pyrimidine-rich (C/T) or purine-rich (A/G) sequences that never contained all four nucleotides. For some pyrimidine-rich templates, a third base (A) was included in minicircles in small amounts (~1:10 bases) to facilitate labeling with aa-dUTP (e.g., Sequence 1). Base pairing energies were calculated for all sequences, and in all cases, the energy was low and on the order of thermal energy (k_B T). A single isolated dATP-dTTP base pair in a long, non-interacting chain (e.g., Sequence 1) is a relatively weak interaction with energy ~1.3 k_B T[16]. Previous work based on Monte Carlo simulations was used to model force-extension data for random and hairpin sequences (poly(dA-dT) and poly(dG-dC)) using standard base pairing rules, and random sequences did not alter the elastic chain behavior[17]. Single molecule images of stretched ssDNA reveal clean and pristine linear polymer backbones, with no discernable evidence of base interactions. It is anticipated that base pairing plays no role
for ssDNA molecules containing user-defined tailored sequences used in this work, in particular Sequence 1. Optical tweezing results from TJ Ha’s group for sequence 1 confirm that there is no base pairing.

### 2.3.6 Fluorescent Dye Labeling and Quantification

We desired to label ssDNA with low amounts of fluorescent dye in order to allow for efficient visualization of single polymers, while also minimizing putative modifications to the native ssDNA backbone. For determination of optimal backbone labeling, three batches of ssDNA (Sequence 1) were synthesized with varying amounts of aa-dUTP, specifically with aa-dUTP:dTTP ratios of 1:9, 3:7, and 3:2. All three samples were labeled with dye, purified and analyzed using bulk and single molecule techniques.

Figure 2.6 contains the results for the dye labeling ratio measurements. The number of dye molecules per 100 bases was experimentally determined using UV and visible wavelength absorption measurements. The theoretical maximum dye labeling ratio was determined by assuming stoichiometric incorporation of aa-dUTP and quantitative completion of the labeling reaction. In general, each sample had a dye labeling ratio less than the theoretical maximum, though we determined similar labeling ratios for all samples (~1 dye:100 bases). However, bulk fluorescence data and single molecule experiments suggest that ssDNA molecules become moderately (~2x) brighter upon increasing amounts of aa-dUTP. One possible explanation is incomplete removal of unincorporated dye molecules. Experimentally measured dye-labeling ratios represent maximum labeling amounts for chain backbones and serve as an effective upper limit for dye incorporation. Overall, a labeling ratio of ~1 dye:100 bases is low and significantly less than the typical labeling ratio for dsDNA (1 dye:4 bp) using intercalating dyes such as YOYO-1.
Bulk level fluorescence measurements in combination with a nuclease assay were utilized to probe for putative dye-dye interactions arising from pi bond stacking for dye molecules along the polymer backbone. The results are shown in Figure 2.7. The nuclease assay showed no dye interactions, as measurements before and after nuclease treatment were within error measurements. This was expected because the degree of labeling for ssDNA polymers was low (~1 dye:100 bases) and far below labeling amounts for previously observed dye-dye interactions using Alexa fluorophores of ~8 dyes:100 bases[18].

2.3.7 Sample Purification

To test the efficacy of our dye purification method, two control experiments were performed with the Biospin 6 columns. In the first experiment, approximately 15 μg of ligated and primed minicircle (MC1) was filtered through 5 successive columns. This experiment was repeated with the same total mass of minicircle, but in the presence of 6M urea, to ensure that the addition of a chemical denaturant did not affect the column performance. The concentrations of the sample filtrates were measured by measuring absorbance at 260 nm using a Nanodrop. The results are shown in Table 2.2. As demonstrated, two columns adequately removed nearly 100% of the starting minicircle material.

In the second control experiment, the same mass of Alexa Fluor 532 dye used in the labeling reactions was filtered through the columns at the same volume as for typical labeling reactions. To quantify the dye removal, the UV-Vis spectra for the sample filtrates was measured using the Nanodrop. The results are shown in Table 2.3. Again, after only two columns, nearly all of the unreacted dye has been removed from the sample. In practice, sometimes additional columns are required. Typically, samples are purified in the Biospin 6 columns until the calculated base:dye ratio no longer changes.
2.3.8 Epifluorescence Microscopy

Fluorescence microscopy was used to directly image single fluorescently-labeled ssDNA molecules. Single ssDNA molecules generated by RCR synthesis are bright, photostable and suitable for single molecule fluorescence microscopy (Figure 2.8). Images of fluorescently-labeled ssDNA were directly compared to images of λ-DNA labeled with an intercalating dye. Currently, λ-DNA is the standard molecule for single molecule polymer studies, and this comparison serves as a useful benchmark for fluorescent labeling studies. Single λ-DNA molecules were labeled with YOYO-1 as described elsewhere, and dsDNA was imaged using epifluorescence microscopy using a mercury lamp as the illumination source.

Figure 2.8 shows images of stretched and coiled ssDNA molecules (Sequence 1) for ssDNA samples with variable dye loadings, with a side-by-side comparison to fluorescently-labeled λ-DNA. Polymer molecules are stretched in planar extensional flow inside a microfluidic flow device. Stretched ssDNA molecules were chosen to be of similar length to stretched λ-DNA (~20 μm contour length for stained DNA). Clearly, fluorescently-labeled ssDNA molecules appear as bright polymer chains suitable for single molecule visualization. Overall, the ssDNA sample synthesized with a 3:2 ratio of aa-dUTP:dTTP showed comparable image quality to λ-DNA. Chain backbones appeared brighter, and the signal to noise levels for labeled ssDNA molecules improved as the aa-dUTP:dTTP ratio was increased, which is generally consistent with the fluorescence data in Figure 2.7. Overall, these experiments serve as a useful guide to generate ssDNA for single molecule studies.

As discussed earlier in this chapter, synthesis of long strands of ssDNA using RCR yields a sample with relatively high polydispersity. To estimate chain length distributions in replicated ssDNA samples, pulsed field gel electrophoresis (PFGE) was used. The results (not shown)
illustrated that ssDNA (Sequence 1) synthesized by RCR using 60 minute reactions produced chains with an average size of ~50 kbases. To further quantify sample polydispersity, single ssDNA molecules stretched to near full extension were directly visualized using fluorescence microscopy. Chain length distributions and a histogram of ssDNA molecule size was constructed (Figure 2.9). In particular, ssDNA polymers were stretched in a stagnation point flow generated in a PDMS-based microfluidic device, which is a useful method for stretching polymers to high degrees of extension (~90% contour length). Polymer length was determined by measuring chain end-to-end distance using image analysis.

As shown in Figure 2.9, the majority of ssDNA molecules were sized larger than ~20 μm (equivalent to the contour length of stained ds-λ-DNA), in agreement with PFGE analysis. Additionally, ~10-15% of molecules exhibited stretched lengths in excess of 30 μm, which is an ideal size range for single molecule polymer dynamics. For single molecule studies, polydisperse samples can be easily analyzed, because the experimenter can select “targeted” polymer chains with specific contour lengths when acquiring data, which overcomes issues with chain length distributions. Alternatively, monodisperse samples of ssDNA can be obtained by denaturing monodisperse pieces of λ-DNA or other dsDNA. This approach has its own challenges, including labeling the polymer backbone with fluorescent dye without using modified nucleotides, and the possibility for base pairing and stacking due to lack of control over the sequence. Although the contour lengths of ssDNA molecules in this work are similar to λ-DNA, ssDNA is extraordinarily flexible. Whereas λ-DNA contains ~150 Kuhn steps, ssDNA contains ~10^4 Kuhn steps for a chain of similar contour length, assuming a bare persistence length of ~0.62 nm for ssDNA with screened electrostatic interactions[19].
2.3.9 Product Separation

The PFGE results for the Hind III digest of λ-DNA filtered through the Sephacryl S-1000 column are shown in Figure 2.10. As demonstrated, larger fragments pass through the column at shorter times due to the fact that individual molecules are too large to diffuse through the gel matrix. Fractions collected at later times include smaller DNA fragments that more readily diffused through the gel. Based on the success of the column in separating dsDNA fragments, the same method was attempted for RCR ssDNA products. The results are shown in Figure 2.11. Based on these results, it appears that the column did not play a significant role in further separating the polydisperse sample that was loaded. This could be due to the bulk of the sample having a higher molecular weight than the intended working range of the S-1000 gel. In order to elucidate the precise reason good separation was not possible, further experiments would need to be conducted.

2.4 Concluding Remarks

This chapter presents work on rolling circle replication and demonstrates the ability of RCR to synthesize long strands of ssDNA. In addition, the fluorescent labeling and characterization of ssDNA products is discussed. Rolling circle replication has advantages over other synthesis methods due to the ability to precisely control sequence composition and incorporate non-natural bases in ssDNA products. In this chapter, the effects of reaction time, non-natural base concentration, and sequence composition were all characterized. As expected, product lengths and yields both increased with increasing reaction times (Figure 2.2). Additionally, the phi29 polymerase enzyme successfully incorporated modified nucleotides with no apparent decrease in product length (Figure 2.3). Although not all designer sequences were replicated successfully, a wide range of product compositions was obtained.
The fluorescent labeling reaction appeared to proceed efficiently, and clear images of stretched ssDNA molecules were obtained using fluorescence microscopy. Labeling ratios for ssDNA are approximately 20 times less than that for λ-DNA, however fluorescent images of ssDNA were of similar quality. Due to the low dye labeling ratio, there was no evidence of dye-dye stacking interactions based on a nuclease assay. Therefore, we believe that our labeling scheme does not alter the backbone structure of the polymer chains, and the dynamic properties of the molecules are not expected to be perturbed from the native state.

Via direct imaging of single ssDNA molecules stretching in flow, the gel electrophoresis results for the synthesis reactions were also confirmed. A large number of stretched molecules appeared to be in excess of 20 microns (34 knt) in length. Ideally, for dynamic experiments, polymer chains with longer contour lengths are crucial for observing stretching pathways and relaxation time behavior at different degrees of extension. Attempts to reduce polydispersity for ssDNA using gel filtration techniques were not entirely successful; however, this problem can be minimized with careful trapping of molecules, allowing the experimenter should be able to “hand pick” molecules from a certain subset of lengths as discussed in Chapter 3.
Figure 2.1 Schematic of rolling circle replication scheme for ssDNA synthesis. DNA polymerase is represented by an oval shape, and stars represent fluorescent dye molecules (Alexa Fluor 532).
Figure 2.2 RCR reaction time was varied between 0.5, 2, 5, 10, 30, 60, and 180 minutes in Lanes 1-7, respectively. NC: negative control with no phi29 DNA polymerase.
Figure 2.3 RCR products for varying ratios of natural to modified nucleotide, with dTTP:aa-dUTP ranging between 1:0, 4:1, 3:2, 2:3, 1:4, 0:1 in Lanes 1-6, respectively. Agarose gels (0.6%) were run for 30 min at 120V. NC: negative controls (no phi29 DNA polymerase).
Figure 2.4 Denaturing alkaline gel electrophoresis for replicated ssDNA products (Sequence 1) with varying dTTP:dUTP ratios. The dTTP:dUTP ratios varied between 1:0, 4:1, 3:2, 2:3, 1:4, and 0:1 in Lanes 1-6, respectively. The lower band corresponds to ssDNA product, which shows similar molecular weight as a function of increasing dUTP. As the dUTP content was increased, some of the ssDNA product showed inhibited mobility within the gel, however, the gel conditions were denaturing, which fully prevents base pairing interactions. The gel was a 0.6% alkaline agarose gel and was run at 60V for 1 hour.
Figure 2.5 RCR products for Sequences 1, 4, 5, 6, and Pyr 6 in Lanes 1-5, respectively, were run in both (a) native and (b) alkaline agarose gel electrophoresis. Sequence 1 yields large ssDNA product and was used in single molecule visualization studies. Sequence 1 migrates at nearly the same mobility in native and denaturing gels, which suggests the absence of base pairing interactions. For other sequences (4, 5, 6), the gels illustrate the sequence-dependent product mobility in native agarose gel electrophoresis. In general, we observed pyrimidine-rich sequences to migrate slowly through native gels, which presumably does not arise due to base pairing, because nearly homopolymeric (dT) ssDNA also did not migrate efficiently in native gels (Lane 5, Pyr 6). Finally, these gels show the difference in product length distributions for purine-rich (Sequence 1) and pyrimidine-rich (Sequences 4, 5, 6, Pyr 6) sequences.
Figure 2.6 Summary of dye labeling ratios as a function of aminoallyl-dUTP ratio. Error bars are minimal and are contained within the size of the data labels.
Figure 2.7 Fluorescent measurement results for labeled ssDNA samples with varying amounts of aminoallyl-dUTP. Three measurements of each sample were taken with a standard deviation shown by the error bars. All measurements had background subtracted as determined by monitoring the emission fluorescence at excitation ranges outside the excitation range of the dye.
Figure 2.8 Direct visualization of fluorescently-labeled ssDNA molecules using fluorescence microscopy. Single molecules of ssDNA and ds-λ-DNA are shown for (a) stretched and (b) coiled configurations. ssDNA (Sequence 1) with variable dye-labeling ratios are imaged.
Figure 2.9 Histogram of ssDNA size distribution using single molecule visualization. (a) Single ssDNA molecules are classified based on size [11, 20] and binned into (b) size distributions over a 73 molecule ensemble.
Figure 2.10 PFG of Hind III digest of λ-DNA. The first and last lanes of the gel contain the original digest as loaded into the column. Lanes 2-6 contain fractions collected from the FPLC at different times during the run. Typically, fractions were collected with around 2 mL of eluted sample in between.
Figure 2.11 PFG of RCR ssDNA product passed through Sephacryl S-1000 FPLC column. Lane 1 contains 1kb DNA ladder with λ-DNA. Lane 2 contains the original RCR product loaded into the column. Lanes 3-8 contain fractions collected at increasing time points during the run.
Table 2.1 RCR template sequences (bold values indicate nucleotides at the primer site)

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Template Sequence</th>
<th># nt</th>
<th>Longest 1 nt stretch</th>
<th>RCR success</th>
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<tbody>
<tr>
<td>1</td>
<td>5'-ACTCTTCT(T)A(T)<em>(T)C(T)</em>(T)CaACTTTCCAT</td>
<td>28</td>
<td>4</td>
<td>Yes</td>
</tr>
<tr>
<td>2</td>
<td>5'-CTTTCTTACCT(T)C(T)ATC(T)C(A(T)_(T)ACTTTCTCCTACT</td>
<td>48</td>
<td>5</td>
<td>Yes</td>
</tr>
<tr>
<td>3</td>
<td>5'-ACTCTTTCTCTTACTCTCTCTACTCTC(T)ATTTCTTTACCTTCTCTCT</td>
<td>66</td>
<td>6</td>
<td>Yes</td>
</tr>
<tr>
<td>4</td>
<td>5'-AGGTGTGTA(A(G)<em>(G)T(G)</em>(G)TATGGGATG</td>
<td>28</td>
<td>4</td>
<td>Yes</td>
</tr>
<tr>
<td>5</td>
<td>5'-GGTAGGTAAT((G)AT)_(G)AT((G)AT)GGATGGTGAATGGA</td>
<td>48</td>
<td>3</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>5'-</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5'-AGGTGAGGAAT(G)<em>(G)T(A(G)</em>(G)T(G)_(G)TATGGGATG</td>
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<td>4</td>
<td>Yes</td>
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<tr>
<td>7</td>
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<td>28</td>
<td>21</td>
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<td>5'-ATAAATT(T)A(A)_(T)ATATTAA</td>
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<td>10</td>
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<td>9</td>
<td>5'-ACACCCCCCCCA(A)<em>3A(A)</em>(C)_(C)AACCACCAACCA</td>
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<td>9</td>
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<td>Pyr 1</td>
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<td>Pyr 2</td>
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<td>Pyr 3</td>
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<td>Pyr 4</td>
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<td>Pyr 5</td>
<td>5'-AGGAAGGAAGAGAGAGAGAGAGAGAGAGAGAA</td>
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<td>Pyr 6</td>
<td>5'-GGAGAA(A)_(A)GAAGAGGAA</td>
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<td>Pyr 7</td>
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<td>5'-AGA(A)<em>(A)GAAG(A)</em>(A)GAAG(A)<em>(A)GAAG(A)</em>(A)GAAG</td>
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<td>9</td>
<td>Yes</td>
</tr>
<tr>
<td>Pyr 10</td>
<td>5'-AGGAGT(G)<em>(G)AT(G)</em>(G)AAAGGATG</td>
<td>28</td>
<td>4</td>
<td>Yes</td>
</tr>
</tbody>
</table>
Table 2.2 Biospin 6 column performance for ligated/primed minicircle 1 (MC 1 L/P)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Column #</th>
<th>Measured Mass (ng)</th>
<th>% Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC 1 L/P</td>
<td>Starting sample</td>
<td>13650</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>136</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>None detectable</td>
<td>&gt;99.9</td>
</tr>
<tr>
<td>MC 1 L/P with 6M urea</td>
<td>Starting sample</td>
<td>13650</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>134</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>None detectable</td>
<td>&gt;99.9</td>
</tr>
</tbody>
</table>
Table 2.3 Biospin 6 column performance for unreacted AF 532 dye

<table>
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<th>Sample</th>
<th>Column #</th>
<th>Absorbance at 532 nm (a.u.)</th>
<th>% Removal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Starting sample</td>
<td></td>
<td>-3.2</td>
<td>-</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td>0.158</td>
<td>95</td>
</tr>
<tr>
<td>Alexa 532 fluorescent dye</td>
<td></td>
<td>0.006</td>
<td>99.8</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>0.003</td>
<td>&gt;99.9</td>
</tr>
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<td>0.003</td>
<td>&gt;99.9</td>
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<tr>
<td>4</td>
<td></td>
<td>0.003</td>
<td>&gt;99.9</td>
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2.6 References


Chapter 3: Automated Hydrodynamic Trap for Single Molecule Polymer Dynamics

3.1 Introduction

Trapping and manipulation of single macromolecules has been an increasingly active area of research over the past 15 years. Common techniques for studying dynamics of single polymers typically rely on the application of external forces including optical [1-5], magnetic [6-9], electric [10-18], and hydrodynamic forces [19-36] to confine and manipulate individual molecules. These tools for studying single polymer molecules have been an invaluable asset towards improved understanding of molecular dynamics; however, in the case of optical, magnetic, and electric trapping, manipulation of individual molecules relies on modification of the molecular structure via attachment of beads—as in the case for optical and magnetic trapping—or requires that the solution be electrically conductive—as in the case for electric trapping—which limits studies to applications where these requirements can be met.

Particle trapping and manipulation using hydrodynamic forces can be assigned to one of two categories: contact [19, 20, 22-27, 31-33] and non-contact [21, 28-30, 34-36] based methods. Contact based methods typically rely on fluid flow to confine molecules against obstacles or channel walls, whereas non-contact based methods utilize stagnation point flows to manipulate individual molecules in free solution. Contact based methods are useful as a high throughput tool and for the study of polymer dynamics in confined geometries; however, they do not enable fine scale manipulation over a broad range of conditions. Non-contact based hydrodynamic particle trapping was first demonstrated by G.I. Taylor in 1934 using the four-roll mill [37]. A computer automated version of the four-roll mill was developed by Bentley and Leal and was capable of
confining small droplets (~1 mm) within 0.5-1 mm of the trap center [38]. Recently, microfluidic analogs of the four-roll mill have been created primarily for the purpose of generating a wide range of fluid flow conditions including simple shear, extensional, and linear mixed flows [39, 40]. Non-contact based hydrodynamic trapping in stagnation point flows with manual feedback control has been used in the past for single polymers to study dynamics in extensional flow [21, 29, 34-36]; however, this approach is inadequate for studying single polymers over extended time periods due to the finite residence time of the molecule near the stagnation point.

Recently, our group presented a microfluidic-based method for manipulating and confining particles using solely hydrodynamic forces [41-43]. Particles were trapped at the stagnation point in planar extensional flow using a computer automated control system. The hydrodynamic trap relies solely on fluid flow with active feedback control to manipulate the particle center-of-mass position to the desired location. This method utilizes a simple two layer microfluidic device without the need for complex synthesis for coupling with external fields.

In this work, we extend the use of the hydrodynamic trap to study single fluorescently-labeled polymer chains. Trapping polymer chains presents new challenges that are not encountered in the manipulation of fluorescent beads: namely, macromolecules undergo a coil-to-stretch transition when exposed to strong external flows, which can complicate the particle detection process. In addition, the fluorescent dyes used in labeling the polymer molecules photobleach on a timescale much faster than that of fluorescent beads, further increasing the difficulty of performing long timescale manipulations of individual particles. The benefit of observing individual polymers for extended time periods is the ability to obtain a similar amount of information from a single molecule as from a large ensemble of a monodisperse sample of molecules. Here we characterize automated hydrodynamic trap performance using double-
stranded λ-DNA as our model polymer. Because λ-DNA dynamics have been thoroughly studied in the past, they provide a useful comparison for our results. We present results for trap performance for a variety of flow and illumination conditions. In addition, we compare results for longest relaxation time and steady state extension obtained from individual molecules to published results where large ensembles were used. In Chapter 5, results are presented for a variety of precision controlled experiments which are enabled by using the automated hydrodynamic trap.

3.2 Materials and Methods

3.2.1 Chemicals

All experiments presented in this work were performed with Lambda DNA (New England BioLabs) fluorescently labeled with YOYO-1 (Molecular Probes). Glucose oxidase, catalase, and β-Mercaptoethanol (Sigma) were used to reduce photobleaching of the dye and photodamage to the DNA. All other chemicals were purchased commercially from Sigma-Aldrich.

3.2.2 Epifluorescence Microscopy

λ-DNA was labeled using YOYO-1 fluorescent dye. A solution containing 0.2 ng/μL of λ-DNA was incubated with 0.2 nM YOYO-1. This staining solution was further diluted by at least a factor of 100 into a viewing solution for single molecule imaging. Fluorescently labeled strands of λ-DNA were imaged in a PDMS-based microfluidic device using an inverted microscope (Olympus IX71) equipped with a 100x oil immersion objective (Olympus UPlanSApo). A solid state laser (Spectra-Physics) with a 488 nm wavelength was used as the excitation source and images were captured by an Andor Ixon EMCCD camera using a custom LabVIEW program. Individual DNA molecules were imaged in a viewing solution consisting of 50 mM Tris (pH 8),
2 mM EDTA, 5 mM NaCl, and 5 mg/mL glucose. Varying amounts of glycerol or sucrose were added to the viewing solution to act as viscosifying agents, and solution viscosities were measured using a Brookfield viscometer. The viewing solution was introduced into the PDMS device via sample tube connected to a pressure transducer (ProportionAir). Pressure driven flow was used to enable precise control over flow rate while allowing rapid start-up and shutdown of the flow. A custom cooling jacket was fitted to the microscope objective enabling precise temperature control of the viewing solution via circulating water bath. A schematic depicting the experimental setup is shown in Figure 3.1a.

### 3.2.3 Hydrodynamic Trap Fabrication

Using standard soft-lithography techniques, we created a two layer PDMS device. An optical micrograph of a sample device is shown in Figure 3.1b. Two master molds, the fluidic and control layer, were created using SU-8 photoresist (Microchem) patterned onto silicon wafers. The photomasks for the master molds were prepared in Adobe Illustrator and printed at the University of Illinois printing services on high resolution, 5080 DPI transparencies. After preparing our silicon masters with the desired channel dimensions, we added trichlorosilane (Sigma-Aldrich) using vapor deposition to prevent PDMS from peeling off our design features. PDMS was mixed in 15:1 and 5:1 base to crosslinker ratios for the fluidic and control layer, respectively. For the fluidic layer, the uncured PDMS was spun onto the silicon wafer at such a speed that the PDMS thickness above the features would be ~20-40 μm. The PDMS for the control layer was poured directly onto the control layer silicon master in a petri dish to form a slab of ~2mm. The two layers were partially cured at 65 °C for 25-30 minutes. After partial curing, the control layer slab was removed and the control valve inlet hole was punched using a 21 gauge luer stub. Then the control layer was aligned with the fluidic layer and the two layers
were cured for an additional 2-4 hours. After the final curing step, the remaining fluid inlet and outlet holes were punched and the PDMS devices were bonded to glass coverslips after oxygen plasma cleaning.

We combined and modified our previously developed techniques for automated trapping of single particles in two dimensions and single DNA molecules stretching in planar extensional flow. Pressure driven flow was incorporated by extending our inlet channel and adding a constriction region. This allowed for working fluid pressure values between 1-3 psi for a 20-100 cP solution viscosities. A valve was positioned above an outlet channel and equal distance from a constriction region relative to the cross-slot. Applying pressure to this valve constricts the outlet channel underneath which effectively manipulates the stagnation point position. We achieved DNA targeting and manipulation using a proportional controller integrated through LabVIEW software as discussed in the following section.

3.2.4 Tracking and Manipulation

Using a custom written LabVIEW program, we performed in-line processing of our image feed. Images were converted to 8 bit binary with preference for bright objects. Particle analysis method from LabVIEW Vision Assistant module was used to determine the x and y center of mass of the stretched DNA molecule. The y-axis center of mass of the DNA molecule was then used to determine the error which is defined as the difference between the DNA position and setpoint or center of the ROI. Both the x and y center of mass coordinates were used to target DNA molecules closest to the center of the ROI. A proportional controller with four error conditions to prevent overshoot was used to regulate the valve pressure to trap the DNA molecule within the stagnation point. The conditions were based on the sign of the error and comparison to the previous error iteration. The ROI coordinates were established so that DNA
molecules above the setpoint resulted in positive error values. In order to prevent ringing of the proportional controller, a gain schedule was incorporated. DNA molecules within a certain threshold from the trap center would implement a lower gain value. As DNA molecules approached the bounds of the ROI a higher gain would be used via gain cofactor. Our method provides the ability to cycle between extension and relaxation of single DNA molecules. A schematic of the general operation of the hydrodynamic trap is shown in Figure 3.2.

3.2.5 Microfluidic Device Characterization

The fluid flow profile was characterized for the microfluidic devices using particle tracking techniques. Fluorescently labeled beads, either 0.53 μm (Bangs Laboratories) or 0.84 μm (SpheroTech) in size, were added to viscous solutions and pumped through the PDMS devices. Solutions were viscosity matched to those used in DNA trapping experiments for accurate determination of fluid strain rates. Images were captured using a CCD camera (AVT Stingray) at frame rates of at least 60 Hz. Individual particle trajectories were tracked and mapped using the ParticleTracker plugin for ImageJ [44]. From the particle position data, instantaneous bead velocities were determined, and we were able to fit the data using the following relationship for planar extensional flow:

\[
\begin{bmatrix}
  v_x \\
  v_y 
\end{bmatrix} =
\begin{bmatrix}
  -\dot{e} & 0 \\
  0 & \dot{e}
\end{bmatrix}
\begin{bmatrix}
  x - x_0 \\
  y - y_0
\end{bmatrix}
\]

(3.1)

where \(v_x, v_y, x,\) and \(y\) (velocities in the x and y direction and x and y positions, respectively) are known values, and \(\dot{e}, x_0,\) and \(y_0\) are fitting parameters. \(\dot{e}\) is the fluid strain rate, and the stagnation point is the position \((x_0, y_0)\). The strain rates in the microfluidic devices were characterized both as a function of fluid pressure and as a function of z-position inside the device. In addition, since each silicon master could produce either two or four devices,
depending on device design, devices from the same master were compared to determine if there was variation from a single master. For all data sets, arrays containing at least 10,000 individual data points were analyzed to minimize the error in the fit.

3.2.6 Single Molecule Data Analysis

Individual video files containing DNA experiments were analyzed using the IDL software and a custom written tracking program was used to locate the ends of DNA molecules on a frame by frame basis for determination of chain length and center of mass position. Chain extension data was typically smoothed using a weighted boxcar average using a three point window.

3.3 Results and Discussion

3.3.1 Microfluidic Device Characterization

A sample image of all detected particle trajectories obtained from our ImageJ analysis is shown in Figure 3.3. From the image, it appears that the streamlines behave as expected for planar extensional flow. A sample strain rate calibration for a single solution viscosity is shown in Figure 3.4. From the results, we can see there is a linear relationship between the applied fluid pressure and observed fluid strain rate. This result is unsurprising and matches our expectations. Since we are working with very dilute solutions in Newtonian fluids, the fluid volumetric flow rate, Q, should be directly related to the applied pressure, ΔP, via the Hagen-Poiseuille equation:

\[
Q = \frac{\Delta P \pi r^4}{8\mu L}
\]

(3.2)

where r is the radius of the channel, μ is the solution viscosity, and L is the channel length.

A sample strain rate calibration as a function of the z-position inside the microfluidic device is shown in Figure 3.5. From these data, we see that the flow profile in the channel is
nearly parabolic and that there is a section of the device about 10-15 μm in depth where the fluid strain rate remains relatively constant. This is an important feature as it allows the experimenter to move about the center of the device during experiments without changing the strain rate to a significant degree (<5%). In extensional flow experiments presented in this work, the relative z-position in the device was carefully monitored so the precise strain rate could be determined. Figure 3.6 shows the results for the strain rate characterizations from different devices taken from the same silicon master. We found that the devices all behaved similarly, such that the dimensions of every device were relatively uniform from master to master.

3.3.2 Hydrodynamic Trap Characterization for Single Polymer Dynamics

To this point, all work reported using the automated hydrodynamic trap has utilized fluorescent beads as the particles of interest to be manipulated. Fluorescent beads are extremely bright, photostable particles that are easily detectable, whereas fluorescently labeled molecules of DNA will gradually photobleach to the point where detection becomes increasingly difficult. In addition, fluorescent beads are symmetric and uniformly labeled, while DNA molecules range from relatively symmetric in the coiled state to highly asymmetric in the extended state with non-uniform brightness that arises due to different chain conformations along the backbone.

In a first set of experiments, trap performance was determined by monitoring DNA chain center-of-mass position as a function of strain rate and signal to noise ratio for extended molecules. In addition, the center-of-mass position was tracked for a single molecule as it underwent transformation from a coiled state to a stretched state (Figure 3.7a). It was important to ensure that trap performance will not be hindered as molecules changed dimensions since this often occurs in single molecule polymer experiments. We observed no evidence for a reduction in trapping capability under stretching conditions as the center-of-mass position of the molecule
varied by less than two microns. Trap performance as a function of strain rate was characterized at a single laser power for strain rates over a range of \( \sim 0.6-3.8 \text{ s}^{-1} \) (Figure 3.7b). As the strain rate was increased, the molecule’s center of mass displacement increased up to a point where molecules could no longer be trapped. We also characterized trap performance as a function of signal-to-background ratio (SBR) while maintaining constant strain rate (Figure 3.7c). As the signal of the molecules approached the background, the center of mass displacement diverged, and we were no longer able to trap molecules. Once the SBR reached values of at least 1.3, stable trapping was possible and we saw incremental improvements in the center-of-mass displacement as SBR continued to increase. It is important to note that there is a tradeoff between trap performance and experimental observation time. It is possible to trap molecules at high strain rates if SBR is high enough, but molecules will photobleach much faster at the required laser power and the experiment may be limited to \( \sim 10 \) seconds. Alternatively, experiments at low strain rates require lower SBR and can be carried out for minutes at a time.

In a second set of experiments, we extended our study to simple dynamics of single molecules. The first measurement we performed was polymer relaxation time from high stretch. Typically, for single molecule relaxation measurements, large ensembles of different molecules are averaged to determine a single relaxation time. In our case, we trapped a single molecule and repeatedly turned on and off the extensional flow, thereby enabling many relaxation events. Figure 3.8 shows an example in which we measured the relaxation time from a single molecule 12 times. An exponential fit was performed on the last 30% of the decay in mean squared end-to-end distance of the ensemble average yielding a time constant of \( \tau=5.8 \text{ s} \), which agrees well with previously reported values for \( \lambda \)-DNA in a 65 cP solution. In addition, when the time constants found from each trajectory were plotted (Figure 3.9) versus run number, there was no trend in the
data, indicating that the elasticity of the molecule is not changing due to being trapped for extended periods of time.

We performed an experiment wherein single molecules were trapped in extensional flow at varying strain rates to achieve different levels of polymer stretch. In previously reported studies of polymer dynamics in extensional flow, experiments typically required manual positioning of a single molecule near the stagnation point followed by sudden startup of flow. Molecules were imaged until they left the viewing area, and steady state extension was determined by averaging the extension of large ensembles of molecules. Using our approach with the automated hydrodynamic trap, we have the ability to hold single chains at the stagnation point for large values of strain (>50) thereby enabling determination of steady state extension via time average of a single trajectory. This is illustrated in Figure 3.10. Each point represents the time average extension of a single trajectory. All the results for our extensional flow experiments are plotted as a function of the Weisenberg number, $\text{Wi}$, which is a ratio of the fluid relaxation time to the experimental time scale, and is determined by:

$$\text{Wi} = \dot{\varepsilon}\tau$$

(3.3)

where $\tau$ is the longest polymer relaxation time. As demonstrated, our results agree well with previous results in which the data consists of large molecular ensembles. In addition, with our precise control over flow conditions, we can manipulate the strain rate while keeping an individual molecule trapped with minimal center of mass displacement. We can either change the strain rate in a random fashion or program a predetermined set of changes. The data shown in Figure 3.11 are for random step changes in the strain rate. In this particular case, we were able to trap the DNA chain for over 3 minutes. An important result from this experiment is that as we move from one strain rate to another and back to the first again, the steady state extension does
not change based on the history of the changes. This result indicates that there is no degradation or physical change in the individual molecule as a result of being trapped for extended periods.

3.4 Concluding Remarks

This chapter presented our efforts to extend the use of the automated hydrodynamic trap to study single polymer dynamics. As a proof-of-principle, we used \(\lambda\)-DNA as our molecule of study, since its dynamics have been well studied in the past. In our first set of experiments, we showed the efficacy of our approach by trapping single molecules as they underwent a coil-to-stretch transition while maintaining center-of-mass position inside the PDMS device. We also characterized the trap performance both as a function of strain rate and signal-to-background ratio. Our results demonstrate that there is a tradeoff between very fine scale particle manipulations and experimental observation time.

In a second set of experiments, we studied simple dynamics of \(\lambda\)-DNA molecules. The results from the relaxation time experiment matched up well with previous published results and provide support for using the hydrodynamic trap to study polydisperse samples, such as the ssDNA sample we synthesize in rolling circle replication reactions. In addition, our extensional flow experiments also provided strong support for the notion that time averaged dynamic measurements reveal the same information that we can gather from large ensemble average measurements of the same experimental conditions. These results for our control system of \(\lambda\)-DNA provide strong support for using the automated hydrodynamic trap as a tool to study the dynamics of our ssDNA and other single polymers.
3.5 List of Figures

Figure 3.1 (a) Schematic showing the experimental setup. Two pressure transducers control the sample fluid pressure and the valve pressure. The PDMS device contains a single inlet and two outlets, which are combined into a single outlet and submerged in viscous waste solution. The height of the waste solution is adjusted relative to the device to eliminate fluid drift due to gravity. A 100x oil immersion objective is used to image the DNA inside the PDMS device. A custom-fitted cooling jacket is connected to a circulating water bath to control the temperature of the fluid around the stagnation point in the device. (b) Optical micrograph of a sample PDMS device. The inlet constriction length and pattern can be altered to adjust the working pressure for the sample fluid.
Figure 3.2 Schematic showing hydrodynamic trap operation for a single particle. As pressure is applied to the control valve, the relative resistance at one outlet increases, and the stagnation point is adjusted forcing the particle to move towards the opposite outlet. This figure was reprinted with permission from [42].
Figure 3.3 Image showing the detected particle trajectories using the image tracker plugin in ImageJ. Each trajectory provides x and y coordinates at differing time points. From this position and time data we can determine instantaneous particle velocities and determine the fluid strain rate.
Figure 3.4 Strain rate curve for a single solution viscosity as a function of the applied pressure. The fluid strain rate is linear as a function of the applied pressure. The intercept does not occur at an applied pressure of 0 psi due to the fact that the pressure transducer requires a threshold pressure to be applied before the pressure is actually applied to the fluid.
Figure 3.5 Strain rate characterization as a function of z-position in the device for several applied pressures. As shown, the flow profile is nearly parabolic away from the walls of the device. Near the center, there is a region where the strain rate remains relatively constant.
Figure 3.6 Strain rate calibrations for different devices taken from the same master. There is a relatively small deviation from device to device, indicating that the dimensions from device to device are relatively unchanged on a single silicon master. TL, TR, and BL stand for top left, top right, and bottom left, respectively. The two devices from the top left position of the master were taken from two different molds of the same master, indicating that the master can be used to fabricate devices several times without affecting the dimensions of the patterned features.
Figure 3.7 (a) Center-of-mass displacement for a trapped molecule going from a coiled to a stretched state. The CoM displacement remains consistent as the trapped particle goes from relatively symmetric to highly asymmetric. (b) CoM displacement for particles at different fluid strain rates. The RMS displacement for strain rates under 3 s⁻¹, is small (<1 μm) in comparison with the size of the extended DNA molecule (typically ~15 μm). At higher strain rates, the RMS displacement increases rapidly with increasing strain rate; however, the maximum strain rate at which stable trapping is possible is highly dependent on the signal-to-background ratio of the polymer molecule. (c) CoM displacement for particles at constant strain rate and varying SBR. Different levels of SBR were controlled by increasing or decreasing laser power. As the SBR approaches one, the ability to detect a molecule is lost, and the particles can no longer be trapped. Above a certain value of SBR, there are diminishing returns with regard to trap stability, as higher SBR will lead to shorter observation times due to increased photobleaching. The minimum SBR required for stable trapping is dependent on the strain rate of the fluid: lower strain rates require lower SBR.
Figure 3.8 Relaxation trajectories from a single λ-DNA molecule. The ensemble consists of 12 individual trajectories, and the average relaxation time is 5.8 seconds, which is in agreement with previous results for solutions of this viscosity (~65 cP).
Figure 3.9 Individual exponential fit time constants for each of the relaxation trajectories shown in Figure 3.8. There is a variation in relaxation times, but there is no discernible trend in the data.
Figure 3.10 Steady state extension curve for λ-DNA. There is good agreement between previous data (red triangles) and data obtained from trapping single molecules (black squares). The inset shows a zoomed in region of the coil-stretch transition, showing a somewhat steeper transition at Wi=0.5.
Figure 3.11 Steady state extension for a single molecule with variable strain rate input. We were able to trap the molecule for over three minutes and over 30 Hencky strain. At points where strain rates are equal at different times, steady state extension values are comparable.
3.6 References


Chapter 4: Relaxation Dynamics of Flexible Polymers*

4.1 Introduction

In Chapter 1, the differences between flexible and semi-flexible polymers were discussed, and a comparison was drawn between the current model system for single molecule polymer studies, λ-DNA, and typical synthetic flexible polymers. The increased flexibility seen in synthetic flexible polymers as opposed to dsDNA has a significant impact on the low force elasticity of the polymer chains as predicted by Pincus [1] and demonstrated experimentally for both ssDNA and polyethylene glycol [2-6]. The nonlinear low-force elasticity behavior seen in flexible polymers will have a significant impact on the dynamics of these chains under non-equilibrium conditions. Recently, both relaxation and stretching dynamics of flexible polymers were simulated [7-9], but at this point, experimental results are limited. In Chapter 2, we introduced a new platform through which we can study dynamics of flexible polymers at the single molecule level using fluorescently-labeled ssDNA [10, 11]. In Chapter 3, we presented a methodology for studying our ssDNA system that can help to overcome some of the issues inherent to our synthesis scheme. Here we present our first dynamic experiments of our ssDNA: determining longest polymer relaxation time. The relaxation time of a polymer system is important as it provides the time scale for the material and is needed to non-dimensionalize time scales when performing other dynamic experiments. In this chapter we present results for optical tweezeing experiments on our synthesized ssDNA to demonstrate that the predicted nonlinear low-force elasticity is present in our samples. Finally, we show molecular weight scaling results determined both using Brownian dynamics simulations and single molecule experiments.

4.2 Materials and Methods

*The simulation results that appear in this chapter were performed by Folarin Latinwo. The samples used for the optical tweezeing experiments were synthesized by Amanda Marciel, and the experiments were performed by Kyung Suk Lee from TJ Ha’s group.
4.2.1 Optical Tweezing Experiments

Optical tweezing experiments were performed on sequences MC1 and Pyr8 (MC8 in figures) to determine the force-extension relationship. The synthesis scheme was modified slightly to create samples amenable to the tweezing experiments. First, 5’ biotinylated primers (IDT) were used in place of 5’ phosphorylated primers to enable attachment to a coverslip surface via biotin-streptavidin linkage. In addition, the reactions were quenched after 12 minutes by spiking the reactions with 3’ digoxigenin labeled dideoxynucleotide (ddUTP) (Roche). A micron-sized bead is linked to the 3’ terminus of ssDNA constructs by digoxigenin-antidigoxigenin linkages, and ssDNA molecules are stretched by manipulating the beads using standard optical trapping techniques [12, 13]. Prior to surface attachment, ssDNA solutions are mixed with urea to denature any bound circular templates. These are washed out after surface attachment prior to experiments. Experiments were conducted in buffer containing 50 mM Tris, 2 mM EDTA, and NaCl ranging from 20-1000 mM.

4.2.2 Brownian Dynamics Simulations

Polymers are modeled using a coarse-grained description of macromolecules, where chains are given by a series of \( N_b \) beads connected by massless springs. Beads serve as contact points with the fluid or centers of hydrodynamic drag, connected by \( N=N_b-1 \) springs that prescribe the average elasticity of the molecules.

4.2.3 Single Molecule Dynamic Experiments

All experimental results shown here were carried out using the MC1 template shown in Table 2.1. The synthesis and purification scheme is as described in section 2.2.1. The ratio of aa-ddUTP:dTTP was changed to 4:1 in an effort to increase the dye labeling ratio. Microscopy on the
samples was performed as described in 2.2.4. Briefly, imaging buffers containing 50 mM Tris (pH 8), 2 mM EDTA, 20 mM NaCl, 5 mg/mL, 140 mM BME, and glucose oxidase/catalase were prepared in ~95% glycerol solutions. Less than 1 μL of our purified, labeled ssDNA sample was mixed with the imaging buffer and introduced into a PDMS device using pressure driven flow. The automated hydrodynamic trap was utilized to confine individual ssDNA molecules for the study of relaxation dynamics up until the point at which molecules were no longer detectable, or they had photocleaved.

4.2.4 Single Molecule Data Analysis

Individual video files containing ssDNA relaxation measurements were analyzed in IDL software as described in 3.2.6 to determine chain end-to-end distance as a function of time. Length estimates for each molecule were performed by measuring the end-to-end distance for the molecules under flow and averaging the extension over several points. In addition, some length estimations were performed by measuring the integrated signal intensity of the coiled molecules in ImageJ. The intensities of two regions of interest of the same size were measured. The first contained the polymer molecule and the second was taken from a region close to the molecule that contained only background noise. The integrated intensity data was averaged for at least 100 video frames to reduce any error in the measurement. In addition, the sizes of the regions of interest were always kept constant from molecule to molecule to standardize the measurements.

4.2.5 Fitting Decay Trajectories to Determine Relaxation Times

Up to this point, single molecule polymer studies of λ-DNA have typically used exponential fits to determine longest polymer relaxation times due to the linear low force elasticity of the molecules. In the case of a nonlinear low-force elasticity, we needed a new approach for fitting
relaxation data. First, we start with a simplified form of the Langevin equation:

\[ dX_t = \frac{F_{\text{elastic}}}{\zeta} \, dt + \sqrt{\frac{2k_BT}{\zeta}} \, dW_t \]  \hspace{1cm} (4.1)

where the two forces acting on the polymer chain are Brownian forces, \( dW \), and the elastic spring force, \( F_{\text{elastic}} \). The spring force for our non-linear system is given by:

\[ F_{\text{elastic}} = f_c \left( \frac{Q}{Q_c} \right)^{\frac{3}{2}} \]  \hspace{1cm} (4.2)

where \( f_c \) and \( Q_c \) are the crossover force and crossover extension, respectively. The crossover extension is defined as the extension at which a molecule is no longer in the power law regime, while the crossover force is the force at the crossover extension [3]. After taking the ensemble average, the Brownian term is zero which results in the following integral:

\[ \int_{X(t=0)}^{X(t)} \frac{dX}{X^2} = C \int_0^t dt \]  \hspace{1cm} (4.3)

where \( C \) is:

\[ C = \frac{f_c}{\zeta Q_c^{\frac{3}{2}}} \]  \hspace{1cm} (4.4)

In order to fit experimental and simulation data using this method, the integral on the left side of Equation 4.3 can be computed numerically and plotted as a function of time. The plot of the integral versus time should be linear with a slope of \( C \), where \( C \) has units of \( 1/(\text{Length}^{0.5} \times \text{time}) \); therefore, to determine a relevant timescale we use \( 1/(\text{Length}^{0.5} \times C) \), where the length scale used is the estimated contour length of the polymer chain, since crossover extension is proportional to molecular weight. We only focus on the last 25% of measured extension to ensure that we are indeed in the nonlinear low force regime. In this way we can develop a curve for molecular weight scaling. We compared this approach to simply fitting the data with an exponential decay to determine if there was a difference in the molecular weight scaling of the relaxation time.
4.3 Results and Discussion

4.3.1 Optical Tweezing Experiments

The results from the optical tweezing experiments are shown in Figures 4.1 and 4.2. The force extension curves were measured in sub-picoNewton increments up to a final force of 20 pN. The data is plotted as fractional extension where the fractional extension is given as $L/(L$ at 20 pN). From the graphs, we can see that for both MC1 and MC8 (Pyr 6 from Table 2.1) there is a nonlinear low-force elasticity for all solution conditions except for the 1M NaCl solution for MC1. These findings are in agreement with the findings from the Saleh group [2, 3], and they validate our approach for synthesizing long strands of ssDNA. In addition, we see no evidence for secondary structure formation or base stacking, which would appear as a plateau in the force-extension curve.

4.3.2 Brownian Dynamics Simulations

The Brownian dynamics simulations we performed were an invaluable tool for aiding our understanding of flexible polymer relaxation dynamics. The simulations allow us to probe a much greater range of molecular weights than is experimentally possible at this point. In addition, we can perform the measurements for a large number of molecules to obtain statistically significant ensemble averages. The molecular weight scaling results from our Brownian dynamics simulations are shown in Figures 4.3 and 4.4. In Figure 4.3, in lieu of using our proposed fitting approach to the decay data, we instead fit an exponential decay to determine the relaxation time. We found a molecular weight scaling of $\tau \sim N^{1.9}$, which is different from the scaling of $\tau \sim N^{1.8}$ we observed when using our new approach. The molecular weight scaling exponent of 1.8 is what one would predict from Zimm theory for polymer chains with linear low-
force elasticity in good solvent with hydrodynamic interactions [14]. A full understanding of these results is currently an area of ongoing research.

4.3.3 Single Molecule Experiments

Although the implementation of the automated hydrodynamic trap in our single molecule studies has enabled us to study individual molecules for much longer times than would otherwise be possible, we are still ultimately limited by the polydispersity of our sample. Therefore, it was important to demonstrate that our chosen analysis method would be applicable even when we could not obtain very large ensembles of decay trajectories as we could in our Brownian dynamics simulations. Figure 4.5 shows a sample Brownian dynamics simulation in which several individual trajectories are plotted as well as the ensemble average of the numerically integrated equation given by Eq. 4.3. As shown, we can see that at low times, each integral term from the individual trajectories follows a very similar line. The slope of that line is the constant, $C$, that we are trying to determine. Based on the fact that the slope could accurately be determined from the average of a small number of individual decay trajectories, we felt comfortable using this approach for our experimental measurements. Figure 4.6 shows an example of experimental data in which we did in fact see a linear portion of the integral term over a range of time. If we focus our fit to that region, we should be able to determine our slope while minimizing any error. We collected over 90 decay trajectories from 14 different molecules for an average of over 6 per molecule. All of the individual trajectories are shown plotted together in Figure 4.7. The time for each trajectory is non-dimensionalized by dividing time by the time constant determined from an exponential fit. The molecular weight scalings for the exponential and power law fits are shown in Figures 4.8 and 4.9, respectively. Using the exponential fit approach, we observed a molecular weight scaling exponent of 1.36±0.15. The
power law fit yielded a molecular weight scaling exponent of 1.53±0.20. Both of these exponents are less than what we observed from our Brownian dynamics simulations.

These results can be associated with several sources of error. First, there is an error associated with the length determination that propagates through to the relaxation time when we multiply the constant, $C$, by a length scale. It is possible that some molecules are at a higher fractional extension than others if the molecules are not experiencing the same dimensionless flow strength, although we attempt to minimize this by trapping molecules in extensional flow at high strain rates as long as possible prior to performing the relaxation measurements. Our attempts at determining length via integration of fluorescent intensity are illustrated in Figure 4.10. As demonstrated, there does not appear to be a correlation between the measured length and the measured intensity. In fact, from the intensity data, it appears dye-dye interactions are causing quenching of the fluorescence. A second major source of error in the data comes from the fact that the spread of relaxation decays from a single molecule can be significant, as was illustrated in Figure 3.9 for λ-DNA. If we can only sample a molecule for a small number of relaxation events, it is possible that the error associated with the measurement is much greater than any error associated with the length measurements. We are currently attempting to define a minimum number of trajectories to achieve a significant confidence level in our measurement using statistical approaches. Finally, error arises due to the fact that we can only measure relaxation times over a small range of molecular weights. In our experimental results, we are only able to measure the relaxation time for molecules that differ in molecular weight by a factor of ~3, whereas an order of magnitude is ideal for these measurements to obtain a good fit.
4.4 Concluding Remarks

In this chapter, we presented results for our flexible polymer system based on single stranded DNA. Using optical trapping methods, we were able to demonstrate that our synthesis scheme could successfully produce macromolecules showing nonlinear low-force elasticity. We also used Brownian dynamics simulations and single molecule techniques in an effort to elucidate molecular weight scaling for longest relaxation time dynamics for flexible polymers. Using Brownian dynamics simulations as our basis, we predict the molecular weight scaling exponent for the relaxation time should be 1.8. Our single molecule experiments, however, were unable to reproduce this value, and we instead observed a molecular weight scaling exponent of 1.53±0.20. We are continuing to make improvements to our experimental techniques and analysis methods that will enable us to make a more accurate statement regarding our experimental findings.
Figure 4.1 Force-extension data for two different ssDNA sequences in different salt conditions.
Figure 4.2 Force-extension curves for two different ssDNA sequences under different salt conditions. This is a zoomed in version of Figure 5.1 plotted on a log-log scale. From the slope of the extension at low force, we observe nonlinear behavior for all molecules except for MC1 under high salt conditions. Under high salt conditions, the solvent becomes a theta solvent, the low-force elasticity becomes linear.
Figure 4.3 Brownian dynamics simulation results for molecular weight scaling of longest relaxation time using an exponential fit approach.
Figure 4.4 Brownian dynamics simulation results for molecular weight scaling of longest relaxation time using an power law fit approach.
Figure 4.5 BD simulations demonstrating that for the integral term in Eq. 4.4, there is a linear region to the decay at short times which can be fit using only a small number of molecules.
Figure 4.6 Single molecule results showing that there exists the same linear region in the numerical integral of Eq. 4.4 that we saw in BD simulations. In the example above, we would only fit the region that is marked as linear.
Figure 4.7 A plot showing all of the individual decay trajectories for ssDNA where each trajectory is scaled by length and plotted vs. dimensionless time. The total number of trajectories is in excess of 90 and is taken from 14 different molecules.
Figure 4.8 Molecular weight scaling of longest relaxation time as determined from single molecule experiments using the exponential fit approach.
Figure 4.9 Molecular weight scaling of longest relaxation time as determined from single molecule experiments using the power law fit approach.
Figure 4.10 Intensity measurements to estimate length for ssDNA molecules. There does not appear to be any correlation between lengths measured by eye and those estimated from total integrated intensity.
4.6 References


Chapter 5: Controlled Dynamic Experiments of Linear and Circular DNA in the Automated Hydrodynamic Trap

5.1 Introduction

In Chapter 3, we introduced the automated hydrodynamic trap, and our efforts to extend its usage to the study of single molecule polymer dynamics. All of the dynamics experiments presented in that work were characterization experiments that could be compared directly to prior experimental studies in which no automated trap was used. One of the benefits of the experimental setup we employ is that we have precise control over our molecule of interest, and while a single molecule is trapped, we can adjust the extensional flow field however we desire. In this chapter, we introduce work that has been enabled by our innovative approach. In the first experiment, we study controlled strain pathways for single λ-DNA molecules. In a second study, we study single λ-DNA molecules exposed to large amplitude oscillatory extensional flow. Finally, we extend the use of the automated hydrodynamic trap to study dynamics of a different class of materials: ring polymers.

5.1.1 Single Polymer Dynamics in Extensional Flow

In Chapter 3, steady state extension data for λ-DNA in extensional flow was presented and compared to prior results. In addition to measuring steady state extension, transient extension was a major area of interest in early single molecule polymer studies [1, 2]. One of the important findings from this early work was the fact that two identical molecules exposed to the same flow conditions could behave differently based on the molecular conformation they adopted in the flow field, or so called “molecular individualism”. Therefore, even though all molecules should eventually reach the same final steady state extension at a given fluid flow strength, the time at
which it takes to get to that point will vary from molecule to molecule based on the adopted conformation. This idea is crudely illustrated in Figure 5.1. For molecules that have their ends on opposite sides of a line perpendicular to the direction of extension, a dumbbell configuration is formed that stretches fairly rapidly since it is oriented in the direction of flow. For molecules that have their ends on the same side relative to the direction of flow, a folded conformation is formed which takes longer to completely unfold before reaching a final steady state extension. The conformations that are adopted typically depend on the flow strength of the fluid: for weak extensional flows that are slightly above the critical strain rate, a molecule’s ends can reorient in the direction of flow and dumbbell configurations dominate. For higher strength extensional flows, a molecule’s ends cannot reorient quickly enough and folded configurations appear at a much higher frequency that approaches 70% of all molecules [2]. In our controlled strain pathway experiment, we are able to control the molecular conformation by implementing a steady ramp up in the fluid flow strength.

5.1.2 Polymer Dynamics in Oscillatory Flows

An increasingly important area of research in complex fluids has been the study of dynamic behavior in oscillatory flow conditions. The primary focus has been the study of polymer solution behavior in non-linear large amplitude oscillatory shear (LAOS). There has been a great deal of useful information garnered from these studies including the ability to develop material signatures and classify complex fluids based on their non-linear response to large deformations [3]. Recently, efforts have been expanded to the area of large amplitude oscillatory extension (LAOE). Using a filament stretching rheometer, the dynamics of both a polystyrene solution and a polydimethylsiloxane (PDMS) network were studied in order to elucidate materials
characteristics [4, 5]. In this work, we extend the study of large amplitude oscillatory extension to single λ-DNA molecules.

5.1.3 Ring Polymer Dynamics

Despite the wealth of information that has been gathered in single molecule studies of polymer dynamics, there is very limited research in the areas of non-linear polymer architectures. An interesting architecture that has practical relevance to polymer processing is the ring structure. There has been a limited amount of study on these molecules, but it is mostly limited to simulations and bulk experimental measurements [6-12]. In 2006, Laib et al. designed a method for obtaining several different sizes of ring and linear polymers using plasmid DNA [13]. In collaboration with Greg Mckenna from Texas Tech University, we have extended the use of the automated hydrodynamic trap to study a sample of ring polymers.

5.2 Materials and Methods

5.2.1 Single Molecule Experiments

The protocols used for our fluorescence microscopy studies are the same as described in section 3.2.2 of this thesis. Briefly, both linear and circular DNA solutions containing 0.2 ng/μL of sample were incubated with 0.2 nM YOYO-1. Staining solutions were further diluted by at least a factor of 100 into a viewing solution for single molecule imaging. Viewing solutions consisted of 50 mM Tris (pH 8), 2 mM EDTA, 5 mM NaCl, and 5 mg/mL glucose as well as 140 mM betamercaptoethanol, glucose oxidase, and catalase to act as oxygen scavenging agents. Sucrose in varying amounts was used to viscosify solutions. Fluorescently labeled molecules were imaged in a PDMS-based microfluidic device using an inverted microscope (Olympus IX71) equipped with a 100x oil immersion objective (Olympus UPlanSApo). A solid state laser
(Spectra-Physics) with a 488 nm wavelength was used as the excitation source and images were captured by an Andor Ixon EMCCD camera using a custom LabVIEW program. The setup was equipped with temperature control as described previously to maintain precise conditions during experiments.

The PDMS device fabrication, automation, and characterization were performed in the same way as described in sections 3.2.3-3.2.5. The acquired single molecule images were opened in IDL and the end-to-end distance was determined on a frame by frame basis with partially automated detection.

5.2.2 Ring Polymer Preparation and Characterization

The ring polymer samples used in these experiments were prepared by Yanfei Li from Texas Tech University using a procedure outlined previously [13]. The work presented here was for circular DNA consisting of 45 (Fos45) and 112.8 kb (K16). The sample preparation method can sometimes lead to linearization of some of the circular product, so we characterized the sample distribution for the 45 kb sample using both gel electrophoresis and single molecule techniques. Finally, we performed both relaxation and steady state extension experiments for both the 45kb and 112.8 kb samples.

5.3 Results and Discussion

5.3.1 Controlled Strain Pathways

In this experiment, we expanded on the idea of variable strain rates presented in Chapter 3, however, here we used predetermined set values instead of randomly changing the values as shown earlier. For this experiment, we wanted to determine the effect of stretching pathways on molecular extension. Two different pathways were designed: a simple step change in the strain
rate from 0 to 1 s⁻¹ and a series of 10 equally spaced increments in strain rate from 0.1 to 1 s⁻¹. Each increment in the second path was maintained for 0.5 units of accumulated strain for a total of 5 strain for the entire group. The strain rate for the single step change was held for at least 5 seconds such that at least 5 units of strain were accumulated. In this way, we were able to design two different paths that each accumulated 5 units of strain while having the same final value of strain rate. Two sets of 10 molecules were used with one group experiencing the single step change and the other group experiencing the stepped ramp. The data for the individual trajectories and the ensemble averages are shown in Figure 5.2. The results illustrate clear differences between the two pathways. For the single step input (Figure 5.2a), the molecules either adopted a dumbbell or a folded conformation with the folded conformation taking a much longer time to unfold and reach steady state as illustrated in Figure 5.1. For molecules exposed to the stepped ramp pathway (Figure 5.2b), there was enough time for chains to orient with the direction of flow at lower strain rates and thereby adopt the dumbbell conformation enabling higher extension at lower accumulated strain. This is illustrated in Figure 5.2c where we see that when each group of molecules has reached 5 units of strain and are at a strain rate of 1 s⁻¹, the average extension for the stepped ramp ensemble ~3 μm higher than the average extension of the single step group. These results underscore the importance of flow pathways for polymer dynamics. There are many other additional pathways that can be implemented to model process dynamics.

5.3.2 Large Amplitude Oscillatory Extension

As a second demonstration of precision controlled polymer dynamics using the automated hydrodynamic trap, we studied molecular extension under conditions of large amplitude oscillatory extension (LAOE). For this experiment, individual λ-DNA molecules were trapped as
the extensional flow field was turned off and on at varying frequencies. The fluid strain rate in
the on state was held constant such that in the absence of oscillations, steady state extension from
molecule to molecule would be the same. In our analysis, we first measured the end-to-end
distance of each molecule as a function of time (Figure 5.3a). Using Origin software, we found
the autocorrelation of the raw data (Figure 5.3b) and took the fast Fourier transform to obtain the
power spectral density (PSD) as demonstrated in Figure 5.3c. If a peak was present in the PSD,
the measured frequency at the peak was compared with the input frequency of the oscillations
(Figure 5.3d). We non-dimensionalized the data by multiplying the frequency values by the
longest relaxation time of the DNA molecules. Our results indicate that as the frequency of the
oscillations increases, there is a critical point at which the molecule no longer has time to relax
an appreciable amount during the “off” state, and the autocorrelation function rapidly decays to
zero. The cutoff point in our experiment occurs around Deborah number, De=5, where De is
defined as:

\[
De = f \times \tau_R
\]  

where \( f \) is the frequency and \( \tau_R \) is the longest relaxation time. In our studies, we were careful to
ensure the sampling frequency was significantly higher than double the input frequency, which is
the minimum required sampling frequency to reconstruct a signal wave. In our measurements,
we have control over the strain rate of the fluid, the relaxation time of the polymer via solution
viscosity, and the input frequency of the oscillations. With such a wide parameter set, it is
possible to develop a phase diagram of sorts to determine the material behavior under a wide
variety of conditions.
5.3.3 Ring Polymer Dynamics

Prior to moving forward with single molecule ring polymer dynamic measurements, we first needed to characterize the sample. Figure 5.4 shows an example gel image of the prepared 45kb product. From the gel, we can see more than one band, which corresponds to the fact that there is a mixture of both linear and circular DNA present in the sample. To verify the results from the gel, we used single molecule techniques to develop a size distribution histogram. A sample histogram for the 45kb sample is shown in Figure 5.5. From the histogram, we can see that approximately 2/3 of the molecules are circular, with the remainder in the linear form. Using our single molecule setup, we can easily select only the circular molecules for our purposes.

As a first dynamics experiment, we studied the relaxation dynamics of our circular polymer samples. For our purposes, we assumed that the longest relaxation time would follow an exponential decay over the last 30% of extension, similar to that of linear polymers [14]. To estimate the last 30%, we assumed that the contour length of the circular DNA was half the contour length of the linear DNA. Using the hydrodynamic trap we collected at least 50 total relaxation trajectories and found the ensemble average. The results for the 45kb sample are shown in Figure 5.6. The measured longest relaxation time for our circular DNA was 1.72 seconds in a 65 cP viscosity solution. Using scaling arguments determined previously from experimental data on linear polymers, we can estimate what the relaxation time for a linear 45kb DNA molecule should be in 65 cP solution with the following [14]:

$$\tau_R \sim L^{1.66}$$  \hspace{1cm} (5.2)

For λ-DNA, which contains 48.5kb, in 65 cP solution, \(\tau_R = 6.1\) seconds. Therefore we can estimate the relaxation time for linear 45kb DNA to be:
which yields a value of 5.39 seconds. The ratio of the estimated linear relaxation time to the measured circular relaxation time of approximately 3.2 is very close to the predicted value of 3 as determined by Brownian dynamics simulations [12]. Using our 112.8kb, sample we measured in the longest relaxation time in four different viscosity solutions: 23.6 cP, 74 cP, 107 cP, and 123.8 cP. The ensemble average decays are shown in Figure 5.7. The scaling of the relaxation time as a function of viscosity is plotted in Figure 5.8 and follows the expected linear increase.

In addition to measuring the longest relaxation time of our circular DNA sample, we were able to use the hydrodynamic trap to develop a steady state extension curve for our sample similar to what we did in Chapter 3 for our control experiment of λ-DNA. The steady state extension curve for our circular DNA is plotted with published results for linear, λ-DNA (Figure 5.9). From the curves, we see that there is a clear transition from the coiled state to the extended state for circular DNA at a critical Wi=0.5. This result again matches previous simulations of circular DNA in extensional flow [11]. An interesting feature to note, however, is that despite the fact that steady state fractional extensions for linear and circular DNA are very similar at high Wi, near the critical Wi, the circular DNA appears to have a shallower transition. This is an interesting result and is the subject of current investigation.

5.4 Concluding Remarks

In this chapter, we demonstrated how the automated trap can be utilized in innovative ways to study single molecule polymer dynamics. Not only can we utilize this precise control to revisit previous studies of linear polymer molecules, we can also extend our studies to new classes of materials including ring polymers. In our controlled strain pathways experiment, we effectively
demonstrated the ability to control molecular conformations based on our prescribed processing conditions. Our experimental observations of \( \lambda \)-DNA dynamics in large amplitude oscillatory extensional flow are a first-of-its-kind demonstration at the single molecule level. Finally, as far as we know, we are the first group to study the dynamics of ring polymers in non-equilibrium conditions at the single molecule level, and we were successfully able to reproduce results that were predicted in previous simulations of these materials.
Figure 5.1 Schematic demonstrating how the initial orientation of a polymer molecule plays a role in its adopted conformation in extensional flow. This figure was inspired by a similar figure from Smith et al. [2].
Figure 5.2 Response of trapped molecules to two different strain paths. (a) Molecules were subjected to a single step in the strain rate from 0 to 1 s⁻¹. (b) Molecules were subjected to a stepped strain rate ramp. The two strain rate paths are designed to both end at a strain rate of 1 s⁻¹. The stepped ramp is designed so that each step corresponds to 0.5 Hencky strain. (c) Comparison of the ensemble average extension as a function of accumulated strain for each path. The stepped ramp reaches higher extension at lower accumulated strain due to the fact that nearly all molecules adopt a dumbbell conformation, which reach steady state extension at smaller strains than folded conformations.
Figure 5.3 Response of single molecules to oscillatory extensional flow. (a) Single trajectory for oscillating extensional flow with input frequency of 0.2 Hz. (b) Normalized autocorrelation functions for several different input oscillation frequencies. As the frequency increases, the autocorrelation decays to zero much faster than for lower input frequencies. (c) Power spectral density for oscillatory extension with an input frequency of 0.2 Hz. (d) Plot of measured Deborah number vs. input Deborah number. A value of zero indicates that there was no measurable frequency from the PSD.
Figure 5.4 Agarose gel image showing the prepared 45kb circular DNA sample. Lane 1 contains a 1kb DNA ladder. Lanes 2-7 show the sample with increased mass loading. The higher band on the gel corresponds to circular DNA, whereas the lower band corresponds to linear DNA.
Figure 5.5 Histogram of the circular DNA sample obtained using single molecule techniques. Approximately $2/3$ of the molecules are in the circular state as compared with the linear state.
Figure 5.6 Longest relaxation time determination for circular DNA. The ensemble was made up of 61 different trajectories, and the relaxation time was determined to be 1.72 seconds in 65 cP buffer.
Figure 5.7 Longest relaxation time determination for K16 circular DNA in 4 different viscosity solutions (23.6 (a), 74 (b), 107 (c), and 123.8 cP (d)). Each ensemble consisted of at least 50 individual relaxation trajectories.
Figure 5.8 Longest relaxation scaling as a function of viscosity for K16 circular DNA. The viscosity scaling follows an expected linear increase with a slope of 0.121 s/cP.
Figure 5.9 Steady state extension curve for circular DNA (Fos45, K16) as compared with linear, λ-DNA. There is a coil-to-stretch transition at $Wi=0.5$, but the transition appears to be more shallow for circular DNA than for linear DNA.
5.6 References


Chapter 6: Conclusions

The overarching goal of the work presented in this thesis was to expand the current field of single molecule polymer dynamic studies to truly flexible polymer systems. We accomplished this utilizing fluorescently labeled single stranded DNA synthesized via rolling circle replication as discussed in chapter 2. We were able to synthesize long, bright strands (>20 μm) of designer sequences of ssDNA. The main drawback of our synthesis approach was that we typically obtain a polydisperse molecular weight distribution in the sample. In chapter 3, we present a methodology, based on the automated hydrodynamic trap, for studying single polymer molecules that can help to alleviate concerns with ssDNA polydispersity. Using λ-DNA we present a series of control experiments that demonstrate the capabilities of this approach. First, we were able to trap and study a single λ-DNA molecule to determine the longest relaxation time. Our observed time scale matched very closely with previous results which utilized large molecular ensembles of different λ-DNA molecules. In addition, we could construct a steady state extension curve where each data point was taken from a single molecule, as opposed to large ensembles.

After developing tools to extend single polymer dynamics to flexible chains, we studied relaxation dynamics of ssDNA from high stretch using both Brownian dynamics simulations and single molecule experiments. Our BD simulations provided a useful measure for comparison to the experimental data. We observed a molecular weight scaling exponent for the longest relaxation time to be 1.8 from simulations, and 1.53±0.20 from experiments. Although these measurements are different, we are continuing to increase our understanding of flexible polymer dynamics. In chapter 5, we discuss how we used the automated hydrodynamic trap to engineer several new dynamics experiments which would not have been possible using other techniques.
We were able to control processing conditions for individual polymer chains to precisely control the adopted molecular conformations. In another experiment, we exposed individual polymer chains to large amplitude oscillatory extension, and tried to uncover the relationship between the frequency of oscillations that we can apply versus what we can experimentally measure. Finally, in a collaboration with Greg Mckenna at Texas Tech University, we were able to classify and study the dynamics of single ring polymers. To our knowledge, this is the first experimental work done at the single molecule level for this class of materials. By studying relaxation dynamics and dynamics in extensional flow, we were able to compare results with previous simulations with good agreement.