Methods for Parallel Amplification of Single DNA Molecules

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Bioengineering

by

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2009
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LIST OF ABBREVIATIONS

DMF: Dimethylformamide
EDTA: Ethylenediaminetetraacetic acid
FITC: Fluorescein isothiocyanate
LPA: Linear polyacrylamide
LRCA: Linear rolling circle amplification
NHS: N-hydroxysuccinimide
PCR: Polymerase chain reaction
RCA: Rolling circle amplification
SDS: Sodium Dodecyl Sulfate
TBTA: Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine
ACKNOWLEDGEMENTS

I would like to acknowledge Professor Xiaohua Huang for his support as the chair of my committee. His guidance throughout my research has proved to be invaluable. I have learned such a wide variety of things working with him and I appreciate everything he has taught me.

I would also like to acknowledge the graduate students working with me in the Huang Lab for all of their help and support. I would like to thank Kris Barbee for teaching me about chemical conjugations as well as microfluidic channel fabrication and slide preparation, Eric Roller for helping me with the TIRF imaging and for setting up the custom program used for imaging, Aric Joneja for helping me with biochemistry questions and all things related to gels, Nora Theilacker for showing me new methods for data analysis, Bob Mifflin for electronics safety lessons, and Ying-Ja Chen and Alex Hsiao for their help with general lab questions.
ABSTRACT OF THE THESIS

Methods for Parallel Amplification of Single DNA Molecules

by

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Master of Science in Bioengineering

University of California, San Diego, 2009

Professor Xiaohua Huang, Chair

The aim of this thesis is to develop two improved methods for amplifying genomic DNA and to order the individual amplified templates into arrays for high throughput and cost-efficient genome sequencing. The first method focuses on the synthesis of functionalized linear polymers for fabricating arrays for DNA amplification. These arrays can be used to improve PCR amplification of single molecules by extending the reaction into three-dimensional space similar to a solution reaction while each reaction is confined within the structure of the array. A method was developed for the synthesis and purification of linear polyacrylamide copolymers
that can be captured and visualized. Several conjugation methods were investigated for functionalizing the copolymers with primers.

The second amplification method utilized linear rolling circle amplification to produce long continuous DNA molecules with multiple copies of the template sequence. These single molecules could be captured onto an array for sequencing. The number of amplified copies in these molecules was characterized by several methods including gel electrophoresis, digestion, probe saturation, and electric field stretching. The amplified products displayed a broad distribution in length. However, they could be captured on a surface and imaged by fluorescence microscopy, and are accessible for downstream applications.

Microemulsions can be used to further control the sizes of DNA single molecule amplicons. A microfluidic device was designed and fabricated for creating uniform microemulsions. Microemulsions with a coefficient of variation around 0.15 could be produced under various conditions. Further improvement in the device will be required to make it more reproducible.
Chapter 1: Introduction

1.1 Genome sequencing technology

Genome sequencing technology is the methods by which the entire DNA sequence of an organism’s genome is determined (Shendure et al., 2008). A diploid human genome consists of twenty-two pairs of autosomes and one pair of sex chromosomes with approximately six billion base pairs. As a result determining the base sequence is quite challenging. However, the importance of whole genome sequencing is a well developed idea.

High throughput genomic sequencing could make it possible to sequence large numbers of individuals in a reasonable amount of time at very low cost. There are a number of applications for genomic sequence information from large populations. Comparative genomics and association studies can be used to better understand the genetic basis of cancer and other complex diseases. However, this quantity of information can only be made available through the development of faster and cheaper sequencing technologies. With these advances, the goal of personalized medicine can become a reality (Schloss, 2008; Shendure et al., 2008).

Despite current next generation technologies Genomic sequencing is a time consuming and costly procedure (Schloss, 2008; Shendure et al., 2008). The overall goal is to sequence a human genome in less than a day for under one thousand dollars. Many improvements in the genome sequencing techniques are necessary to realize this goal.

The process of genomic sequencing begins with a sample of genomic DNA which is fragmented and processed to construct a genomic library. Next, each piece of the DNA fragments is separated and amplified into monoclonal colonies. Sequence detection of each colony is done in parallel to obtain raw sequence data. Finally, the raw sequence data is assembled and analyzed to reveal the genomic sequence. The work described in this thesis
focuses on the development of new methods for the massive parallel amplification of genomic DNA.

1.2 Methods for DNA amplification

Next generation sequencing instruments utilize several different approaches for amplifying template DNA to a level sufficient for detection. Current methods for creating amplified clusters of single DNA sequences include water-in-oil microemulsion PCR (Dressman et al., 2003; Diehl et al., 2006), the in situ amplification of single DNA molecules on a surface by bridge PCR (Adessi et al., 2000; Fedurco et al., 2006), polony PCR (Mitra et al., 1999) and rolling circle amplification (RCA) (Lizardi et al., 1998). These methods result in separate clusters of DNA attached to micron beads, a glass slide, or trapped inside a gel. Amplified clusters of DNA are important for increasing the signal produced from a single base so that it can be detected with conventional fluorescence microscopy and used for sequencing.

Emulsion PCR

Emulsion PCR uses water in oil emulsions as mini reaction chambers for PCR amplification (Dressman et al., 2003; Diehl et al., 2006). In this method, primers are attached to magnetic micron sized beads which are mixed together with the template molecules and the components for PCR to form an aqueous phase solution. After stirring the aqueous phase together with the oil phase, emulsions are formed. A certain percentage of the emulsions contain one bead and one template, while others may contain just one or the other or neither. The emulsions are temperature cycled and each template is amplified by PCR, extending the primers attached to the beads in the process. This process produces beads with many copies of a unique DNA sequence. Then the emulsions are broken and the beads recovered with a magnet and attached to a slide for sequencing. Emulsion PCR is used in the 454 and ABI SOLiD
sequencing instruments to create beads covered with thousands of copies of single template sequences of DNA.

Bridge PCR

In bridge PCR, the method used in the Illumina/Solexa Genome Analyzer, individual DNA molecules are amplified by PCR with primers attached to the surface of a glass slide (Adessi et al., 2000; Fedurco et al., 2006; Johnson et al., 2007; Turcatti et al., 2008). First DNA primers are attached by their 5’ end to the surface. After hybridization of the template, the primer is extended. This results in a full complement of the template bound to the surface. In an alternate mechanism, the template DNA is attached to the surface by its 5’ end at the same time as the primers. After the target DNA is attached to the surface, by either method, it is able to bend over, and hybridize to another primer on the surface, forming a bridge. In the next cycle, this primer is extended, resulting in yet another copy of the template. Just as in solution PCR, thermocycling of the surface leads to an exponential increase in the number of that specific sequence. However, the product is localized in an area limited by the physical length of the DNA. Although this method forms colonies from amplification of single template molecules, these colonies are randomly distributed on the surface without any order at all. The sizes of the colonies formed are not uniform and are generally small (only 1000 copies). These issues make it more difficult to analyze images for sequence information and ultimately result in lower throughput and shorter read lengths.

Polony PCR

Another method developed for amplification is Polony PCR (Mitra et al., 1999). A polyacrylamide gel containing the components for PCR, including the template and primers, is poured on a glass slide. Throughout temperature cycling the long PCR products are trapped
inside the matrix of the gel localized near the original template. In this way, a single template DNA results in a colony of as many as \(10^8\) copies of that sequence. This PCR colony is called a ‘Polony’. Modifications made to attach the primers to the matrix of the gel help to localize DNA even further by attachment to the gel.

In general, the length of the DNA template that can be amplified by these methods is shorter than a few hundred bases (Shendure et al., 2005).

1.3 Research Design

The goal of the following set of experiments is to utilize and modify some of the best features of these techniques to produce an ordered array of amplified DNA. The first idea is to use a microfabricated array of polymers as a support for DNA amplification on the surface. Linear polymers decorated with primers are attached to the surface of a coverslip in an array. Then primers are extended by PCR with the efficiency of a solution reaction and the resulting colony is confined by the structure of the array. A method is developed for synthesis and purification of linear polyacrylamide copolymers that can be captured and visualized. Conjugation methods such as CLICK chemistry and reductive amination may be useful methods for functionalizing the copolymers with primers. These conjugation chemistries can be tested by conjugation of two linear molecules and their separation by length.

Another method of amplification that could be used to reach this goal is linear rolling circle amplification, used to create single molecules of amplified DNA. These single molecules coil into a sphere-shaped structure and can be captured onto an array. The number of amplified copies in these molecules can be characterized by several methods. These methods include (1) gel electrophoresis where product strands are separate based on their lengths, (2) restriction enzyme digestion where LRCA products are cut at the repeats to count copies, (3) probe
saturation where the point at which all of the repeats are saturated with a probe is determined, and (4) electric field stretching where each molecule is stretched for direct measurement by fluorescent imaging.

Microfluidic devices can be used for creating uniform microemulsions where LRCA can be completed in a controlled environment. Uniform microemulsions provide a technique for further control of the sizes of DNA single molecule amplicons. Microfluidic filtration membranes provide an array of T-junction interfaces between water and oil so that many emulsions can be created at one time. A device is designed and fabricated to hold a microfluidic filtration membrane at the interface of the dispersed aqueous phase and the continuous oil phase to create uniform microemulsions. Various flow rates can be used to produce microemulsions with a low coefficient of variation.
Chapter 2 : Microfabricated Polymer Arrays for DNA Amplification

2.1 Introduction

In this method, various chemistries are used to decorate long linear polymers with primers and capture them onto an array patterned glass cover slip for localized amplification of genomic DNA single molecules. First, long linear polymers are synthesized with various functional groups for subsequent conjugation with primers and the surface. Polyacrylamide polymers are synthesized by chemically linking monomers together through a chain reaction. Functional copolymers are created by incorporation of modified monomer units (Efimov et al., 1999; Rehman et al., 1999). These polymers can be functionalized after synthesis as well by chemical conjugation of with the functional units (Ostaci et al., 2008). Through various chemical conjugation reactions, the polymers are decorated with the appropriate primers and are captured onto the surface of a functionalized glass cover slip patterned with an array of wells. A single template DNA molecule is hybridized to a primer in a well of the array and temperature cycling allows the extension of the primer to replicate the target sequence. Then the template is washed away and amplification of the target sequence continues within the confined space of the array feature. Figure 2.1 illustrates the concept and the general reaction procedure. The result is localized amplification of the template sequence.
Figure 2.1 Single molecule DNA amplification on polymer arrays. A DNA molecule is hybridized to a primer tethered to the linear polymer in a feature of the array. The molecule is amplified by PCR within the physical confinement of the feature.

This method is similar to the polony PCR method except that amplification is limited by the area of the feature rather than the dense gel structure. The polymers are linear rather than crosslinked. As a result, the access to the amplified colonies for downstream sequencing reactions will be improved. It is also similar to the bridge PCR method except that the structure of the linear polymers extends the total area for amplification into the third dimension so that the primers are spread throughout the three-dimensional space rather than restricted on a two dimensional surface. The resulting amplification is more similar to a solution reaction than a surface reaction. This allows for a more efficient amplification of the single DNA molecules due to the fact that the primers are much more accessible and are more likely to come into contact with the template. The resulting colony of amplicons is better controlled than in the polony PCR method because it is spatially confined to a feature in the array and much more efficient than the bridge PCR method because it provides a three dimensional reaction space.
This set of experiments aims to develop a procedure for polymer synthesis and decoration. The polymers must be synthesized with multiple functionalities so that they can be coupled to different molecules. For DNA amplification, the polymers must have two primers attached. They must also have some functionality to allow capture onto the surface. There are many options for functionalizing these polymers.

2.1 Rationale and Design

Linear Polyacrylamide Synthesis

There are various methods for creating the long linear polymers necessary for this method of amplification. The method explored in these experiments is to synthesize and use linear polyacrylamide (LPA) copolymers synthesized by a chain chemical reaction.

Polyacrylamide gels are a common part of almost every biology lab because they are used to separate pieces of DNA based on their size. These gels are simple to make and as a result, with only a few easy steps, polyacrylamide gels are made on a daily basis. Long linear polyacrylamide copolymers can be synthesized just as easily by a similar method. In general, polyacrylamide gels are made by mixing a solution of acrylamide monomers and crosslinking units in a certain proportion and initiating the polymerization chain reaction to create a dense gel of crosslinked polymers. If this chain reaction is initiated without the crosslinking units, long linear polymers result from the linkage of multiple monomers. Functionalized monomers can be included in the reaction to produce long copolymers with various functionalities. These copolymers can be further modified with primers and captured for DNA amplification as illustrated in Figure 2.2.
Figure 2.2 Linear Polyacrylamide Synthesis and Functionalization for Array. Functionalized monomers are used for synthesis of linear polyacrylamide molecules that can be conjugated to primers and captured onto a feature in an array for DNA amplification.

In order to increase the efficiency of the polymerization chain reaction oxygen is removed from the system so that polymerization can continue without stopping. This should help produce polymers with more consistent lengths. In a gel, the length of each polymer is insignificant because each is linked to the other. When synthesizing linear polymers for amplification purposes, a consistent range of lengths is necessary for controlled capture on the array. If the chain reaction continues without stopping the length of the copolymers can be controlled by the overall concentration of monomers available for synthesis.
Functionalization

Chemistries

There are several conjugation methods and surface chemistries that can be used to decorate the polymers and attach them to the surface. One of these is the CLICK chemistry, shown in Figure 2.3, which forms a covalent linkage between an azide and an alkyne (Kolb et al., 2001). This one-step process is very simple and uses copper as a catalyst.

\[
\text{Azide}^+ + \text{R}_2\text{Alkene} \xrightarrow{\text{CuI, TBTA}} \text{Triazole}
\]

(2.1)

Figure 2.3 CLICK Conjugation Chemistry.

Even more options exist for coupling amine-containing molecules with other functional groups including isothiocyanate, N-hydroxysuccinimide (NHS) esters, and aldehydes. The chemical reactions are shown in Figure 2.4 These are the most commonly used covalent conjugations because they are fairly simple and have high yields.
A noncovalent linkage that works well and is often used is streptavidin-biotin binding. Streptavidin is a protein with four binding sites for the vitamin biotin. This means that one streptavidin molecule can potentially link four biotin-containing molecules together. If one of these biotins is on the surface and another on the polymer then the two are linked. This binding is even stronger if two or more biotins are contributed by each molecule. This is a very commonly used noncovalent conjugation.
Components

Amine-functionalized acrylamide monomers are stable in several chemical reaction environments and are commercially available therefore they are good starting molecules for further functionalization. NHS functionalized acrylamide monomers are also available as starting molecules for further functionalization however these are not as stable as the amine monomers because they are hydrolyzed in water. Amine and NHS acrylamide units can be modified before polymerization to create the desired functional groups on the monomers to be polymerized or on the polymer after polymerization. Since NHS amine coupling reactions must be done in a non-aqueous environment, any monomers which are functionalized with this chemistry must be modified before polymerization because the acrylamide copolymer is not soluble in organic solvents and is therefore unavailable for reaction in this kind of environment. In contrast, reactions such as CLICK chemistry and reductive amination of amine compounds with aldehyde containing molecules as well as biotin avidin binding are compatible with an aqueous reaction environment. Therefore, these conjugation methods can be used to functionalize the polymer after it has been synthesized.

In this set of experiments, biotinylated monomers are produced by reacting NHS-Biotin with amine functionalized monomers in organic solvent resulting in an amide bond that subsequently links the biotin to the acrylamide unit (Reaction 2.3). When incorporated in the polymerization reaction these monomers provide a functionalized unit for capturing the polymer on a streptavidin coated surface.

Azide functionalized monomers are created in the reverse conjugation by reacting NHS labeled acrylamide monomers with a molecule made up of an amine linked to an azide
(Reaction 2.3). These monomers are used for CLICK chemistry conjugation of alkyne labeled units to azide units on the polymer.

In a similar reaction, fluorescent monomers are produced by reacting fluorescein isothiocyanate (FITC) with the amine functionalized monomers to form an isothiourea bond (Reaction 2.2). The resulting monomers are linked to a fluorescent molecule and therefore are useful for making the polymer visible.

**Synthesis**

Linear polyacrylamide (LPA) copolymers are synthesized in a simple procedure. Acrylamide monomers are mixed together based on a specific ratio so that the functionalized units are incorporated at a predicted frequency. Then the solution is isolated from the outside environment and oxygen is removed by bubbling Nitrogen or Argon through the solution. Ammonium Persulfate (APS) initiates the polymerization reaction which proceeds to link acrylamide monomers together in a chain reaction until there are no monomers remaining.

After the synthesis, the LPA can be purified by washing with acetone. Since the long polymer is insoluble in acetone it will precipitate out to form a pellet at the bottom of the tube. On the other hand, any remaining acrylamide monomers are soluble in acetone and so they are easily removed with the supernatant. After a number of watches the purified LPA can be dried and redissolved in water.

The LPA synthesis is tested by capturing the purified product on beads and imaging. The LPA is captured on streptavidin coated magnetic beads by the biotinylated monomers included in the polymer synthesis. Then the beads, with captured polymer, will be visible when imaged on the microscope, because of the fluorescein-labeled monomers incorporated in the polymer.
The oligonucleotide primers are added to the polymer after polymerization to increase the quantity and efficiency of the conjugation. In this way, a more precise number of primers can be used and there is less waste. The present set of experiments develops a method for linear polyacrylamide copolymer synthesis and tests two methods for functionalizing the polymer with primer. One method uses CLICK chemistry with azide-functionalized polymer linked to a hexynyl primer. The second method uses reductive amination to link the amine-functionalized polymer to formylindole primers.

**Click Conjugation**

Copper-catalyzed azide-alkyne cycloaddition is the most well known and widely used CLICK chemistry conjugation (Kolb et al., 2001). In this reaction, an azide and terminal alkyne are linked through a 1,3-dipolar cycloaddition using a copper as a catalyst to form a triazole. This reaction proceeds at room temperature in various solvents with a very high selectivity. TBTA is a stabilizing ligand for Cu(I) which protects it from oxidation and disproportionation and enhances its catalytic activity (Chan et al., 2004).

CLICK chemistry conjugation can be tested by linking a long azide-amine molecule to hexynyl-primers and detecting the product progression through a gel at varying reaction times. As the conjugation takes place, the resulting linked molecule is longer and therefore runs slower through a gel. As a result, the primer band will disappear as reaction time increases and a new band will appear higher on the gel.

For this conjugation method, azide functionalized monomers are incorporated in the LPA synthesis. Then click chemistry conjugation is used to attach hexynyl-primers to the azide functionalized LPA (Reaction 2.1). The result is a long linear polymer decorated with primers designed for amplification.
To test the conjugation of primers to LPA a time series of the reaction can be separated on a gel to determine the primers location. Primers which are not conjugated will run quickly through the gel due to their short length. However, the LPA will remain trapped in the well of the gel and any conjugated primers will remain with it. So with an increase in reaction time we expect to observe a decrease in free primers as they are linked to the polymer and an increase in conjugated primer in the well.

**Reductive Amination Linkage**

A carbonyl compound reacts with an amine compound to form a Schiff base. Subsequent reduction of the product to form alkylamine linkages is known as reductive amination. Reduction of the Schiff base is completed by adding a reducing agent such as sodium cyanoborohydride or sodium borohydride.

Reductive amination can be tested by linking an amine-modified primer to an aldehyde modified primer. Each of the primers must be purified so that they contain no free amine in the solution. Then they are reacted together and reduced to covalently link the two pieces of single stranded DNA. Sodium borohydride is used as the reducing agent in these experiments because it is less toxic than sodium cyanoborohydride and the DNA should be tough enough to withstand the reducing strength of the sodium borohydride. The conjugated product is easily separated on a polyacrylamide gel where the product length is the sum of the primer lengths. The efficiency of the reaction can be determined by how much of the primer is converted to product. If the primer band disappears then the reaction is complete.

When amine-functionalized monomers are incorporated in the LPA synthesis, reductive amination can be used to conjugate formylindole modified primers to the polymer (Reaction 2.4). This is another way that long linear polymers can be decorated with designed primers.
Array

The linear copolymers may be assembled on an array. Biotinylated glass cover slips are patterned with an array of wells using photolithography. The primer decorated LPA copolymers can be captured to the functionalized surface that is exposed at the bottoms of the array of wells. Then the excess LPA is washed away and the resist is removed, resulting in an array of polymer patches.

Amplification

Linear copolymers attached to a surface and functionalized with primers act as a three dimensional space for PCR amplification much like a solution. Template DNA molecules are captured on the array by hybridization and after one cycle of PCR extends the capturing primer, the template is washed away. Now a number of PCR cycles can amplify the sequence locally over and over extending the primers on the polymer.

2.2 Methods

Materials

The oligonucleotides were obtained from either Operon Biotechnologies, Inc. or Integrated DNA Technologies Inc.. The sequences of oligonucleotides used are as follows.

Amine labeled primer Am-P53: 5’-[AmC12]GAACAGCTTTTGGAGGTGCTTTGTGCCTGCTGGAGACCGGCGAC-3’; Formylindole labeled primer FI-C2C156P1: 5’-(Formylindole)-TTTTTTTTTTT
GCTGGCTGCGGTACCATCATATACGTCGT-3’; Hexynyl labeled primer Hxynl-C2C156P1: 5’-TTTTTTTTTTTTTTTGCTGGCTGCGGTACCATCATATACGTCGT-3’. All chemicals are ACS analytical grade.

Linear Polyacrylamide Synthesis

Fluorescent monomers were produced by conjugating N-(3-Aminopropyl) methacrylamide hydrochloride to Fluorescein isothiocyanate (FITC). The solution containing
100 mM Fluorescein isothiocyanate, 50 mM N-(3-Aminopropyl) methacrylamide hydrochloride, 100 mM Triethylamine (TEA) in Dimethylformamide (DMF) was mixed and the reaction was carried out overnight. Ethanolamine was added to 75 mM to inactivate any excess FITC molecules.

Biotinylated monomers were produced by conjugating N-(3-Aminopropyl) methacrylamide hydrochloride to N-hydroxysuccinimide (NHS) labeled biotin. The solution containing 50 mM N-(3-Aminopropyl) methacrylamide hydrochloride, 100 mM Biotin-XX SSE, 100 mM Triethylamine in DMF was mixed and the reaction was carried out overnight. 75 mM Ethanolamine was added to the reaction to inactivate any excess NHS-Biotin. Azide-functionalized monomers are produced by mixing 50 mM N-Acryloxyxuccinimie with 100 mM 11-Azido-3,6,9-trioxaundecan-amine in DMF.

Linear polyacrylamide copolymers were synthesized by mixing 4% acrylamide monomers with the appropriate ratio of functionalized acrylamide monomers along with 200 mM potassium phosphate, pH7 and 0.1% N,N,N',N'-Tetramethylethylenediamine (TEMED). For amine-functionalized LPA used 1,000 : 10 : 2 : 1 of acrylamide monomers : amine monomers : fluorescein monomers : biotin monomers. For azide functionalized LPA use 1,000 : 10 : 2 : 1 of acrylamide monomers : azide monomers : fluorescein monomers : biotin monomers.
Figure 2.5 Setup for Linear Polyacrylamide Synthesis. Argon was bubbled through a large volume of water. Then the argon was bubbled through the polyacrylamide monomer solution through a needle inserted though the septum cap of an air tight vial. The air was released through another needle.

The setup used for the synthesis is illustrated in Figure 2.5. The reaction mix of monomers was transferred to an air-tight vial with septum cap from Pierce. Argon was bubbled through the liquid for fifteen minutes to remove all oxygen from the system. Before the gas was introduced to the system it was bubbled through water in a large glass beaker with a side port, for fifteen minutes or more. This kept the gas humidity level high and free of oxygen. Then, at low flow, the gas line was connected to an 18G 1½ needle for input into the solution. The needle was inserted into the solution through the septum cap. An open ended 18G 1½ needle was inserted through the septum cap into the air above the solution for the gasses to escape so pressure doesn’t build up. After fifteen minutes of bubbling through the solution all the oxygen was removed from the system, the needles were removed, first the vent needle then
the input needle. Next 10% ammonium persulfate (APS) is added to the solution with a 34-gauge needle and a small volume glass syringe to a final concentration of 0.02% to initiate the polymerization reaction. The reaction was allowed to proceed in the dark at room temperature for about twelve hours, more or less. The solution was centrifuged down to the bottom of the tube and the vent needle was inserted to eliminate any pressure difference. Then the acrylamide copolymer solution was transferred to a new tube.

Linear polyacrylamide copolymers were washed and extracted with acetone. To purify the linear polyacrylamide, one volume of acetone was added and mixed well. The mixture was centrifuged at max speed for five minutes then as much solution as possible was removed without disturbing the polymer pellet. The pellet was washed three more times with acetone. The tube was placed in a vacuum desiccator to evaporate the remaining acetone and dry the polymer. The polymer was resuspended in water.

The synthesized LPA was captured on magnetic beads to test FITC and biotin functionalization. 10 µl of 0.1% Dynabeads MyOne Streptavidin C1 beads and 20 µl of linear polyacrylamide were mixed in 20 µl of 1X binding and washing buffer (5 mM Tris-HCl (pH 7.5), 0.5 mM ethylenediaminetetraacetic acid (EDTA), 1 M NaCl) and placed on a shaker for 1 hour. The beads were pulled to the side with the magnet and the supernatant was removed. Then the beads were washed three times with 50 µl of 1X binding and washing buffer. The beads were resuspended in 20 µl of 4X SSC-T (600 mM Sodium chloride, 60 mM Sodium citrate, 0.05% Triton X-100) and imaged on the Zeiss Axiovert microscope with the 40X objective and FITC cube. The intensity of these beads was imaged with the same conditions and compared to a control set of Dynabeads MyOne Streptavidin C1 with no LPA captured. Bead locations were noted by bright field imaging.
**CLICK Conjugation**

CLICK chemistry was tested by the conjugation of a long azide functionalized molecule to a hexynyl-primer. 100 µM of 11-Azido-3,6,9-trioxaundecan-amine, 10 µM Hxynl-C2C156P1 and 400 µM Copper Iodide (CuI), 400 µM tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA) were mixed in dimethyl sulfoxide (DMSO) and the CLICK reaction was carried out for 24 hours. The reaction was compared to controls, with no azide, no CuI and no TBTA, in which the reaction should not occur. Products were separated on a 20% polyacrylamide gel by applying 400V for 48min. DNA bands were stained with SYBR Gold dye and imaged on the Bio-Rad Gel Doc.

Assuming all of the azide functionalized monomers were incorporated in the polymer the final concentration of the azide was approximately 5.63 mM. 563 µM of the azide, in the LPA solution, was mixed with 400 µM CuI and TBTA in DMSO. Then 2µM of the hexynyl primers was added to each solution in a time series, a set amount of time before the products were separated on a 15% polyacrylamide gel. DNA bands were stained with SYBR Gold dye and imaged on the Bio-Rad Gel Doc.

**Reductive Amination Linkage**

Formylindole primer, Fl-C2C156P1, and amine primer, Am-P53, were mixed together at 1 µm concentrations with ratios of 1:1, 5:1, and 1:5. The reductive amination buffer, 100 mM sodium acetate (pH 4.7), was used to fill to the remaining reaction volume. One set of samples was reacted for 24 hours after mixing and another set was reacted for 2 hours after mixing. After the reaction time 5 M sodium borohydride in 1 M sodium hydroxide was added to a final concentration of 0.05 M in two aliquots separated by five minutes. The products were
separated on a 20% polyacrylamide gel by running at 400V for 30 minutes. DNA bands were stained with SYBR Gold dye and imaged on the Bio-Rad Gel Doc.

Array

Glass cover slips were functionalized with a biotin surface through a multi step process. First the slides were cleaned extensively. The process began with a 30min micro-90 soak with sonication then a 30min acetone soak with sonication followed by a 30min methanol soak with sonication, to clean the slides. Next they were treated with a 1:1:5 solution of NH₄OH:H₂O₂:H₂O for 60 min at 85°C. The slides were rinsed well with water then treated with a 3:1 solution of H₂SO₄:H₂O₂ to activate the OH groups on the slide. Following activation, the slides were rinsed very well with water then with methanol and baked at 110°C for 15 minutes to dry. Next the slides were functionalized with an amine via Gama Aminopropyl Triethoxy Silane. A 2% solution of Gama Aminopropyl Triethoxy Silane (GAPTS) was prepared in a 20:1 solution of Ethanol:H₂O. This solution was reacted for 5 min to allow for hydrolysis and condensation. The slides were placed in the solution and reacted for 5 min to functionalize with amine. Then the slides were rinsed quickly with acetone three times for two minutes each and cured at 110°C for 10 min. 1 mM NHS-PEG-Biotin (mw 5000) and 100 mM Triethylamine (TEA) were mixed in DMF. The solution was spotted onto one side of a cover slip and covered with another inverted cover slip forming a sandwich. The two were kept separate with a smaller #1 coverslip at one end. These pairs were incubated for 1 hour at room temperature. Then they were separated and rinse with acetone for 5 minutes and inactivated with a wash in 1% NH₄OH and 0.1% sodium dodecyl sulfate (SDS) for 15 min. Finally they were rinsed thoroughly with water then twice with acetone for 5 minutes each and dried with a vacuum and stored there until used.
Biotinylated glass cover slips were patterned with an array of wells using photolithography. A thin layer of resist was spun onto the coverslip and baked to harden. Then a mask was used to protect a patterned area and the rest of the resist was exposed to light. Then the resist was developed to reveal the array pattern.

2.3 Results

Linear Polyacrylamide Synthesis

Linear polyacrylamide copolymers were captured onto MyOne streptavidin coated beads and imaged with bright field and fluorescent illumination. As shown in Figure 2.6, the control beads with no LPA were slightly fluorescent on their own but beads with the captured LPA were much brighter. Bright field images of the same field show the locations of the beads.

Figure 2.6 LPA captured on MyOne beads. The bright field images (left) and fluorescent images (right) images of control beads with no copolymers captured (top) and beads with biotin and FITC functionalized LPA captured by the streptavidin(bottom).
**CLICK Conjugation**

Click conjugation of hexynyl primers to a long molecule with an azide at one end slows the progress of the primers through the gel. Figure 2.7 shows the gel analysis of the product from the reaction. The control lanes show only one band at the primer location whereas the conjugation reaction lanes show two bands. There is one band at the primer location and another band which is slowed slightly by the excess length of the linker.

**Figure 2.7 CLICK Chemistry Test.** This gel shows the conjugation of hexynyl primer with a long azide molecule. The lanes were loaded as follows: 1) 25bp Ladder, 2) CLICK reaction, 3) CLICK reaction, 4) Control with no azide, 5) Control no Cul, 6) Control no TBTA, 7) CLICK reaction, 8) CLICK reaction.
The time series gel testing CLICK conjugation of primers to LPA showed no decrease in primers over time. Reaction times of 15 minutes, 60 minutes, and all time points in between showed equal amounts of primer in the band at the bottom of the gel and no increase in primer trapped in the well.

**Reductive Amination Linkage**

The gel presented in Figure 2.8 shows the separation of the two primers from the product of the reductive amination procedure. The formylindole primer shows up as a band at 60 bases and the amine primer is a band at 53 bases. Therefore, the conjugated product is at 113 bases. Although the pH of the buffer and the reaction time were varied, each experiment shows results similar to the ones displayed.
2.4 Discussion

Linear Polyacrylamide Synthesis

This set of experiments demonstrated that linear polyacrylamide copolymers can be synthesized with different functional groups including biotin, for capturing with streptavidin, and FITC, for visualization with the microscope. After the capture of fluorescently labeled LPA,
the streptavidin coated beads have intensity values about eight times greater than the control beads with no captured LPA. This shows that biotin functionalized monomers as well as fluorescent functionalized monomers were incorporated in the polyacrylamide chain reaction to form long copolymers.

Excess monomers were separated from copolymers by acetone precipitation. A visible pellet after synthesis confirmed that long polyacrylamide polymers were synthesized and they are not soluble in acetone. Since the fluorescent monomers can only be captured if they are linked to biotin monomers through the synthesis of copolymers, it is evident that the fluorescence on the beads must be from the copolymers rather than the monomers.

Although linear polyacrylamide copolymers can be synthesized easily, is very difficult to control the synthesis process. LPA is synthesized by a chain reaction which can stop and restart at any time. As a result, the product length is very hard to control. Inconsistencies in the polymer lengths make it unpredictable in the features of the array. If it is too long it may not be isolated to one feature of the array. Long copolymers may be able to span the space between the features destroying the confinement property of this technique. The images of LPA captured on beads show some long tails and blobs of polymer coming off of some of the beads. This may be due to longer products which are only partially captured to the bead.

Long linear polyacrylamide copolymers were captured into the array of streptavidin coated wells on a biotinylated coverslip. Like the capture on the beads there were many large blobs of fluorescent polymer that were not captured inside the wells. Instead, the copolymers were sticking all over the resist. When the resist was removed the copolymers were washed away as well. This indicates that the LPA was not captured on the biotinylated coverslip surface.
Further optimization of the method for capturing the polymers onto the arrays will be required for more efficient capture of LPA into the wells. Improvements could be made for blocking the resist to keep streptavidin from sticking everywhere and to prevent LPA capture all over the surface. The resist removal process could be optimized as well. It is possible to pattern the array with a variety of resist solutions which can be removed by several different solvents. The resist might also be removed at different stages in the capture process such as before the LPA is captured but after the streptavidin coats the array. These changes may help to create a more uniform array of linear copolymers.

Another modification to be made to this procedure is to synthesize the copolymers at an even lower monomer concentration to help reduce the number of excessively long polymers. The product length is limited by the availability of the building blocks. Removing oxygen should keep the reaction going. With less stops and starts, the process should be more consistent. With more coherent polymer lengths, the array could be produced with much greater accuracy.

It might also be beneficial to try to synthesize the copolymers on the surface of the array. This could be done by functionalizing the coverslip with acrylamide monomers which could be the starting point for the synthesis reaction. Photocleavable crosslinking units included in the synthesis create a gel like substance. Then the polyacrylamide is linearized by cleaving the crosslinkers and washing away and polymers not attached to the surface. Building the polymers in the array eliminates the need for complicated capture procedures and might also produce shorter more controlled polymers.

**CLICK Conjugation**

An extended reaction time is required for conjugating hexynyl primers to the LPA. Even after one hour of reaction time, the amount of primer conjugated to the LPA copolymer was not
enough to detect. The test of conjugation of the hexynyl primers to a long azide labeled molecule shows that after 24 hours of reaction, the migration of some of the hexynyl primers was retarded by the long azide-labeled molecule. However, even with an extended reaction time, the conjugation did not have a very high efficiency. This may be because the primers were not 100% labeled with the hexynyl group. If this were the case and only a small portion of the oligos was functionalized with the alkyne then a low yield would be expected for this experiment. It is also possible that the reaction conditions were not optimal. Further experiments might prove CLICK chemistry to be a good method for conjugation of primers to copolymers. The experiments described above show that click chemistry is a slow process. Consequently, CLICK chemistry is not a good choice for surface conjugation. It may work for conjugation of hexynyl primers to azide functionalized LPA copolymers. However, there are other conjugation chemistries which can make the link between primers a copolymers much faster and with a greater efficiency.

**Reductive Amination Linkage**

Tests of the reductive amination conjugation method showed that the linkage was not efficient and the product yield was very low. There was a small almost undetectable amount of product at one hundred thirteen bases, in the reaction with excess amine primer. The amine- and formylindole-labeled primers that were not conjugated to one another were way overloaded in the gel and yet the product was barely visible. A band higher up on the gel around one hundred twenty bases appeared to be the product but it is more likely a dimer of the 60 base formylindole primer. This band is still visible on a gel where increased concentrations of the formylindole primer were separated alone.
This reaction can be catalyzed by hydrogen ions in a low pH buffer. However if the pH is too low the amine is protonated and is not available to react with the aldehyde. The reaction may be more efficient at a much higher pH as well. This reaction was tested in both high and low pH buffers. In all cases the product was the same. Therefore, the low yield may be caused by some other factor.

Sodium borohydride was used as the reducing reagent in this set of experiments. It is possible that this reducing agent is too strong and is reducing the formylindole primer before it gets a chance to react with the amine primer. A better choice of reducing agent might be sodium cyanoborohydride because it is milder and is less likely to reduce and inactivate the formylindole primer. This conjugation method was tested with both a short and long reaction time before the reducing agent is added. Given sufficient reaction time most of the molecules should react and then be locked into place when reduced. However, the efficiency was not increased with increased reaction time either before reduction or with an extended reduction time. The efficiency of the reaction remained steady even with various changes in the procedure. One possible problem causing a low product yield may be that the formylindole primer is not efficiently modified. If there is only a fraction of the primers available for reaction then the yield would be very low.

In general reductive amination is used for conjugating proteins to various molecules and the procedure is done with very high concentrations. In comparison, these reaction conditions have very low concentrations of the two reaction molecules. This means that these components are much less likely to come into contact with each other and react. Consequently, this conjugation method may not be suited for coupling low concentration primers to LPA copolymers.
Although the CLICK chemistry and reductive amination methods did not work well for the conjugation of primers and polymers, these methods are used for many other applications with very good yield. This indicates that these processes may need only to be optimized for attaching primers to linear polyacrylamide copolymers.

**Further Experiments**

Another long linear polymer, which might be used for anchoring primers in a three dimensional space, is DNA. This polymer is synthesized by a DNA polymerase which covalently links DNA bases together. DNA polymers are understood very well, they are flexible, and can be controlled precisely. They also provide a very simple method for primer conjugation. For this technique primers are designed to have a section at the 5’end that is complimentary to the DNA polymer sequence. The remainder of the sequence to the 3’end is the primer necessary for amplification. These primers can be linked to the DNA polymer by photocrosslinking the two strands of DNA.
Chapter 3: Amplification of DNA Single Molecules by Linear Rolling Circle Amplification

3.1 Introduction

Linear Rolling Circle Amplification (LRCA) is an isothermal technique for DNA replication which utilizes the strand displacement properties of certain DNA polymerases (Lizardi et al., 1998). In this method, the target DNA is a single stranded circular piece of DNA, as illustrated in Figure 3.1. A short primer is hybridized to a known portion of the circle sequence and polymerization is initiated by incubation at the optimum temperature. As the polymerase synthesizes the complimentary sequence around the circle it eventually comes back around to the 5’ end of the primer. Then the polymerase displaces the primer as well as the synthesized complementary strand and continues to synthesize around the circle in a nonstop process. Each time the polymerase makes its way around the circle it produces another copy of the complementary DNA sequence. These copies are linked together as one continuous linear molecule made up of tandem repeats of the compliment of the template sequence. LRCA replicates circularized DNA into true single molecules which are concatemers of the sequence of interest. Single molecules of DNA amplified by LRCA can be utilized as isolated colonies of the template sequences.
Figure 3.1 Rolling Circle Amplification. A known primer sequence is hybridized to a circular template DNA molecule. Then the primer is extended from the 3’ end by a strand displacement polymerase such as Phi29. The complimentary sequence is synthesized and displaced multiple times to produce a long single DNA molecule of multiple linked copies of the target sequence.

There are several advantages to using rolling circle amplification as opposed to cyclic methods. Rolling circle amplification has a greatly reduced error rate since any errors in synthesis are isolated and are not propagated throughout the rest of the amplification. Another advantage is that this process is isothermal. An additional factor which speeds the process is the highly processive enzymes used in synthesis. Phi29, the enzyme chosen for these experiments, has a very high processivity. Once the enzyme binds, under ideal conditions, it can synthesize up to seventy kilobases before stopping.(Blanco et al., 1989) Other methods for amplification are also limited by the length of template DNA that can be amplified. Rolling circle amplification on the other hand, can amplify a long template just as easily as a short template. This is very important for further applications of the amplification sequence.
Rolling circle amplification is a versatile technique because it can be performed in solution or on a surface. Because of the linkage between template copies this amplification method provides a means for sequence isolation. The simplest and most efficient way to amplify DNA is in solution where all of the reaction components come in contact with each other frequently. Given sufficient space, each circle amplified will result in a strand of DNA which is isolated from all others. The template concentration can be controlled such that each amplified product does not tangle with any others.

If the RCA products are synthesized on the surface they are spatially isolated from one another based on the initial loading density of the template. The loading density of the template can be controlled so that each amplified product does not contact any of its neighbors. As it is synthesized the DNA coils and folds into a random sphere shaped structure. The approximate size of the LRCA product can be estimated by the Flory radius. Calculations of the product size can be useful for predicting the volume necessary for isolated amplification. Since the DNA is a polymer it behaves in a predictable way. It twists and folds into a random worm like configuration. The Flory radius is the radius of the random conformation of polymers (Flory et al., 1950) The equation relates the radius to the length of each unit times the number of units raised to some constant value empirically determined based on the type of polymer. For DNA this number is 0.6 and the length of each single stranded DNA base is about 5 Å. These values can be used in the equation to calculate the average radius of the DNA polymer. Figure 3.2 shows the Flory radius of the LRCA product of 78 and 156 base circles calculated from the number of copies of the original sequence.
Figure 3.2 Flory Radius vs. Copy Number. This graph shows the approximate radius, calculated by $R_F = a n^\gamma$, where $a$ is the length per unit, $n$ is the number of units, and $\gamma$ is a constant equal to 0.6. The radius of LRCA polymer product of 78 and 156 base circles is calculated based on the number of copies of the original sequence.

Once the radius is determined the volume occupied by each LRCA product can be calculated. Based on the volume per molecule the maximum concentration that allows for separation of amplified products can be determined. The maximum concentrations are represented in Figure 3.3 for two different circle lengths. From the calculations, the maximum concentration allowed for production of one thousand copies of a 78 base circular template in solution without entanglement is 5 nM and for a 156 base circle is 1.4 nM.
Figure 3.3 Maximum Concentration for Separated Amplification. This graph shows the maximum concentration of circle that would allow production of a given number of copies of 78 or 156 base circle by LRCA without intermolecular entanglement.

Since the LRCA coils into a spherical structure rather than remaining in a long string of DNA the shape of the product can be used to help in the array assembly process. If the LRCA products have a low size variance the colonies of DNA may be assembled in an array based on their size. If this is the case it can be assumed that there is one product per feature because the size of each product is sufficient to block the feature on the array from further products binding.
A biotinylated primer is hybridized to the circular template. Then Phi29 polymerase amplifies the target sequence by LRCA to form single molecule colonies. These colonies can be captured onto a streptavidin coated array where the sequence can be detected.

A simple method for capture of the LRCA product on the surface is to use a 5’ biotinylated primer in the amplification process. Then the product is labeled at one end with a biotin which can be captured on a streptavidin coated surface. The general concept and method is illustrated in Figure 3.4.

### 3.2 Experimental Design

**Linear Rolling Circle Amplification**

The procedure for amplifying circular DNA with rolling circle amplification has been previously developed. However the process is adapted and further developed through these
experiments for the synthesis of consistent products. Phi 29 polymerase has a high synthesis rate and an optimal synthesis temperature of 30°C. As a result the reaction mix is kept at 4°C until the starting time of the reaction. At this low temperature the rate of synthesis is very slow and should not contribute significantly to the product length. A time series is produced by varying the synthesis time is controlled by the time the reaction mix is incubated at the optimum temperature. A zero minute reaction should show any synthesis that occurs before the time series begins and can be used as a control. Phi 29 polymerase can be inactivated quickly by heating to a very high temperature to stop the reaction. Experiments are conducted with low reaction concentrations so that each circle can have the space necessary for isolated amplification. The concentration values chosen are determined by the Flory radius calculations and the target number of copies.

Characterization of the products at each time point can reveal important information such as the rate of synthesis as well as the number of copies, size, and intensity of the resulting LRCA product. A known rate of synthesis makes the process adjustable for obtaining products of a known length. The number of copies of the template and the variation in that number are critical for later sequencing steps and the physical size and variance are important when counting on size exclusion for assembly on an array. It is also important to know if the intensity of the resulting RCA products is sufficient for detection in later sequencing steps. This set of experiments develops a procedure for rolling circle amplification and uses several different methods for characterization of the resulting products.

**Gel Separation**

The RCA product can be quantified by electrophoretic separation on an alkaline agarose gel. Long linear single strands of DNA are negatively charged. Consequently when they are
introduced to an electric field they migrate toward the positive electrode. The progress of this migration is retarded by loading the DNA in a porous gel. As a result the DNA migrates through the gel at a rate based on its size. Comparison with a ladder, a set of DNA segments with known lengths, allows for the length of the product to be determined. Once the product length is known the average number of copies can be calculated. Since the LRCA product is single stranded it is run on a denaturing gel. The high pH of an alkaline gel helps to separate the single stranded DNA and to remove any nonspecific binding between the strands so that they migrate more consistently. One problem with alkaline gels is that they heat up over time. Since LRCA products are so long the gel must be run for an extended amount of time to separate the various lengths. As a combined effect of these two properties, these gels must be run on ice or in a cold room to keep the gel from melting and to keep the alkaline conditions consistent.

**Digestion**

The RCA product can also be quantified by digestion as shown in Figure 3.5. In this technique a complimentary primer sequence is hybridized along the length of the RCA product at each repeat of the sequence. Then a portion of each copy of the template will be double stranded. A restriction enzyme which has a recognition site contained in that double stranded area is added and it cuts the linear RCA product into equal pieces. Gel separation of the digested DNA will result in the formation of a bright band at the same length as the circle because each copy has the same length. The amount of DNA can be calculated by comparison of this band with a mass ruler and the average copy number can be determined.
Figure 3.5 Quantification by Digestion. First a complimentary primer is annealed to the repeats in the LRCA product to produce double stranded restriction sites for a digestion enzyme to cut the product into sections. The sections are separated and counted on a gel to determine the average number of copies per circle.

Probe Saturation

A method of probe saturation was also used for estimating the average copy number of the RCA product. In this method, illustrated in Figure 3.6, a fluorescent probe was mixed with the RCA product in a series of concentrations. The probe hybridizes along the length of the linear RCA product. Next the mixture is run through a microcon filtration system which retains the long RCA product as well as any hybridized probe in the top of the filter and allows any of
the short probe molecules to flow through with the solution. In samples where the amount of probe exceeds the number of RCA products, the excess probe will be in the filtrate. The probe has a fluorescent molecule attached which absorbs light at a specific wavelength. A spectrophotometer is used to measure the absorbance of the filtrate which can be used to calculate the excess amount of probe and thus the amount of probe that was hybridized to the linear RCA product. The expected absorbance pattern of the filtrate and the fluorescence pattern of the retentate are illustrated in Figure 3.7. With increased probes per template the point of saturation can be determined. This amount is equal to the ratio of copies per template.

**Figure 3.6 Probe Saturation Schematic.** A fluorescently labeled probe is hybridized to the LRCA product in varying concentrations. Then the excess probe is separated from the product and bound probe by microcon filtration. The absorption spectrum indicates the amount of probe in the filtrate solution.
**Figure 3.7 Expected Absorbance and Fluorescence of Filtrate and Retentate.** As the number of probes per template increases there will be no excess probe in the filtrate until the product is saturated then the excess probe will increase. In contrast the retentate solution will show increased fluorescence up to the point of saturation then the fluorescence will remain constant. These changes are detected due to the fluorescent molecule attached to the probe.

**Electrophoretic Stretching**

Another method was developed for direct quantification of the LRCA products by electrophoretic stretching and measurement of physical length. In this technique the LRCA products are tethered to the surface at one end via biotin-avidin binding and then the product is physically stretched out by an electric field as illustrated in Figure 3.8. Images of the stretched LRCA product can be used to measure each product independently. A 5’ biotinylated primer is used in the amplification process so that the long strands of tandem repeats have a 5’ biotinylated end. Consequently the linear strands of DNA can be immobilized by one end on a
**Figure 3.8 Electric Field Stretching.** The biotinylated LRCA product is hybridized to a Cy3 probe, incubated with streptavidin, and captured on a biotinylated coverslip. Then an electric field is generated across the surface to stretch the LRCA product. Fluorescent imaging allows the measurement of each product length directly.
streptavidin coated glass cover slip enclosed by a microfluidic channel. Fluorescent probes are hybridized to the LRCA product to make it visible under the microscope. Then the channel is filled with buffer and an electric field is generated by two platinum electrodes positioned across the channel. As a result the negatively charged DNA is pulled toward the positive electrode as in electrophoresis. However, because it is tethered at one end the DNA is not totally free to move and so it is stretched out by electrophoresis to its full length. This allows for direct measurement of the physical length of each individual piece of DNA. Consequently the copy number of each RCA product can be calculated from the physical length to which it is stretched and a distribution can be plotted to find the variation in the lengths.

A fluidics device was designed to include platinum electrodes inside the microfluidic channel perpendicular to the length and as close to the biotinylated cover slip as possible. The setup is shown in Figure 3.9. This device is a polycarbonate block machined to fit as an insert in the microscope stage. Fluid ports are drilled and tapped on the top surface along with reservoirs above the electrode channel intersection. The electrodes are located in two small groves on the underside of the block and are threaded through holes to the top of the block where they are wired to terminals. The electrodes are covered with RTV Silicone Rubber to fill the groove and the excess is cut off so the bottom surface is flat. Next a double sided tape gasket is used to form the channels which are sealed on the bottom by a biotinylated cover slip.
Figure 3.9 Device for Electric Field Stretching. A polycarbonate block, with fluid ports and reservoirs machined in the top and a groove for platinum wires on the bottom, acts as a top for the microfluidic channel. A double sided tape gasket makes the connection to the biotinylated coverslip that forms the bottom of the channel. The platinum wires are connected to terminals on the top of the device so that a voltage can be applied through the solution in the channel.

An electric field is applied by switching on a Bio-Rad power supply to create a voltage difference of 160 V/cm across the channel. This power supply has an automatic safety off switch if the current is below a certain value. Because the channel has such a high resistance, 1 MΩ, the current is very low, around one hundred microamps, and automatically shuts down the power supply. A box was designed with a dummy load to allow an acceptable current at all times. This resistor is connected in parallel to the channel of the stretching device so that the
voltage remains constant and known across both resistances. A relay was inserted into the
dummy load box to allow automatic on and off switching of the voltage to the stretching device.

Another safety feature built in to the power supply detects any rapid change in the
resistance of the circuit and shuts off the voltage. When the channel relay is turned on the
resistance change is dramatic enough to trigger this switch. As a result this feature must be
deactivated before running each experiment. This is accomplished by pressing 6 and STOP
simultaneously and changing the rapid change in resistance to off.

Image Analysis

Visualization of the LRCA products on the microscope can provide vital information
about the final product. Images of the LRCA products captured on a surface can be analyzed to
determine the product density. Images can also indicate whether or not the intensity is
sufficient for detection. These parameters are very important for later sequencing applications.

The size of the LRCA products can be analyzed visually as well. Individual LRCA products
with fluorescent probe hybridized are clearly distinguished as individual molecules captured on
the surface of a coverslip. Since these products contain many copies of the probe sequence
there are many dye molecules bound to each product. These dye molecules are clearly visible
on the microscope. The number of pixels which are lit up by each product can be counted and
the size calculated. The only shortfall is if the product is smaller than the diffraction limit.
Because the imaging process is diffraction limited any particle smaller than that limit still lights
up the same number of pixels. However anything larger than this limit and the size can be
calculated directly based on the objective and camera specifications.

Since DNA is a dynamic molecule it is constantly changing. Under different
environmental conditions the ball of LRCA may expand or contract. One factor which may be
very important in determining the size of the product is salt concentration. It is know that DNA relaxes in low salt environments and binds tighter in higher salt environments. As a result the size of the LRCA product may vary based on the amount of salt in the buffer. This may also affect the intensity of each product by changing the amount of probe which is hybridized. As the salt concentration decreases the DNA relaxes and some of the probe may break away from the LRCA product. Thus changing the intensity of each product by decreasing the number of dye-probe molecules bound.

3.2 Methods

Materials

The oligonucleotides were obtained from either Operon Biotechnologies, Inc. or Integrated DNA Technologies Inc.. The sequences of oligonucleotides used are as follows.

Sequence for Circle1 (Circle1): 5’-TCAGCTGTGATCATCAGAACTCACCTGTTAGACGCCACCAGCTCCA
ACTGTGAAGATCGCTTATCGCATGTCCTATC-3’; Primer1 for amplification of Circle1 (C1P1): 5’-CACAGCTGAGGATAGGACAT-3’; Biotinylated primer for amplification of Circle1 (C1P1-2XBio): 5’-[BIOTEG][BIOTEG]CACAGCTGAGGATAGGACAT-3’; Complimentary primer for digestion of Circle1 LRCA product (CompPrim1): 5’-ATGTCCTATCCTCAGCTGTG-3’; Sequence for Circle2 (Circle2): 5’-TAGCACGGACGCACGTATATGATGGTGTTACCCAGACCGCATCACCAGACTGAGTATCTCCTTACGTGCTGTTTTCCATCC-3’; Primer1 for amplification of Circle2 (C2P1): 5’-GTCCGTGCTAGAAGGAAACA-3’; Fluorescent probe for LRCA product from Circle1 (C1det1Cy3): 5’-Cy3-TCAGAAGTACCTTATTCGCATATCTTATCTAGCAGGGAGCTGCTGTCCTATGCATCTCCT-3’; Combined sequence of Circle1 and Circle2 (Circle156): 5’-TCAGAAGTACCTTATTCGCATATCTTATCTAGCAGGGAGCTGCTGTCCTATGCATCTCCT-3’.
All reagents are ACS analytical grade. All enzymes and DNA ladders were obtained from New England Biolabs.

**Linear Rolling Circle Amplification**

A reaction solution of 25 nM Circle, 50 nM Primer, 1X Phi29 reaction buffer, and water was mixed for the gel separation, digestion, and probe saturation experiments. A solution of 1 nM Circle, 2 nM Primer, 1X Phi29 reaction buffer, and water was mixed for the electrophoretic stretching experiments. These reaction mix solutions were incubated the in a heat block at 65° for two minutes. Then the whole block was removed from heat and allowed to cool down slowly to room temperature. Then the reaction mix was placed on ice and 1 mM dNTP’s, 100 µg/ml Bovine Serum Albumin (BSA), and 0.15 µM Phi29 polymerase were added and mixed well. Each sample was incubated at 30° for the allotted amount of time then the polymerase was inactivated by heating the sample to 80° for three minutes.

**Gel Separation**

A time series of LRCA products was created by varying the synthesis incubation time. These samples were run in separate lanes of a 0.8% Alkaline Agarose Gel next to a Lambda Hind III DNA Ladder, each in 1X Ficol loading buffer (30% Ficol 400, 100 mM TrisCl, 100 mM EDTA, Bromophenol Blue, and Xylene Cyanol). The gel was run at 60V for two hours on an ice bath. The bands of DNA were stained with SYBR Gold dye and imaged on the Bio-Rad Gel Doc.

**Digestion**

The time series LRCA products were annealed to CompPrim1 in a 1000:1 primer to circle ratio by heating to 65° for five minutes then ramping down to 60° for five minutes, 50° for five minutes, 40° for five minutes and cooling to 4°. Next the samples were incubated at 37° for 90 minutes with both PvuII-HF restriction enzyme and the corresponding buffer. Then the samples
were run on a 15% acrylamide gel next to the Gene Ruler Ultra Low (GRUL). The gel was stained with SYBR Gold dye and imaged on the Bio-Rad Gel Doc. Quantity One gel analysis software was used to measure the intensity of the DNA in each band, compare it to the standards, and determine the quantity of DNA. Then the average copy number was calculated by the increase in the quantity of DNA per circle.

**Probe Saturation**

The five minute extension LRCA product was annealed to a varying amount of the Cy3 labeled fluorescent probe C1det1Cy3 in 4X SSC-T (600 mM Sodium chloride, 60 mM Sodium citrate, 0.05% Triton X-100) by heating to 65° for five minutes then ramping down to 60° for five minutes, 50° for five minutes, 40° for five minutes and cooling to 4°. Each solution was spun through an YM100 Microcon filter at 5G for 10 minutes at 4°. The filtrate was diluted into a cuvet and the absorbance spectrum was measured on a spectrophotometer. The absorbance was recorded and the value at 550nm was plotted vs. the amount of probe per template.

**Electrophoretic Stretching**

A device, for electric field stretching of DNA, was machined out of a 12 mm thick polycarbonate sheet on a CNC Milling machine. Platinum wire was stretched through the groves on the bottom of the device and threaded through the vertical holes to the top. On one end the wire was wrapped around a screw in the polycarbonate block then it was stretched tightly through the groove to the other end and wrapped around a banana terminal on that side. The groove around the wire was filled with RTV Silicone rubber. A 10:1 wt:wt solution was mixed with the electric mixer and spun down in the centrifuge at least three times to fully mix. A gasket was cut on a Robo Craft cutter to form channels in a piece of Scapa 702 double sided tape. The biotinylated coverslips were derivitized through the process described in Chapter 2. A fluidics
chamber was assembled by aligning the channels in the tape gasket with the fluid ports and reservoirs on the polycarbonate device and sticking one side of the tape directly to the device. Then a biotinylated coverslip was sealed to the other side of the tape gasket to enclose the channels.

After the channel was assembled, the reservoirs were plugged with a rubber gasket and the channels were prewet with buffer (50 mM TrisCl, 125 mM KCl, 0.02% Triton X-100). Next the channels were filled with BSA solution (5% BSA in 2X binding and washing buffer and 1% Tween) and incubated at room temperature for five minutes to block the surface from sticking. The channels were washed with one volume of buffer. The time series LRCA products were annealed to a Cy3 labeled fluorescent probe with 2000 probes per circle by mixing 0.5 nM LRCA product plus 1 µM C1det1Cy3 in 4X SSC-T (600 mM Sodium chloride, 60 mM Sodium citrate, 0.05% Triton X-100) and by heating the mixture to 65° for five minutes then ramping down to 60° for five minutes, 50° for five minutes, 40° for five minutes and cooling to 4°. Then a 10:1 solution of streptavidin:LRCA was premixed and incubated for 1 hour to prepare the time series samples for the surface. After the buffer wash each channel was filled with a time series sample and incubated at room temperature for 30 minutes. Then the channel was washed extensively with twenty-five volumes of buffer to remove any excess LRCA which had not bound to the surface. Next the channels were filled with 0.5X TBE (45 mM Tris-borate and 1 mM EDTA) with 0.02% TritonX-100 and the reservoirs were opened and filled as well to prepare for stretching.

An electric field was applied by switching on the Bio-Rad power supply to create a voltage difference of 160V/cm across the fluidics channel. The dummy load was connected in parallel to the device and a relay was used to switch the voltage, in the fluidics channel, on and off automatically.
The surface was imaged on a Zeiss microscope with a 100X oil objective NA 1.46 and 532nm laser illumination for total internal reflection (TIRF) imaging. The LRCA products were stretched while videos and images were captured with an Andor camera. A custom software program was used to control the microscope, camera, TIRF angle, and lasers as well as the automatic switching of the voltage on and off.

**Image analysis**

LRCA product prehybridized with probe is captured on the biotinylated surface of a coverslip and imaged on the Zeiss microscope with a 100X oil objective NA 1.45 with 532nm TIRF illumination. Various qualities of the image can be analyzed such as the intensity of the LRCA products and their size under various conditions.

### 3.3 Results

**Gel Separation**

Three different circles were amplified by LRCA and the time series products were separated by length on alkaline agarose gels. The gels in Figure 3.10 show the separation of LRCA time series products from Circle1, Circle2 and Circle156. The bands are compared to the ladder to determine the length of DNA produced for each incubation time. Graphs show the intensity peak of the bands in each lane and the corresponding length for that position.

Circle 1 and Circle 2 are 78 base long circular sections of DNA while Circle 156 is a combination of Circle 1 plus Circle 2 with a total length of 156 bases. The number of copies of each circle in linear amplified product can be determined by dividing the total number of bases by the length of the circle. The rate of synthesis is determined by dividing the total number of bases by the synthesis time.
Figure 3.10 Gel Separation of LRCA Products. Gel and intensity plots of (A) Circle1, (B) Circle2, and (C) Circle156 LRCA products. Lanes were loaded as follows for (A) and (B): 1: 0 min, 2: 1.25 min, 3: 2.5 min, 4: 5.0 min, 5: 10 min, 6: 20 min and for (C) 1: 1.25 min, 2: 2.5 min, 3: 5.0 min.
Table 3.1 Gel Separation Results for Circle1.

<table>
<thead>
<tr>
<th>Time(min)</th>
<th>1.25</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bases</td>
<td>10450.3</td>
<td>12349.3</td>
<td>12930.9</td>
<td>13324.3</td>
<td>14376.3</td>
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<tr>
<td>Copies</td>
<td>134.0</td>
<td>158.3</td>
<td>165.8</td>
<td>170.8</td>
<td>184.3</td>
</tr>
<tr>
<td>Rate (b/s)</td>
<td>139.34</td>
<td>82.33</td>
<td>43.10</td>
<td>22.21</td>
<td>11.98</td>
</tr>
</tbody>
</table>

Digestion

After digestion of the LRCA time series products, the cut pieces of DNA were separated on the gel shown in Figure 3.11. The gene ruler and DNA ladder act as standards for quantification. It is clear that the amount of DNA in each lane increases as the LRCA reaction time increases.

Figure 3.11 Digestion Quantification Gel. This gel shows the amount of DNA digested from each LRCA time point sample. The samples were loaded as follows: 1) GRUL, 2) 25bp Ladder, 3) 2min, 4) 2.5min, 5) 5min, 6) 10min, 7) 20min, 8) 40min, 9) GRUL, 10) 25bp Ladder.
The quantity of DNA in each band is determined by analysis with Quantity One.

Comparison of the bands with the molecular weight ladder allowed the amount of cut DNA to be determined. The average number of copies of the circle, shown in Table 3.2, can be calculated from the resulting quantity of DNA and the original quantity of circle.

**Table 3.2 Digestion Results for Circle1.**

<table>
<thead>
<tr>
<th>Time(min)</th>
<th>2</th>
<th>2.5</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bases</td>
<td>1638</td>
<td>1606.8</td>
<td>1778.4</td>
<td>1692.6</td>
<td>1801.8</td>
<td>1786.2</td>
</tr>
<tr>
<td>Copies</td>
<td>21.0</td>
<td>20.6</td>
<td>22.8</td>
<td>21.7</td>
<td>23.1</td>
<td>22.9</td>
</tr>
<tr>
<td>Rate (b/s)</td>
<td>13.65</td>
<td>10.71</td>
<td>5.93</td>
<td>2.82</td>
<td>1.50</td>
<td>0.74</td>
</tr>
</tbody>
</table>

**Probe Saturation**

The absorbance spectra, of the filtrate from the probe saturation sample, are measured to detect the number of fluorescent probes in the solution. The spectra are plotted, in Figure 3.12, for each sample. Because Cy3 dye has a peak absorbance at 550nm this is the wavelength of interest for this experiment. The absorbance at 550nm increases with increase in probe concentration as expected. These values are plotted in Figure 3.13 to show the correlation between absorbance and the ratio of probes to template.
**Figure 3.12 Absorbance Spectra of Probe Saturation Filtrate.** This graph shows the spectra of the filtrate from samples with varying ratios of probes to template. As the ratio increases, the absorbance of the sample shows an increase in the peak at 550nm of Cy3 absorption.

**Figure 3.13 Filtrate Absorbance at 550nm.** The peak absorbance at 550nm is recorded and plotted vs. the number of probes per template.
Electrophoretic Stretching

The images of normal and stretched LRCA time series products are shown in Figure 3.14. They were analyzed with Image J and MATLAB to determine the intensity of each spot and the lengths of the stretched molecules. The time series of LRCA synthesis shows increased length of the stretched product with increased reaction time.

Measurements of the pixel length of the stretched LRCA products can be converted to the approximate length in bases and thus the number of copies can be determined. These results are presented in Table 3.3.

Table 3.3 Electrophoretic Stretching Results of Circle1.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>10</th>
<th>20</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bases</td>
<td>2092</td>
<td>6938</td>
<td>12714</td>
</tr>
<tr>
<td>Copies</td>
<td>27</td>
<td>89</td>
<td>163</td>
</tr>
<tr>
<td>Rate (b/s)</td>
<td>3.5</td>
<td>5.8</td>
<td>3.5</td>
</tr>
<tr>
<td>CV</td>
<td>0.91</td>
<td>0.82</td>
<td>0.88</td>
</tr>
</tbody>
</table>
Figure 3.14 LRCA Product Images. These fluorescent images show bright spots of LRCA products (left) and the elongated lines of products stretched with 160V/cm (right). A) 10min LRCA sample: 300ms exposure, 12 gain, 18 mW B) 20min LRCA sample: 100ms exposure, 12 gain, 18 mW C) 60min LRCA sample: 40 ms exposure, 32 gain, 24 mW.
3.4 Discussion

Linear Rolling Circle Amplification

The reaction conditions work well for amplification of the circular template molecules. Since the extension temperature of Phi29 polymerase is so close to room temperature, the reaction mixture and tubes were precooled on ice before adding the polymerase. Then they were mixed and moved quickly to the set extension temperature to start the extension time. A zero minute control was processed in parallel with the time series samples. This sample, identical to the others, was moved directly from the ice bath to the 80degree inactivation. This control shows if any amplification takes place before the reaction time began. Following this procedure there was no amplified product in the control sample indicating that the time series method is successful.

Concentration of circle and primer are kept relatively high for detection on the alkaline agarose gel. However the reaction works equally well if not better at lower concentrations because there are less intermolecular entanglement issues. The processing of each individual template molecule may be cut short if the polymerase becomes entangled within the ever growing product. Then the components necessary for further synthesis, such as space or dNTP’s, are not readily available for the process to continue.

The following quantification experiments show that by varying the reaction time LRCA products can be produced with different lengths. Phi29 polymerase synthesizes very fast so the product length was detectable at even as little as 1.25 minutes. However the lower reaction times had lower amounts of product and required concentration to be detected on the gel. A spin vac. dried the samples and they were redisolved at a higher concentration. So increased reaction time not only increased the product lengths but also the amount of amplified product.
Gel Separation

Gel separation is the standard method for quantifying the lengths of DNA samples. As a result this was the first method used to determine the average lengths of the LRCA products. The time series products of three different circles were separated by electrophoresis on alkaline agarose gels. Each resulted in products of similar length for the same reaction time.

The limitation of this method is that long DNA is not separated as well as short DNA and so it is very difficult to distinguish between the very long RCA products. Some of the products are so tangled together that they remain trapped inside the well and do not even enter the gel.

Digestion

Digestion of the LRCA time series products showed a noticeable increase in the amount of 78 base fragments as well as the 156 base dimmer with increased reaction time. However the quantification calculations based on the mass ruler reveal close to the same number of copies in each sample. This may be because the bands of the increased time samples are blackened in the middle, a phenomenon with an unknown cause.

Probe Saturation

The probe saturation experiment provided no conclusive results. The absorbance values decreased with probe concentration as expected. However they passed zero and gave negative values. These values show that the amount of fluorescence was too close to the detection threshold. This method of quantification might work with more sensitive absorbance detection. Or if the concentration of the total sample could be increased without changing the ratio of probes per template there would be much more probe for detection. However this would require the use of a lot of reagents and therefore would be very expensive. A cheaper way to increase the concentration of the sample would be to measure the absorbance of the more
concentrated solution. The spectrophotometer requires a minimum volume of 500µl so the samples are diluted before they can be measured. In comparison a NanoDrop spectrophotometer requires only 2µl sample volume.

**Electrophoretic Stretching**

Initial experiments with low voltage were not successful at stretching the LRCA products that were tethered to the surface and further research suggested that long strands of DNA could be stretched by higher voltages. After the voltage applied to the channel was increased, many of the LRCA products were stretched. However there still seemed to be an issue with product mobility. Several of the products appear to be firmly stuck to the surface while others are bouncing around in the solution and are merely tethered to the surface at one end. The bright spots of product that are stuck to the surface do not stretch out but the ones moving in solution do. The number of products free for stretching seems to be dependent on the channel preparation procedure.

Electrophoretic stretching of the LRCA products showed a wide variety of product lengths. For separated amplification of the individual circular template molecules, the concentration of the reaction must be kept low to keep the LRCA products from intermolecular entanglement. Even at 1nM concentration several of the RCA products are knotted into bright spots that never stretch out. Lowering the concentration further may help to decrease the entanglement and allow better separation of the products. However a low concentration will not decrease intermolecular entanglement which may still occur. Surface amplification of the circle may help to keep the long products from entanglement. Since one end of the product is immobilized on the surface the product will likely unwind from that location rather than balling up so tightly.
Many of the products are not completely stretched. There are several very bright spots where the LRCA product is tethered. These spots never stretch out but many of them do have a long string or loop that does stretch from that point. The product here is so tangled that it can only stretch out partially in the electric field.

**Further Experiments**

The method of linear rolling circle amplification works well to produce many copies of a target DNA sequence that are linked by the nature of the synthesis. However the product length and therefore the number of copies of each circle vary greatly. Synchronization of the synthesis process could help to produce more consistent product lengths. This may be done by preincubation of the circle primer with the polymerase at a very low temperature so that they can bind while synthesis is still very slow. Then when the temperature increases all of the polymerase molecules will begin synthesis at the same time resulting in molecules of with more consistent lengths. Although the sizes of the LRCA products are quite variable there are many large molecules. If the synthesis procedure can be tuned to produce products of consistent lengths then they could be assembled onto an array.

Long strands of DNA are dynamic molecules that move and change their conformation based on their surroundings. One very important factor that affects DNA conformation is the salt concentration. Increased salt concentrations cause DNA to stick together and become more compacted. As a result, trying these reactions at lower salt concentrations may allow for better stretching. However this may also cause the products to lose their probes due to the fact that the high salt helps keep them hybridized. This would cause the DNA to fade and it might not even be visible if the probe doesn’t stick to the product. So caution must be taken to find a salt concentration at which the products are free for stretching yet still visible.
Another method, for producing LRCA products that are free for stretching, is to amplify the circle on the surface. If one end of the product is tethered to the surface the product is less likely to become entangled. As the polymerase synthesizes the product the circle rolls away from the surface resulting in longer freer molecules. The density of the products can be controlled by the amount of circle and primer captured onto the surface. Surface amplification by LRCA may provide an even better method for producing consistent colonies of a template sequence.
Chapter 4: Microfluidic Filtration Membranes for Producing Uniform Microemulsions

4.1 Introduction

It is common knowledge that water and oil do not mix, they are immiscible. This is the principle behind emulsions. Emulsions are droplets of water suspended in a solution of oil or droplets of oil suspended in water and microemulsions are micrometer sized emulsions. Microemulsions can be used as small reaction chambers for isolated PCR amplification of target DNA sequences on beads (Dressman et al., 2003; Diehl et al., 2006). These microemulsions are produced by mixing a solution of water in oil until the water is broken into droplets suspended in the oil. This process results in microemulsions with a wide range of sizes. Since each emulsion has a different size the volume changes and the contents varies. As a result the reaction is difficult to control and many of the emulsions are wasted. This thesis works to develop a method for producing microemulsions with a uniform size. Having control of the size of the reaction chambers makes it easier to control whatever reaction the emulsions are used for.

Uniform water in oil microemulsions can be used to further control the linear rolling circle amplification of template DNA. Microemulsions, created with the LRCA reaction mix as the aqueous phase, act as tinny reaction chambers for the product synthesis. When the temperature of the emulsion solution is raised to the optimum the polymerase will replicate the circular template until either the free dNTP’s are all incorporated or there is no more space for further extension. As a result the size of the emulsion and concentrations of key components can be used to control the length of the LRCA product precisely. These benefits require emulsions with very consistent volumes.
4.2 Experimental Design

Uniform microemulsions can be produced by adding small drops of aqueous phase into a cross flow of oil phase. This is accomplished with a t-junction geometry where aqueous phase is introduced at a right angle to a continuously flowing oil phase (Nisisako et al., 2002). The throughput of this geometry can be increased dramatically by introducing the aqueous phase through an array of pores in a membrane into a bulk oil phase (Kobayashi et al., 2002). Each pore acts as a t-junction for microemulsion production. A microfluidic device was designed to allow the aqueous phase to flow through a microfiltration membrane, positioned so that the continuous oil phase flow is perpendicular to the drops of aqueous phase flow, as illustrated in Figure 4.1. The aqueous phase droplets that come through the pores are sheared off by the oil phase flow to form microemulsions. The size of these emulsions is dependent upon the size of the pores as well as the flow velocity of each phase and therefore it can be controlled precisely.

Figure 4.1 Microemulsions for LRCA Control. Uniform microemulsions can be created with a microfiltration membrane at the interface between an aqueous phase and oil phase. The pores of the membrane provide an array of t-junctions where the disperse phase is introduced to the continuous phase and sheared off at a precise volume. If the aqueous phase contains the components for LRCA the product length can be controlled by the volume of the microemulsion.
A fluid flow block and membrane adaptor device, designed to hold a microfiltration membrane at the interface of the aqueous and oil phases, can be used to create uniform microemulsions. In this design, shown in Figure 4.2, the membrane is glued to the tip of a fitting with a fluid port at the top and a cone that opens up the aqueous phase flow behind the membrane. The surface of the fitting is machined precisely so that the membrane surface is 100µm below the side edges of the fitting and the other two sides are machined with ramps to direct the oil phase flow past the membrane. This fitting is screwed into a block so that the membrane is positioned in the middle of the oil phase flow. A flat bottom fitting is screwed in from the opposite side of the block all the way until it touches the side edges of the membrane adaptor and forms the bottom of the channel. This channel is in line with the oil phase fluid flow ports and forms a T-Junction at the aqueous phase interface with the oil phase.

**Figure 4.2 T-Junction Device and Membrane Adaptor.** The T-Junction device has oil phase flowing from the side and aqueous phase flows from the top. The membrane adaptor is screwed into the device and the edges are pressed flat against the bottom piece to form a 100 µm channel for oil flow below the membrane.
Fluid flow is set up with fittings and tubing and is powered by syringe pumps connected to the solution reservoirs. These syringe pumps are computer controlled and the flow of each phase can be set to precise values. Since the size of the microemulsions is dependent on the fluid flow rates, the following experiments test various combinations of aqueous phase and oil phase velocities to determine which produces the best microemulsions.

Microemulsion size is also dependent on the pore size of the microfluidic filtration membrane. Filtration membranes are available with 2.5 µm and 5.0 µm pore diameters. Tests of emulsion production at the same flow rates with each membrane should produce microemulsions of different sizes.

The number of emulsions produced by this device per second is dependent on the number of pores in each membrane as well as the flow rates of the continuous and dispersed phases. These microfiltration membranes contain at least 100 pores and therefore can produce many emulsions in parallel. The high throughput of this device could be another great improvement over other methods for producing uniform microemulsions.

4.3 Methods

Device Fabrication

A CNC milling machine was used to drill and machine the device. The fluid flow block and membrane adaptor were made out of a Teflon block and rod respectively. The membrane adaptor surface was machined as in Figure 4.3. The height of the oil phase flow channel is controlled by the depth of the recess machined for the membrane. A low density 2.5 µm or 5.0 µm microfiltration membrane purchased from Aquamarjin was secured to the tip of the adaptor with a very thin layer of five minute epoxy. Then the T-junction device was assembled with the membrane aligned in the crossflow of the continuous phase. Upchurch ¼” 28 fittings and 1/16”
diameter Upchurch tubing were used to set up the fluid flow system. Teflon tape was wrapped around each fitting to help seal the connection and keep the device from leaking.

![Diagram of Membrane Adaptor Dimensions]

**Figure 4.3 Membrane Adaptor Dimensions.** A Teflon cylinder is held securely in an aluminum square and positioned in the vice so that one corner is at (0, 0). The membrane adaptor tip is machined into the surface based on the given dimensions. Then the fluid flow holes and cone are drilled in the center.

**Microemulsion Production**

The oil phase (4.5% Span 80, 0.4% Tween 80, 0.05% Triton X-100, 95.05% Mineral Oil) flow was powered by a 2.5 ml syringe pump, controlled by PumpLink. A large bottle filled with the oil mixture and sealed with a pressure cap was used as a reservoir for the oil. The pressure cap contained ports for Upchurch ¼” 28 fluid fittings. One of these fittings was supplied with
pressurized air to keep the contents of the bottle under pressure and the other fitting
collected a piece of tubing in the solution to the valve on the syringe pump. In this way, the oil
phase syringe was filled with solution under pressure. The aqueous phase (10 µM Fluorescein,
10 mM Tris-Cl Buffer pH 8.0, 0.1% Triton X-100, in Water) flow was powered by a 500 µl syringe
and pump, controlled by PumpLink.

These two syringe pumps were operated independently at various flow rates. With the
microstepping function enabled the flow of the fluid was steadier because the velocity was
controlled more precisely by microsteps rather than steps. One step equals eight microsteps so
the movement of fluid was eight times finer. Aqueous phase velocity was varied from the
lowest limit of eight half steps per second, V8, up to V16 and V32. Several experiments were
done with oil phase velocities from V80, V120, V160, to V200. The aqueous phase flow was
always started a few seconds before the oil phase flow and continued a few seconds after to
ensure no backflow of oil through the membrane. After several runs through the device to
prime the system, a sample of the outflow was collected in a test tube.

Microemulsion Quantification

A drop of the outflow fluid was imaged on a glass slide using the Leica microscope with a
10X objective. Because the aqueous phase contains fluorescein, fluorescent illumination lit up
the microemulsions and they were easily distinguished from the oil phase which was not
fluorescent. Images were analyzed with Image-J. With this program a threshold was set and the
images were converted to black and white images. Then the microemulsions were identified as
circular objects and the diameters were measured in pixels. Each pixel on the Hamamatsu
camera is equivalent to 6.54 µm. With a known 10X magnification and the camera
specifications, the size of the microemulsions was calculated precisely.
4.4 Results

The emulsions produced by the T junction microfiltration membrane varied from run to run. The microfluidic filtration membranes are very delicate and they are easily broken. Many of the experiments resulted in large droplets of aqueous phase in the oil solution indicating a broken membrane. However, sometimes the emulsions produced were consistent with the predictions. Some of the best results are presented in Figure 4.7. These emulsions were produced with an oil phase velocity of \( V_{120} \) (10.0 µl/sec), \( V_{160} \) (13.3 µl/sec), \( V_{200} \) (16.6 µl/sec), and an aqueous phase velocity of \( V_{8} \) (0.67 µl/sec). The diameter of each microemulsion was determined and plotted along with the average diameter and standard deviations. These plots are shown in Figure 4.4, Figure 4.5, and Figure 4.6. The statistics of these measurements are summarized in Table 4.1.

![Figure 4.4 Diameters of Microemulsions Produced by Oil V120.](image-url)
Figure 4.5 Diameters of Microemulsions Produced by Oil V160.

Figure 4.6 Diameters of Microemulsions Produced by Oil V200.
Figure 4.7 Images of Microemulsion. Fluorescent images of water in oil microemulsions produced by the T-Junction device with varying oil phase velocities (A) 10.0 µl/s, (B) 13.3 µl/s, (C) 16.6 µl/s, and aqueous velocity, 0.67 µl/s.
Table 4.1 Microemulsion Statistics. This table shows the average diameter and standard deviation of microemulsions produced by a given oil velocity. The coefficient of variation is calculated from these statistics and the number of microemulsions counted are noted.

<table>
<thead>
<tr>
<th>Oil Velocity (µl/s)</th>
<th>Average Diameter(µm)</th>
<th>Standard Deviation</th>
<th>Coefficient of Variation</th>
<th>Number Counted</th>
</tr>
</thead>
<tbody>
<tr>
<td>120</td>
<td>31.9</td>
<td>5.6</td>
<td>0.18</td>
<td>391</td>
</tr>
<tr>
<td>160</td>
<td>27.4</td>
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<td>0.12</td>
<td>280</td>
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<tr>
<td>200</td>
<td>27.6</td>
<td>4.2</td>
<td>0.15</td>
<td>315</td>
</tr>
</tbody>
</table>

4.5 Discussion

Microemulsions produced by this device were fairly uniform and these initial tests and set up reactions are very encouraging. However, the emulsions produced by this device were not reproducible due to the variability of the device throughout the design process. Some experiments produced very nice looking small fairly uniform microemulsions while others produced large and inconsistent microemulsions. Aqueous phase velocity was set at the lowest limit of the syringe pump at 0.67 µl/s (V8) and produced the most reliable microemulsions with this flow rate. The aqueous phase flow rate was set and the oil phase flow rate was varied to test the dependence of diameter on flow rate. A correlation between the fluid flow velocities and the microemulsion diameter could not be determined from these experiments. Each condition produced microemulsions with diameters within one standard deviation of the other conditions. There was no noticeable trend as expected with oil phase velocities of 10.0 µl/s (V120), 13.3 µl/s (V160), 16.6 µl/s (V200). It is possible that the change in the oil phase flow rate was not significant enough to affect the microemulsions diameters. Testing the set up with much higher and much lower velocities might help to reveal a trend.

No correlation between membrane pore diameter and microemulsion size that could be determined from this set of experiments because a consistent data set was not obtained for
membranes of both sizes. The microfiltration membranes are very delicate and break easily and so many experiments were not valid.

Air bubbles were a major issue in the formation of uniform microemulsions with the filtration membranes. Air present in either the aqueous phase or oil phase resulted in very strange and inconsistent double emulsion like structures. A large air bubble caused the aqueous phase and oil phase to mix at the wrong interface and create nonuniform emulsions. In order to solve this problem several changes were made in the design and setup. First the device was machined to minimize any areas where air pockets might form. Ramps were machined to direct the oil phase flow through the channel without any dead areas. A cone was machined above the membrane to remove the dead space in the aqueous phase flow and reduce the probability of a bubble trapped there. The oil phase syringe was filled with pressurized fluid to reduce the formation of bubbles in the fluid pick up. Then several flow sequences were run to prime the system and remove any air bubbles before a sample was collected. Further improvements would be to use pressurized flow for both solutions so that they are entirely continuous.

Another major problem encountered was the frailty of the membranes. These microfiltration membranes are only 1µm thick and can only withstand pressures up to 0.5 MPa (about 72 psi). Some of the membranes were broken in the device assembly process while others were broken in the process of making the emulsions.

Increased precision in handling the membranes resulted in less breakage during assembly. However, the procedure is still difficult. The membranes used in these experiments were glued to a filtration support and had to be cut out with an xacto knife before they could be assembled into the device. New membranes for further experiments can be ordered without
the support. This will remove the tedious step of cutting them out and will reduce the risk of breaking them.

Some of the membranes were broken because the side supports on the initial versions of the membrane adaptor were not wide enough to hold the membrane up and keep it from coming into contact with the bottom of the device. Enlarging the diameter of the membrane adaptor created a larger surface area for the side supports and helped to hold the membrane at a set channel height, above the bottom surface. This design modification eliminated the breakage of the membrane by contact with the bottom surface.

Opening up the aqueous phase flow behind the membrane also helped to decrease the number of membranes broken. This modification reduces the force of the fluid on the back of the membrane and allows it to slow down before it hits the pores. Without the cone machined in the membrane adaptor the aqueous phase would hit the membrane in one area and not another causing variation in the fluid flow at each pore. Increasing the area of the flow makes it more consistent over the whole membrane. This design modification seemed to help significantly in keeping the membrane intact and producing more uniform microemulsions.

Some more modifications could be made to improve the system and reduce the number of broken membranes. Any small particle will break the membrane if forced through by the flow. Therefore it might also be beneficial to filter the aqueous phase fluid before it enters the system. This would minimize the chances that a particle would break through the membrane. Another improvement might be to try using pressure driven flow. That way the pressure on the membrane will never be forced over the maximum pressure and the membrane would likely stay intact.
Pressure driven flow may also provide a method for more precise control over the size of the microemulsions. In these experiments the aqueous phase velocity was set at the lowest possible velocity for this set up. Pressure driven flow would allow even lower flow rates for the aqueous phase flow. This may help to decrease the variation in microemulsion diameters.

Pressure driven oil phase flow could be better controlled as well. The benefit would be that the oil phase flow could be truly continuous rather than stepwise. A major improvement would be that it would not be limited by the volume of the syringe but could have a large reservoir of oil solution. There would be a virtually unending supply of the continuous phase. However one difficulty is that the oil phase is fairly viscous and requires a high pressure to move at an acceptable rate. But with larger diameter shorter tubing sections this may not be an issue.

Pressure driven flow might also help to reduce the air bubbles introduced into the fluidics system. Since some of the air bubbles are introduced via the syringe pump setup because the fluid is pulled into the syringe faster than it can move. Removing this part of the setup would also eliminate the air bubbles. In addition the solution would be under pressure and any dissolved gases would likely be eliminated creating a bubble free fluid.

**Further Experiments**

New membrane options include high porosity membranes as well as membranes with pore diameters down to 0.2 µm. Higher porosity membranes would help to increase the throughput of this device drastically. And with smaller pore sizes the microemulsions created with these filtration membranes should have corresponding smaller diameters.

Changing the aqueous phase and oil phase recipes may also influence the microemulsion diameter. Some resources suggest that the amount of surfactant in the two
phases can affect the size of the emulsions produced in other devices. This may have a similar affect in the membrane device as well.

Some further changes that could be made would be to use microfabrication methods to produce a T junction or some other geometry for producing microemulsions. Once uniform microemulsions are produced reliably they can be used as reaction chambers for the linear rolling circle amplification process.
References


