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PREPARATION OF DNA-STRUCTURES IN SINGLE MOLECULE EXPERIMENTS

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ABSTRACT

The study of biomolecules on a single molecule level enables the investigations of dynamic, mechanistic as well as heterogeneous behavior of the biomolecules. For studying the features of DNA-protein interactions involved in DNA-repair, on the single-molecule level, the combination of microfluidics, surface preparations and fluorescence microscopy is used by the single molecule biophysics group at the physics department at the University of Gothenburg. In addition, traditional biochemical techniques, Polymerase Chain Reaction and agarose-gel electrophoresis, are also used to produce and analyze various types of DNA used in the single molecule experiments. The work is interdisciplinary and combines physics, surface science, biochemistry and micro/nano science, which motivated me to finish my master thesis project in the field of single molecule biophysics.

In order to investigate the repair of the double-stranded break (DSB), we tried to simulate the homologous recombination (HR) process in vitro to study the interaction between DNA and the protein Rad51 through single molecule experiments. The objective of this thesis project was to prepare DNA structures to mimic the damaged DNA with processed DSBs. The damaged and processed DNA structures are double-stranded DNA (dsDNA) with a 3'-single-stranded DNA (ssDNA) overhang of various lengths. These DNA structures will work as the substrates for the study of the DNA-protein interaction that are involved in the repair of DSBs.

Short pieces of ssDNA (140nt) were designed and bought directly. Long pieces of ssDNA (5kb) were produced via Polymerase Chain Reaction (PCR) followed by lambda-exonuclease treatment using lambda-phage DNA (48 kbp) as template. The optimal conditions were acquired after repeated experiments and the results were verified by agarose gel electrophoresis. Finally, these ssDNA molecules were ligated to the original lambda-DNA. Fluorescently labeled Replication Protein A (RPA) that preferentially binds ssDNA, was used to confirm that the DNA samples contained the desired structure, dsDNA with 3'-ssDNA overhang. The interaction between DNA and RPA was studied in microfluidic flowcells using fluorescence microscopy where the DNA samples were attached to a functionalized lipid bilayer surface within the microfluidic flowcells.

Keywords: ssDNA, RPA, lambda-exonuclease, microfluidics, lipid surface, fluorescence microscopy

CONTENTS

Chapter I Introduction

In living cells, DNA stores the essential genetic information to life. The DNA has to maintain its integrity and stability during replication and transcription. However, many environmental factors, such as chemical insults, stalled replication forks or ionizing radiation, can lead to many kinds of DNA damages. If not repaired properly, DNA damages can cause mutation, apoptosis, and even cancer.[1] Among DNA damages, the most dangerous one is the double-stranded break (DSB), which means that both strands of the DNA molecule are cleaved. In addition, DSBs can induce chromosomal rearrangement, which means that genes are disrupted in this process, causing hybrid proteins or inappropriate activation of genes.[1, 2] Homologous recombination (HR) is one of the most important DSB-repair mechanisms. HR, as a DNA repair process, can be found in all forms of life, which offers high-fidelity, template-dependent repair or tolerance of complex DNA damages, such as DSBs.[3]

During DSB repair, the key protein in HR is Rad51 which is the eukaryotic RecA homolog and highly conserved from yeast to humans. Rad51/RecA protein is a class of DNA recombinases that catalyzes the pairing and exchange of complementary DNA strands during HR.[4, 5, 6] In mammals, when a DSB has been identified during HR, the ends of DNA molecules are being processed by 5' to 3' exonucleases to produce long single-stranded DNA (ssDNA) 3' overhangs(fig1.1).[7] At first, these ssDNA overhangs are bound by Replication Protein A (RPA) in the nuclear environment. RPA is a ssDNA-binding protein in eukaryotes. [8, 9, 10] After RPA binding to these ssDNA overhangs, Rad51 is assembled to replace RPA forming an extended right-handed helical nucleoprotein filament on the damaged ends (fig1.2).[3, 6, 9]

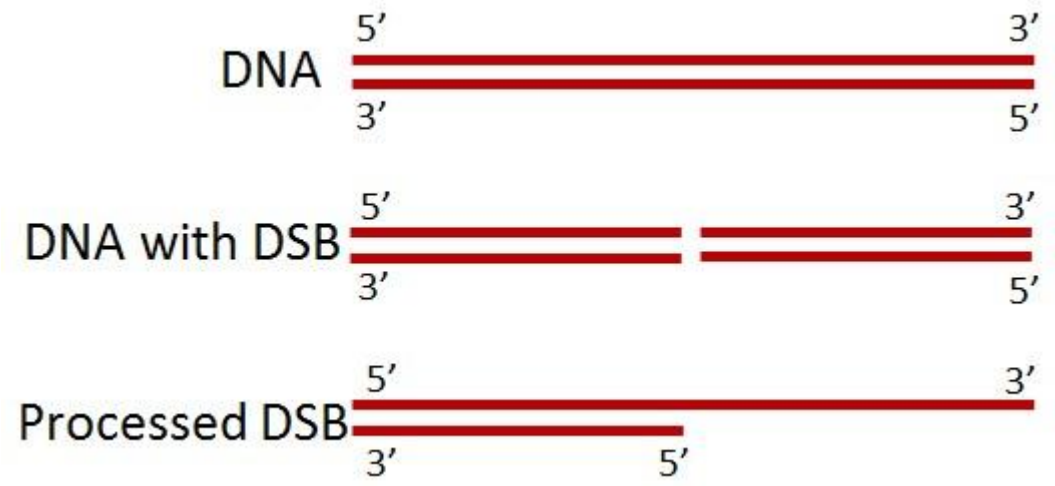

Fig1.1: Schematic description of DSB

In the end of HR, the protein filament search for homology in a complementary strand and start to pair up and replicate the other strand to leave a fully repaired DNA strand without any loss of genomic information.[3, 6, 11] The process of HR in a cell is very complicated, and includes various types of proteins. However, some steps in the HR pathway can be performed in vitro with one or a few proteins. In the lab we will investigate the interaction between DNA and Rad51 protein to study this DSBs repair pathway by single molecule experiments and the process can be simulated as shown in fig1.3.

Fig1.2: Filament formation of Rad51 and RPA on ssDNA. After RPA binding to ssDNA, Rad51 replaces RPA and forms a continuous right-handed helical filament.

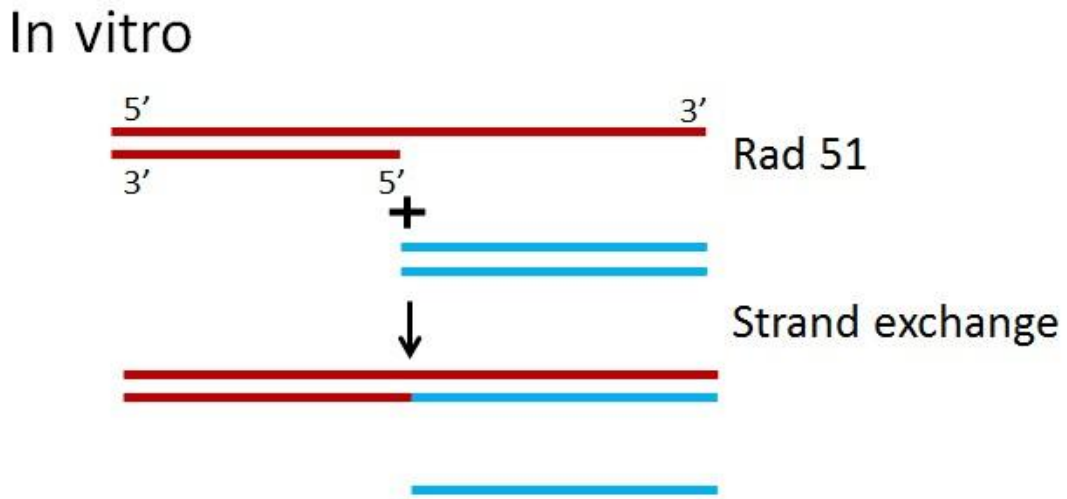

Fig1.3: The process of strand exchange as it occurs in vitro.

To study this repair pathway and the interaction between DNA and Rad51 by single molecule experiments, in this project, my goal was to produce the appropriate DNA structures to mimic the damaged DNA with processed DSBs as shown in fig1.1. The DNA molecules with a 3' ssDNA overhang will act as the substrate for future research of DNA-Rad51 interaction. The DNA structures are composed of dsDNA with ssDNA overhang at different lengths. Short pieces of ssDNA have been designed and purchased directly. Long pieces of ssDNA are produced by Polymerase Chain Reaction (PCR) followed by lambda-exonuclease treatment.

The DNA strands used within this study is based on lambda-DNA which has a 5' 12nt

overhang in each end. At first, one end of lambda-DNA is biotinylated, and the other one is attached to ssDNA overhangs of different lengths (fig1.4). There are three types of DNA structures that was tested in this work. The first one is the dsDNA with a 12nt ssDNA overhang. When lambda-DNA is biotinylated in one end, the end without biotin will leave a 12nt ssDNA overhang automatically. The second one is the dsDNA with a 128 nt ssDNA overhang. Short pieces of ssDNA with 140nt are ligated to the biotinylated lambda-DNA so that the DNA molecule has a 128nt ssDNA overhang. The third one is the dsDNA with a long ssDNA overhang. Using lambda-DNA as template, long pieces of ssDNA with around 5000nt are produced and ligated to the biotinylated lambda-DNA as well. To confirm the attachment of the ssDNA to the dsDNA we will use fluorescent RPA that has been labelled with green fluorescent protein (GFP). RPA has a higher affinity for ssDNA than for dsDNA and binding of RPA will be used to detect the presence of ssDNA in the ends of the dsDNA structures.

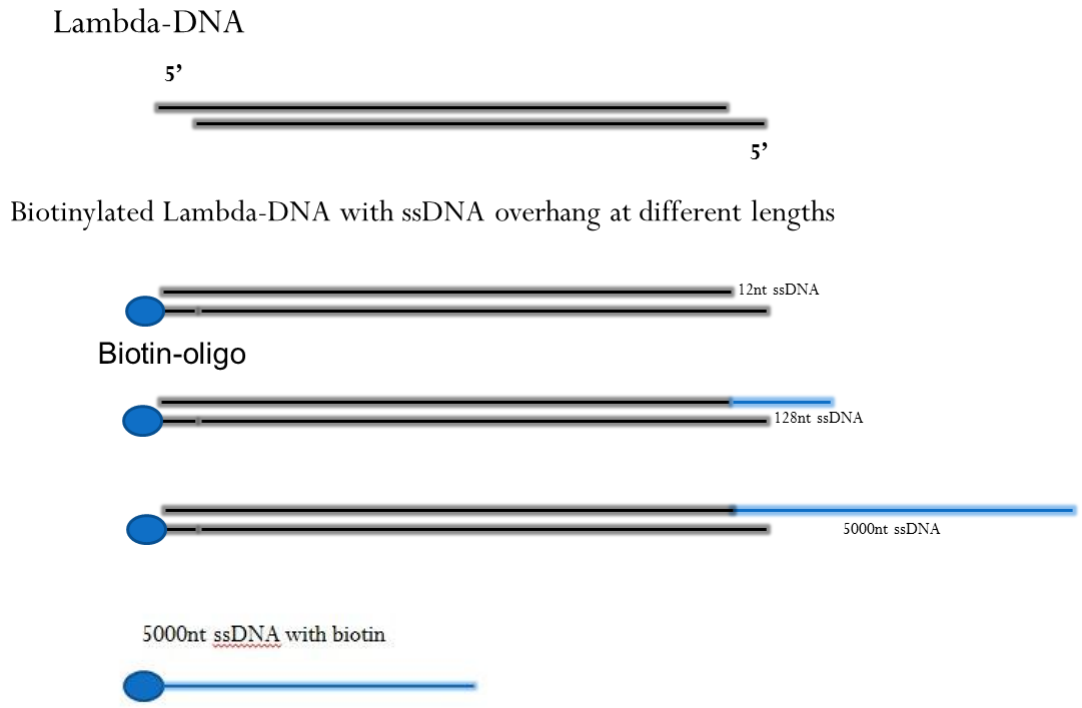

Fig1.4: The structures of original lambda-DNA and the desired DNA substrates.

Chapter II Theoretical Background

2.1 Polymerase Chain Reaction (PCR)

The Polymerase Chain Reaction is a biological technique to amplify desired DNA fragments through designing specific primers for known DNA sequence. An integrated PCR process contains several components and steps. An original DNA as template includes the target DNA sequence. Two primers, forward primer and backward primer, with around twenty nucleotides are complementary to the 5' ends of each strand from the DNA target. An enzyme called DNA polymerase adds free nucleotides to the 3' end of DNA strand and catalyze the DNA synthesis.[12] In our project, we use *Taq* polymerase which is a thermostable DNA polymerase. Many nucleotides with triphosphate groups are called deoxynucleoside triphosphates (dNTPs) which provide the building-blocks for the polymerization of a new DNA strand. Buffer solution containing necessary chemical ions, like Mg^{2+}, provides a suitable and stable environment for optimal activity of the *Taq* polymerase. Finally, the whole reagents are reacted in a thin-walled tube by a thermocycler.[13, 14]

The total procedure of a PCR reaction starts with an initial denaturation, follows repeated temperature changes called cycles, and terminates with a final extension around 10-20 minutes at extension temperature. Every cycle can be described simply by three steps, denaturation, annealing and extension. The denaturation often occurs at 94-98° C and dsDNA are separated into single strands in this step. The annealing temperature is related to the design of primers, which allows primers to bind to DNA single stands. The temperature of extension is strictly set up at the temperature where the *Taq* polymerase has its optimum activity. At the step of extension, new DNA molecules are synthesized. [15, 16]

The target DNA is amplified exponentially to billions of copies in a PCR reaction after 20-40 thermo-cycles generally. The principle of PCR reaction procedure is shown in figure 2.1.[17] In the first cycle, the entire dsDNA is separated to two single strands by heat treatment, then the primers are annealed to complementarily hybridize with the 3' end of desired region, and two new dsDNA are synthesized from primers by incubating with DNA polymerase and the mixture of the four dNTPs at optimum environment (Fig.2.1A). From the second cycle, the newly synthesized DNA fragments serve as templates in their turn, and the entire cycle is performed over and over again. After severalcycles, the predominant DNA product is fixed to the length between the two original primers, which is the desired length of the fragment, and the remainder of original DNA strands is very few, which can be ignored (Fig. 2.1B).[17]

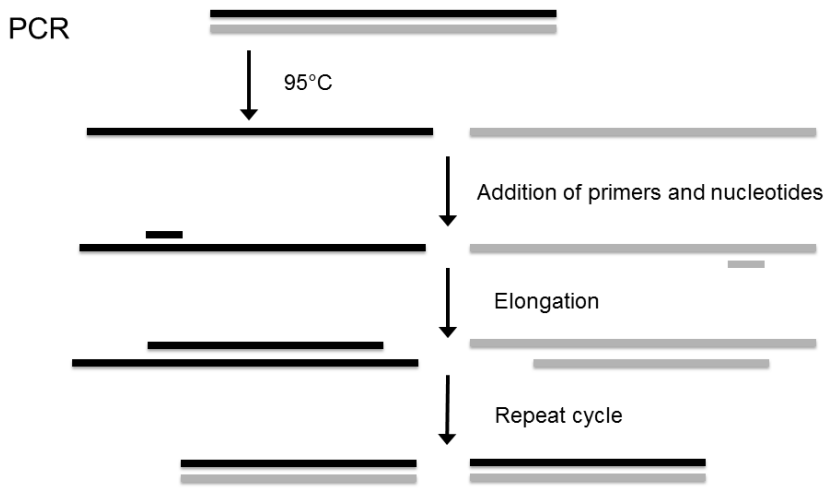

Fig. 2.1: The principles of the PCR reaction procedure.[17]

2.2 Agarose Gel Electrophoresis

Gel electrophoresis is the easiest and most common way to estimate the size of linear DNA and RNA fragments by separating them by length, or separate proteins by charge. Agarose is a kind of material to make gel matrix in lab, because it is extremely easy to prepare and non-toxic.[18] The gel matrix as a sieving medium allows negatively charged nucleic acid molecules to migrate toward the catode under an electric field, and the nucleic acid molecules are separated by different moving speed according to their different lengths. Shorter molecules move faster than longer molecules and gather in lower position of gel matrix, because they are smaller and it is easier for the smaller molecules to go through the gel matrix.[19]

In DNA separation, agarose gel is typically used at concentrations of 0.5% to 2.0% to separate and estimate the size of DNA fragments, especially long DNA fragments, from 50 base pair to several millions of bases. In this project, the agarose gels we used are made in 0.7%, which show a good resolution of large 5-10kbp DNA fragments, and even for lambda-DNA (48kbp).[18]

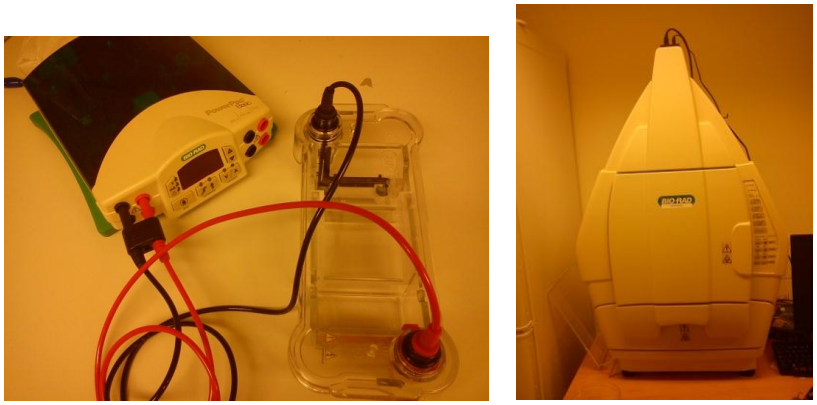

Fig.2.2: The instruments used in gel electrophoresis.

The necessary equipment for agarose gel electrophoresis is shown in figure2.2. A chamber connected to the power supply provides the place and electric field for electrophoresis. A plastic tray is the place where the gel is being cast. Sample combs are to form sample wells in the gel when molten agarose is poured. Electrophoresis buffer usually uses Tris-acetate-EDTA (TAE) or Tris-borate-EDTA (TBE). In this project, we use 1x TBE buffer to provide a stable liquid environment for the whole system. Before the sample is loaded, we need to add loading buffer to the sample which contains a chemical with high density (e.g. glycerol or sucrose) to make the sample loaded easier. The loading buffer also contains negatively charged dyes which help monitoring the electrophoresis process. After the electrophoresis is completed, a kind of fluorescent dye, Ethidium Bromide, is used for staining nucleic acids in buffer and making them convenient to detect by transilluminator because Ethidium Bromide can intercalate between the bases of nucleic acids and fluoresce under ultraviolet light. Ethidium Bromide can also stain ssDNA. The transilluminator is an ultraviolet lightbox which is used to illuminate stained DNA in gels.[20]

2.3 Lambda-Exonuclease Treatment

Lambda-exonuclease is a highly processive enzyme that acts in the 5’ to 3’ direction and catalyzes the removal of 5’ mononucleotide from duplex DNA. The substrate needed for lambda-exonuclease treatment to produce ssDNA is dsDNA with only one 5’-phosphorylated end. That is realized by designing one primer without a 5’ phosphate (fig2.3). Although lambda-exonuclease also degrades ssDNA with non-phosphorylated ends, it is at a reduced rate.[21] Therefore, we designed the backward primer with 5’-nonphosphorylated end for protection and tried to strictly control the concentration and time of reaction to seek optimal condition to obtain ssDNA from dsDNA in as high amount as possible.

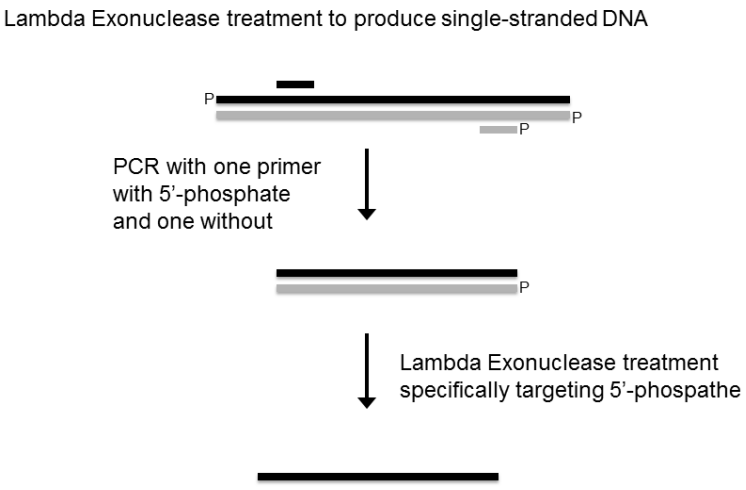

Fig2.3: The principles of lambda-exonuclease treatment.[22]

2.4 Phosphorylation

Phosphorylation is the process that adds a phosphate (PO_4^{3-}) group to a protein or other organic molecule, such as ssDNA in this project. We used T4 Polynucleotide Kinase (T4 PNK) to perform this process in this project. The enzyme catalyzes the transfer and exchange of P from ATP in the reaction solution to the 5'-OH end of polynucleotides (fig2.4). Hence, ATP is the necessary material for the reaction. The addition of 5'-phosphates to the ssDNA produces by lambda-exonuclease treatment provides the preparation for subsequent ligation.[23]

Fig2.4: The principle of phosphorylation of the ssDNA strand.

2.5 Biotinylation

In the biotinylation, biotin is attached to the beginning of the original lambda-DNA (fig2.5). Biotin is a small protein (MW=224.31) so that biotinylation is unlikely to destroy the natural function of molecule. In this project, there are two reasons why we use biotin. One is for protection of the ssDNA and the other is for attachment to the surface within the microfluidic flowcells that is further described below.[24]

Instead of using a non-phosphorylated 5' end of dsDNA for protection of ssDNA in the lambda-exonuclease treatment the DNA can be protected by a biotin. On the other side, the attachment of DNA molecules to lipid surface needs to be realized by biotinylation. Biotin binds specifically to neutravidin with an extremely high affinity. When the lipid surface is prepared, a neutravidin solution is added before loading the DNA sample. Biotinylated DNA molecules attach to the surface by biotin binding to neutravidin.[24]

Fig2.5: The principle of biotinylation

2.6 Ligation

Ligation refers to the process that connects two ends of DNA molecules using DNA ligase which is a specific kind of enzyme that repair DNA molecule by forming two covalent phosphodiester bonds between 3'-OH end of one nucleotide and 5'-phosphate end of another (fig2.6).[25] Therefore, before the ligation process, the ssDNA molecule obtained from lambda-exonuclease treatment is treated by phosphorylation. For the ligase reaction, ATP is required, which is supplied by the ligase buffer.

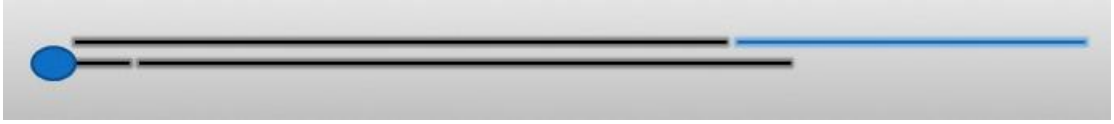

Fig2.6: The principle of ligation

2.7 Single molecule experiments

The single molecule experiments is enabled by the investigation of immobilized biomolecules on a surface, such as DNA and protein, which require that their biological integrity is not destroyed.[26] In this project, the main technique is the microfluidics. Microfluidics deals with fluids in the micro domain where the fluids have a laminar flow. Microfluidics technology is for example used in enzymatic analysis, DNA analysis and clinical pathology.[27]

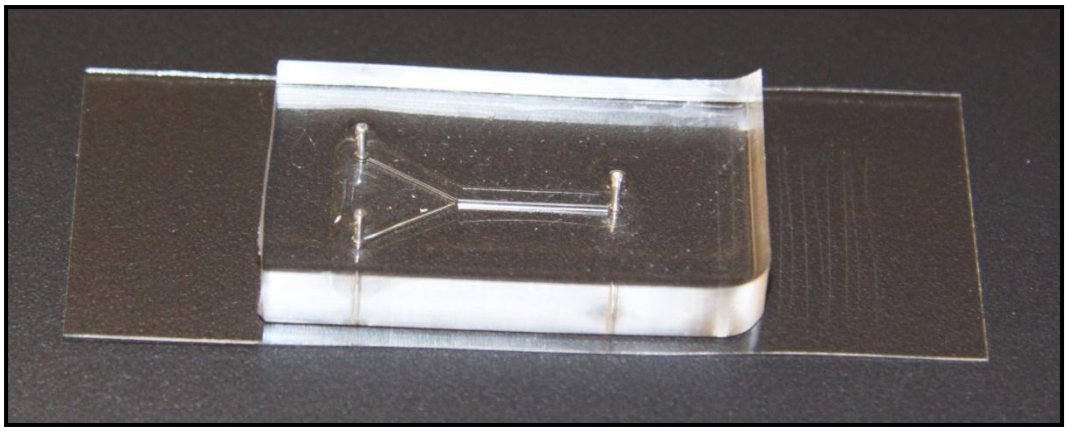

Fig2.7: The microfluidics device used in this project.

In this case, the microfluidics device used have a Y-shaped PDMS (polydimethylsiloxane) sample chamber which is fabricated using soft lithography,[28] and the fused silica slide was mechanically etched to produce barriers for lipid diffusion. On the bottom of the chamber, lipid bilayers are formed, which provide a microenvironment that is similar to the natural environment within the cell. In addition, the lipid bilayer minimizes the nonspecific interactions between the molecules and the surface. At the same time, the surface is functionalized to offer solid anchor points for the molecules, which does not affect their biological

properties.[26]

The lipid bilayer surface is functionalized as shown in figure 2.8. The big circles are lipid vesicles with biotin which are assembled on the surface and the lipid bilayer surface with biotin is formed. The small dots represent neutravidin which is a biotin-binding protein and provide the anchor point for DNA molecules on the surface. Biotinylated DNA molecules are immobilized on the surface by binding to neutravidin molecules.[7, 26] When the buffer flow is applied, the DNA molecules is stretched on the flow direction under hydrodynamic force. The details about surface preparation will be descripted in Chapter III.

As RPA highly favors binding to ssDNA over dsDNA, it is used to detect and confirm that the DNA molecules contain the desired ssDNA part in the end of the molecules. For detection using fluorescence microscopy, GFP is attached to RPA. We hope that RPA only bind to ssDNA and not dsDNA. If so, from the microscopy, we would see a bright point when the flow is off, and a short bright line jump a distance toward flow direction when the flow is on. The short bright line would jump back to become a point in quondam position when the flow is off again. The distance between the bright point and short line is dark and the length should be of the same length as the original lambda-DNA.

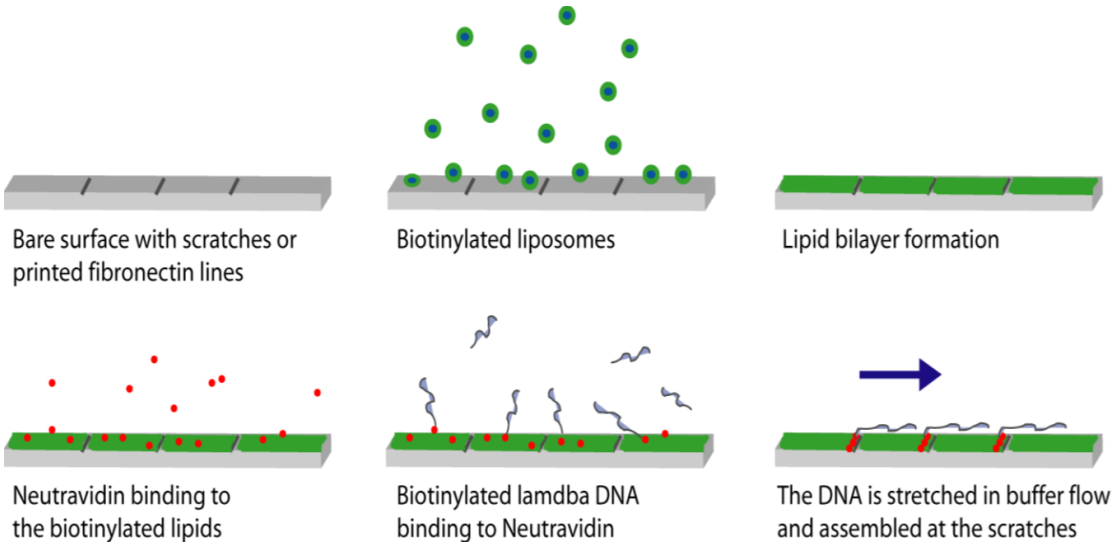

Fig2.8: The principle of surface functionalization. The glass surface slide is etched. The lipid bilayer is formed by biotinylated liposomes. Biotinylated lambda-DNA binds to neutravidin binding to the biotinylated lipids. The DNA molecules are stretched in flow and assembled at the barriers.[26]

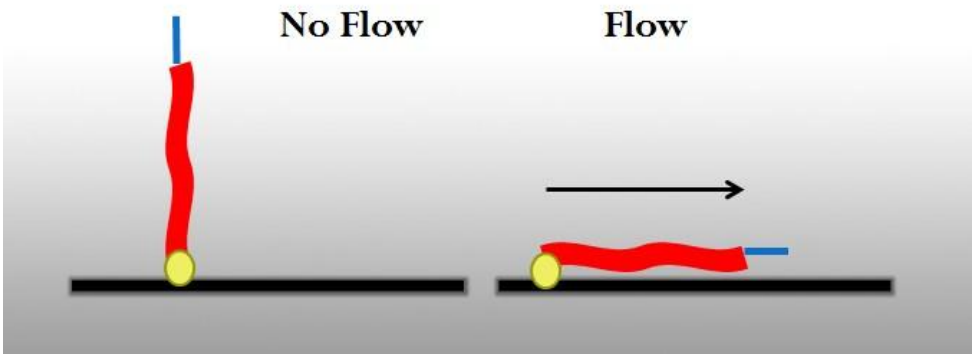

Fig2.9: Supposed DNA action on surface. The thick part represents dsDNA and the thin line represents the ssDNA overhang part.

Chapter III Experiments

3.1 Double-Stranded DNA Amplification by PCR

In this project, we used diluted lambda-DNA (*Promega, 465ug/ml*) as template for the PCR reaction and its sequence is shown in reference 29. The lambda-DNA is linear and double-stranded (48502nt) with 12nt single-stranded overhang in every 5' end. Its contour length is 16 μm long. In order to obtain long pieces of ssDNA at around 5kbp in the following lambda-exonuclease treatment step, primer1 as backward primer was designed to GGGCGGCGAC CTCGCGGGTT TTCGCTATTT AT from the first nucleotide and contained 32nt with 5'-OH end. Primer2 as forward primer was designed to GTGTCTCCCG GACGTCATCC from the 5101st nucleotide and contained 20nt. Therefore, the obtained dsDNA has 5120bp with only one 5'-phosphate end. Primer3 was designed with the same sequence as primer1 but with biotin at 5' end. Original lambda-DNA was diluted 100times to 4.65ng/ul by adding TE buffer. Two PCR reactions using primer1 and primer3 respectively as backward primer and primer2 as forward primer was performed in order to produce 5kbp dsDNA with and without biotin. The reagents for the successful PCR are shown in table 3.1. The conditions for both of them are shown in table 3.2. The unsuccessful trials will discuss in Chapter IV.

Table 3.1A: Reagents for PCR reaction to produce 5kbp without biotin

Volume	Reagent	Final Concentration
5ul	Accu Taq LA 10X Buffer	1X
2.5ul	dNTP Mix (10mM)	500uM
1.6ul	Template lambda-DNA (4.65ug/ml=4.65ng/ul)	0.1488ng/ul
3ul	Primer 1 (10uM)	600nM
3ul	Primer 2 (10uM)	600nM
34.4ul	Milli-Q Water	----
0.5ul	Accu Taq LA DNA Polymerase Mix	0.05unit/ul

Total Volume: 50ul

Table 3.1B: Reagents for PCR reaction to produce 5kbp with biotin

Volume	Reagent	Final Concentration
5ul	Accu Taq LA 10X Buffer	1X
2.5ul	dNTP Mix (10mM)	500uM
1.6ul	Template lambda-DNA (4.65ug/ml=4.65ng/ul)	0.1488ng/ul
3ul	Primer 3 (10uM)	600nM
3ul	Primer 2 (10uM)	600nM

34.4ul	Milli-Q Water	----
0.5ul	Accu Taq LA DNA Polymerase Mix	0.05unit/ul

Total Volume: 50ul

Table 3.2: The conditions for PCR reaction

Description	Temperature	Time
Initial denaturation	98°C	30sec
For cycles 1-30		
Denaturation	94°C	5-15sec
Annealing	65°C	20sec
Extension	68°C	20min
Final Extension	68°C	10min

The reagents were prepared in an eppendorf tube by 4 times, and then separated into four thin-walled PCR tubes to make sure that every tube has the same samples. The samples were placed in a thermocycler which was set up the program as below:

Program: Lid: 105°C Vol: 50ul
1 98° 00:00:30
2 94° 00:00:15
3 65° 00:00:20
4 68° 00:20:00
5 Goto 2, 29 times
6 68° 00:10:00
7 4° forever
8 end

After running over night, the results were verified by agarose gel electrophoresis, which is shown and discussed in Chapter IV.

3.2 Single-Stranded DNA Produced by Lambda-Exonuclease

The concentrations of 5kbp dsDNA and 5kbp dsDNA with biotin were estimated at 600nM which was limited by the concentration of primers. Although the lambda-exonuclease (*BioLabs M2062 5000U/ml*) preferred the 5'-phosphorylated dsDNA, it would also digest ssDNA without phosphate ends at a greatly reduced rate. To avoid digestion ofssDNA two sets of experiments were prepared. One for 5kbp dsDNA, and the other group was for 5kbp dsDNA with biotin. Because the process was easy to control for 5kbp dsDNA with biotin, the concentration of 5kbp ds DNA with biotin was double compared to 5kbp dsDNA without biotin. After repeated experiments where different conditions were tried out, the most successful conditions for the lambda-exonuclease treatment are shown in table 3.3&3.4.

To make sure that every tube has the same condition, the reagents were prepared

together in an eppendorf tube and separated into six thin-walled PCR tubes for incubation. To find the optimal conditions, six tubes were incubated for 30mins, 45mins, 1hr, 1.5hrs, 2hrs and 4hrs respectively. After incubation, the enzyme was killed at 75°C for ten minutes. The results were verified by agarose gel electrophoresis, which is shown and discussed in Chapter IV.

Table 3.3A: The reagents of lambda-exonuclease treatment for 5kbp dsDNA

Volume	Reagent	Final Concentration
4ul	Lambda-exonuclease (5unit/ul)	0.4unit/ul (20unit)
1.5ul	5kbp dsDNA (600nM)	18nM
39.5ul	Milli-Q Water	----
5ul	Lambda-exonuclease Reaction Buffer 10X	1X

Total volume: 50ul

Table 3.3B: The reagents of compare group for 5kbp dsDNA:

Volume	Reagent	Final Concentration
1.5ul	5kbp dsDNA (600nM)	18nM
43.5ul	Milli-Q Water	----
5ul	Lambda-exonuclease Reaction Buffer 10X	1X

Total Volume: 50ul

Table 3.4A: The reagents of lambda-exonuclease treatment for 5kbp dsDNA with biotin

Volume	Reagent	Final Concentration
4ul	Lambda-exonuclease (5unit/ul)	0.4unit/ul (20unit)
3ul	5kbp dsDNA with biotin (600nM)	36nM
38ul	Milli-Q Water	----
5ul	Lambda-exonuclease Reaction Buffer 10X	1X

Total volume: 50ul

Table 3.4B: The reagents of compare group for 5kbp dsDNA with biotin:

Volume	Reagent	Final Concentration
3ul	5kbp dsDNA with biotin (600nM)	36nM
42ul	Milli-Q Water	----
5ul	Lambda-exonuclease Reaction Buffer 10X	1X

Total Volume: 50ul

3.3 Agarose Gel Electrophoresis

To get good separation, a 0.7% gel was made by 0.28g agarose that was added to 40ml 1X TBE buffer. Every 5ul sample was prepared by adding 1ul loading dye. Every well in the gel was loaded by 5ul sample with loading dye. The gel was run at 70 voltages for 2hrs. After running, the gel was stained in the 1X TBE buffer (150ml) with 5ul ethidium bromide for 35minutess. In the end, the gel was illumined by

UV-light and an image was obtained.

3.4 Phosphorylation

After lambda-exonuclease treatment, the ssDNA product was collected and dialyzed in 10mM Tris-HCl at pH7.8 @ 25°C overnight to change the chemical environment of the solution. The concentration of ssDNA after dialysis was calculated and estimated at around 5nM.

At phosphorylation, 625ul ssDNA (5nM), 5ul T4 PNK (10,000U/ml) and 70ul ligase buffer were mixed and separated into 14 thin-walled PCR tubes. All tubes were incubated at 37°C for 30mins. Here the ligase buffer was used to replace T4 PNK buffer because the ligase buffer contains ATP which is necessary in phosphorylation. The contents in lambda-exonuclease reaction buffer, T4 polynucleotide kinase reaction buffer and DNA ligase buffer are displayed in Appendix.

After incubation, the T4 PNK enzyme was inactived at 65°C for 20mins. All samples were collected and dialyzed in TE buffer .

3.5 Biotinylation

For the process of biotinylation, 129ul lambda-DNA (aliquot that gives 1nM in 1ml), 20ul oligonucleotide (around 20X excess use "oligo-bio-R"), 100ul 10X ligase buffer (*Promega*) and 743ul Milli-Q water were mixed carefully by turning the tube and heated at 80°C for 10mins. After heating, the heating block was turned off and the tube was left in the heating block to cool down slowly to room temperature, which took around 2hrs.

After cooling on the ice for 15mins, 8ul T4 DNA ligase (*Promega*) was added in the solution and incubated at room temperature for more than 2hrs. The ligase was inactivated at 75°C for 10mins. Finally, the solution was dialyzed against TE buffer overnight.

3.6 Ligation

The short ssDNA fragment (140nt oligo) was boughtlyophilyzed, which was dissolved to 100uM in stock by adding TE buffer. Then the 140nt ssDNA was diluted to 1uM by adding TE buffer.

In the step of ligation of short ssDNA, 300ul biotinylated lambda-DNA (1nM), 12ul 140nt ssDNA (1uM, around 40X excess over biotinylated lambda-DNA), 36ul 10X ligase buffer (*Promega*) and 4ul Milli-Q water were mixed and heated at 80°C for 10mins. After heating, the heating block was turned off and the tube was left in the heating block to cool down slowly to room temperature.

Before 8ul T4 DNA ligase (*Promega*) was added, the solution was cooled on ice for 15mins. Then the solution was incubated at room temperature for more than 2hrs, and the ligase was inactivated at 75°C for 10mins.
For the ligation of long ssDNA pieces, 40ul biotinylated lambda-DNA (1nM), 320ul 5kb-ssDNA (5nM, around 40X excess over biotinylated lambda-DNA), and 40ul 10X ligase buffer were mixed carefully and heated at 80°C for 10mins. After heating, the heating block was turned off and the tube was cooled down slowly to room temperature at the heating block.

After cooling on the ice for 15mins, 8ul T4 DNA ligase (*Promega*) was added in the solution and incubated at room temperature for more than 2hrs. Then the solution was incubated at room temperature for more than 2hrs, and inactivated the ligase at 75°C for 10mins.

The success of the ligation was tested using single molecule experiments using fluorescence microscopy, which is shown and discussed in Chapter IV.

3.7 Surface Preparation

The whole microfluidics system was produced as shown in fig2.9. The glass surface was prepared with barriers for the assembly of the DNA molecules (fig2.10). The whole system was flushed by lipid buffer at first. The lipid solution (0.4mg/ml liposomes with 0.01% biotin) was injected into the system three times every 10mins to form a biotinylated supported lipid bilayer. After the third time, the whole system was incubated for 1hr and was after that rinsedwith lipid buffer to remove excess liposomes.

The 15ml BSA buffer was made of 1.2ml Tris-HCl (0.5M), 15ul $MgCl_2$ (1M), 15ul DTT (1M), 1.3ml BSA (2.3mg/ml) and 12.5ml Milli-Q Water. All reagents were mixed and filtered by 0.2um filter. The whole microfluidic system was blocked by BSA buffer for 30mins first. For neutravidin binding to the biotinylated lipid bilayer, the neutravidin solution suspended in BSA buffer (18ul/ml) was incubated in system for 3mins and rinsed by BSA buffer.

The biotinylated DNA sample was added in BSA buffer and incubated in the microfluidic system for 30mins so that the DNA was able to bind to neutravidin. BSA buffer was used to rinse away excess free molecules again. Finally the protein RPA-GFP solution suspended in BSA buffer (10nM RPA and 2mM ATP) was pushed into the system and the flow was controlled by a syringe pump at the rate of 20ul/min. The detection of RPA-GFP binding to DNA was made usingfluorescence microcopy. The results is shown and discussed in Chapter IV.

Chapter IV Results and Discussion

4.1 Double-Stranded DNA Amplification by PCR

Every tube from the PCR amplification of dsDNA without and with biotin was verified by agarose gel electrophoresis, because we cannot guarantee that each tube with the same sample would contain the appropriate product. The results are shown in fig4.1. There are 15 lines in a gel. Lambda DNA-HindIII digest (*BioLabs, 500ug/ml*) was used as size marker in the line2, 8 and 14. From top to bottom, the bands mean 23130bp, 9416bp, 6557bp, 4361bp, 2322bp and 2027bp respectively. When the ladder sample was prepared, 0.5ul original sample was diluted in 14.5ul MQ water and added 3ul loading dye (*6X Blue*). The template was prepared by 10ul adding 2ul loading dye and loaded in well 3 and 9 as comparison. In the left side of gel, 5kb-dsDNA samples standing in line 4, 5, 6 and 7 came from the four tubes for dsDNA without biotin. Every sample was prepared with the concentration as size marker, 0.5ul PCR production was diluted in 14.5ul MQ water adding 3ul loading dye. In the right side of gel, 5kb-dsDNA-biotin samples standing in line10, 11, 12 and 13 came from the four tubes for dsDNA with biotin. Every sample was also prepared by 0.5ul PCR production diluted in 14.5ul MQ water and adding 3ul loading dye. Every well was loaded in 5ul sample.

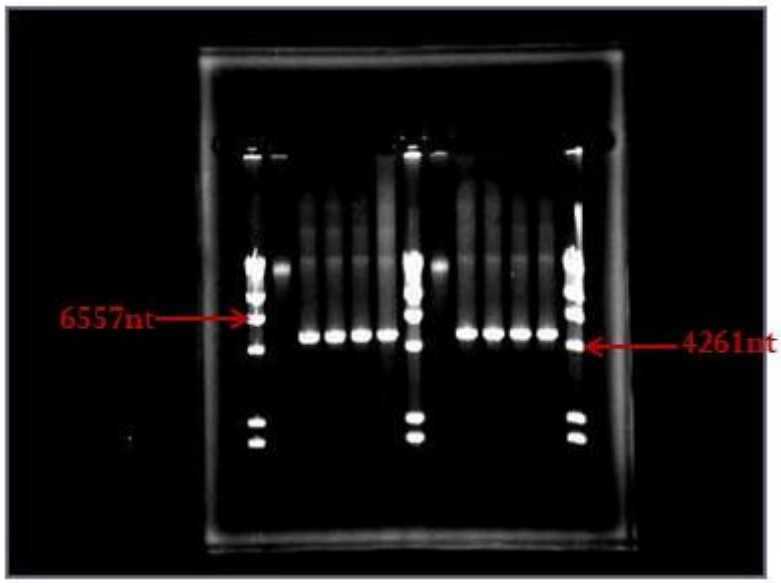

Fig 4.1: The results of dsDNA amplification by PCR.

From the figure 4.1, it can be concluded that the bands from PCR products are in the

position a little bit higher than the band of 4361nt. The PCR products were designed to be 5120bp. Therefore, it can be concluded that the PCR production for 5kb-dsDNA without and with biotin were successful for every tube. However, there are some light bands left which is probably the template DNA. The template DNA will be digested by the lambda-exonuclease treatment.
After verification, the successful tubes were collected. The concentrations of 5kb-dsDNA without and with biotin are estimated to 600nM, which is limited by the concentration of primers. The concentration may be less than 600nM because experimental errors might exist during the whole experimental procedure.

Note that the polymerase we used in PCR is Accu Taq LA DNA Polymerase Mix which was suitable for the amplification of long DNA fragments.

After successful amplification of the 5 kbp dsDNA, the same conditions were applied in a PCR reaction for amplification of longer dsDNA products of 15 kbp. The results are shown in fig4.2. The band of the desired 15kbp dsDNA should be concentrated in the middle position of 23,130bp band and 9,416bp band. However, it was shown that there were multiple products in the tubes. 2 different attempts were made where the annealing temperature was adjusted, but both of them failed. The problem was probably due to the design of the primers. The difference in melting temperature between the two primers should not exceed 3°C. In this case, the difference between the melting temperatures of the two different primers were larger which probably caused the amplification of multiple PCR products as seen in figure 4.2.

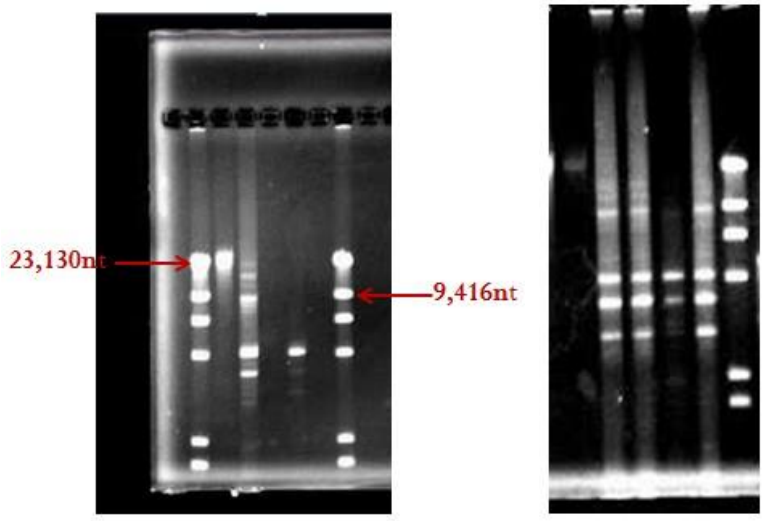

Fig4.2: The result of 15kbp dsDNA amplification.

4.2 Single-Stranded DNA Produced by Lambda-Exonuclease

To obtain the optimal conditions to produce 5kb ssDNA by lambda-exonuclease treatment, the sample was separated into 6 tubes for incubation at 6 different times. However, for practical reasons, the volume of final tube was slightly less than the

others' (50ul), and incubated for 4hrs. The results of the ssDNA-production are shown in fig4.3. The samples in the wells were prepared by 5ul of the reaction from each tube together with 1ul loading dye. The results are shown in figure 4.3.

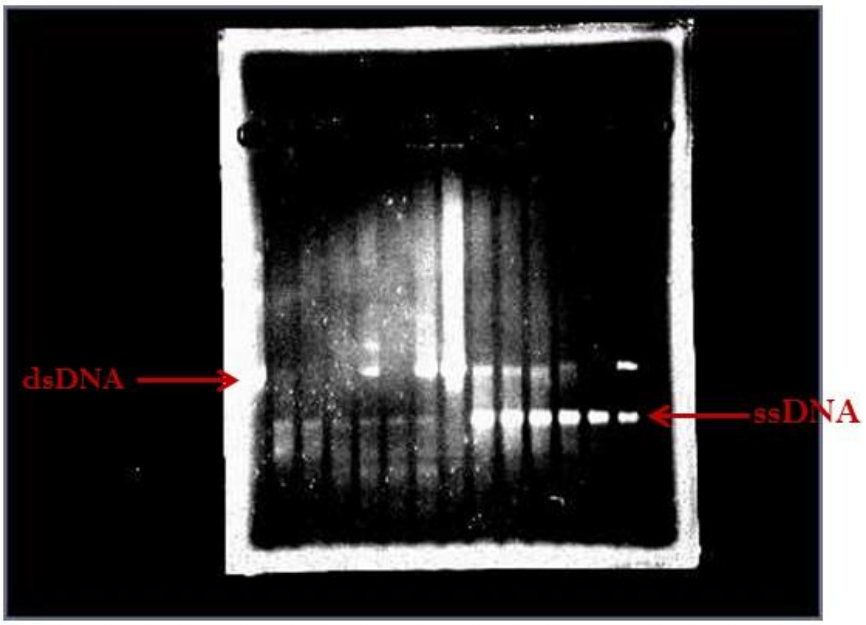

Fig 4.3: The results of the 5kb ssDNA production.

From line 1 to line 7 counting from the left side of gel shows the results of production of 5kb-ssDNA without biotin. Line 1 is the control dsDNA. Line 2 to line 7 is the results from the tubes incubated for 30mins, 45mins, 1hr, 1.5hrs, 2hrs and 4hrs respectively.

Theoretically, with time increasing, less and less dsDNA should remain. However, if the incubation time is too long, the ssDNA will also be digested by the lambda-exonuclease. In the fig4.2, it is shown that in line 2, the concentration of ssDNA is the highest. For the higher incubation times less and less DNA remains. Therefore, it was concluded that the best incubation time for production of ssDNA without biotin, was 30 minutes. The reaction conditions (enzyme concentration, amount of dsDNA and sample size) were also optimized in the same way as shown for the incubation time optimization (data not shown). The optimal conditions were found to be 1.5ul dsDNA (600nM) in 50ul reaction volume with 20 units enzyme incubated for 30mins.

Attempts were made with higher concentrations of dsDNA and longer incubation time to produce samples with higher concentration of ssDNA. However, after multiple trials, the attempt failed.

In the right side of gel in figure 4.3, line 8 is the comparison group for 5kbp-dsDNA with biotin. Line 9 to line 14 is the results from the tubes from lamda-exonuclease

treatments of biotin-5kbp-DsDNA that was incubated for 30mins, 45mins, 1hr, 1.5hrs, 2hrs and 4hrs respectively.

As mentioned in chapter III, the concentration of the dsDNA used in this reaction is the double compared to the above reaction with 5kbp without biotin. Because the 5' end of the dsDNA is protected by biotin it is easy to control the production of ssDNA with biotin, as ssDNA will not be degraded. With increasing time, the amount of dsDNA is decreasing and ssDNA is increacing. From the fig4.3, in line 13, the dsDNA with biotin disappear totally. Hence, it is concluded that the optimal condition for ssDNA with biotin is that 3ul dsDNA (600nM) with biotin in 50ul reaction volume with 20units enzyme incubated for 2hrs.

A hypothesis is provided that for the case of dsDNA without biotin, it will be also an appropriate condition that 3ul dsDNA (600nM) in 50ul reaction volume with 20units enzyme incubated for 2hrs. However, through my trials, it is supposed that this condition need to be more strictly controlled in repeated experiments to testify.

It can be found that there are aberrances in line 7 and line 14 for 4hrs incubation time. The dsDNA both without and with biotin remain in high amounts. That is caused by the reason that the sample size of the final tube is less than the others'. It was again found that the reaction efficiency is greatly related to the reaction volume.

According to the above conclusion, using the condition that 1.5ul dsDNA react in 50ul reaction volume including 20units enzyme with incubation for 30mins, the ssDNA could be produced efficiently. The sample was prepared and separated into 7 tubes. Every tube was verified by agarose gel electrophoresis after the reaction procedure and the results are shown in fig 4.4. Line 2 and 14 are the comparison group of undigested dsDNA. Line 3 to line 9, show the results from the 7 tubes. Line 9 is the final tube, which contains less sample size. Line 10 and 11 shows a reference sample with 5kb-ssDNA, line 12 and 13 shows 5kb-ssDNA with biotin.

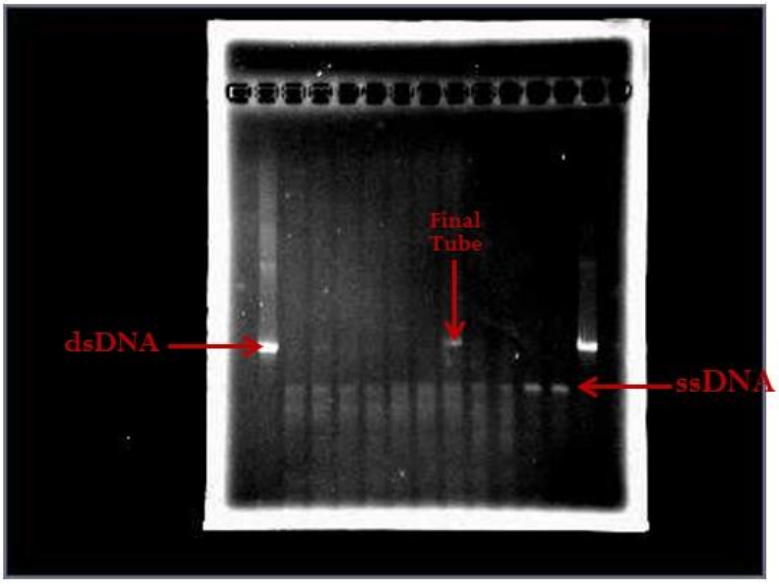

Fig 4.4: The results of the production of the 5kb-ssDNA.

All ssDNA left from the lambda-exonuclease treatment were collected and dialyzed in 10mM Tris-HCl at pH 7.8 @ 25°C over night to change the chemical environment. The sample volume was increased after dialysis because of the difference between concentration of ions in interior reaction buffer and exterior. Therefore, the final concentration of ssDNA was decreased and estimated to 5nM.

After dialysis in 10mM Tris-HCl at pH 7.8 @ 25°C, the 5kb-ssDNA samples were phosphorylated and dialyzed again in TE buffer. After phosphorylation the 5kb-ssDNA was ligated to the lambda-DNA according to the protocol and experimental procedure described in chapter II and III.

4.3 Single molecule experiments; ssDNA and RPA-GFP interaction

The three different DNA constructs; 12nt, 128nt and 5kb-ssDNA ligated to lambda-DNA were tested in single molecule experiments where they were exposed to RPA-GFP. In the two cases of 12nt and 128nt ssDNA overhangs, the results from microscopy did not perform as well as expected. The result of the biotinylated lambda-DNA with a12nt overhang is shown in fig4.5. The DNA did bind RPA-GFP but the were not selectively bound to the short ssDNA.

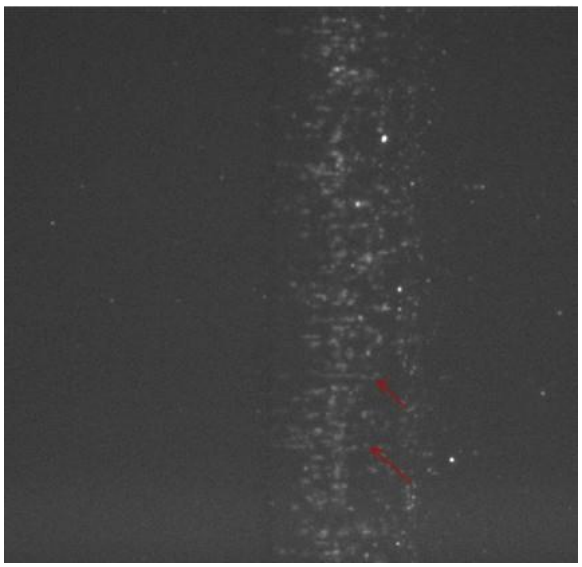

Fig 4.5: The interaction between DNA-12nt and RPA.

The same results were obtained in the lambda-DNA-140nt case as well. The result is shown in fig4.6. The biotinylated lambda-DNA was ligated with a 140nt short ssDNA fragment. The new DNA molecule should have a 128nt ssDNA overhang, which would bind RPA and the biotinylated end should be tethered to lipid surface. From the picture, it can be seen that the visible part (indicating bound RPA-GFP) in the molecule is uncontinuous but definitely longer than 128nt. It is concluded even in this case that the 128 nt ssDNA overhang is too short for selective binding of the RPA. The binding of the DNA to the lipid surface can be confirmed by RPA, but the binding of RPA cannot distinguish between the dsDNA part and the ssDNA. This situation could be improved by using DNA with longer ssDNA overhangs or decreasing the RPA amount in the system. We also cannot exclude the situation that the DNA would be damaged during the experiment operation and procedure and then RPA will bind to the damaged parts of the DNA.

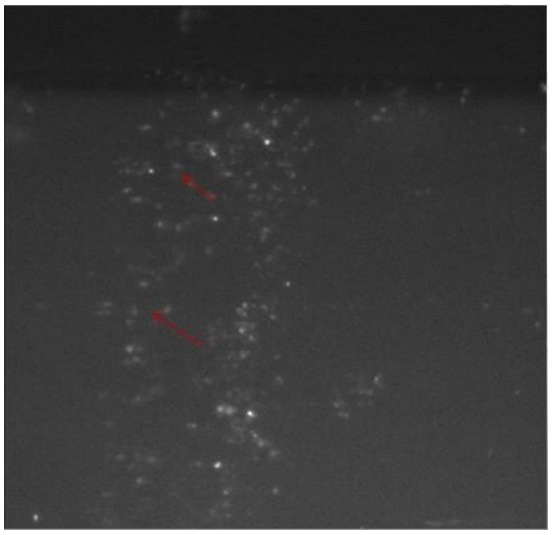

Fig 4.6: The interaction between DNA-140nt and RPA

The 5kb-ssDNa ligated to lambda-DNA was then introduced into the microfluidic flowcell and exposed to RPA-GFP. The results are shown in fig4.6. The two situations using flow and no-flow is illustrated in the figure. It is clearly shown that RPA was bound in high amounts on the ends of the DNA. The flow conditions compared to the no-flow conditions show that the bright spot where the RPA is bound to the end of the DNA move around 12 μm, which is the approximate length of the DNA at this flowrate. Not all Dna molecule showed this behavior. There were RPA-spots bound to the surface. The reason for this behavior could be either that the ssDNA bound to the dsDNA is atteched to the lipid bilayer or that unbound ssDNA is inserted or bound into the lipid bilayer. To improve this behavior two things can be performed; a dialysis step after the ligation can be introduced and the RPA can be bound to the ssDNA prior to introducing the DNA to the lipid bilayer. This would probably reduce the boun RPA-GFP spots on the surface.

It should be mentioned that the 5kb-ssDNA with biotin that was produced has not yet been tested in the single molecule experimental setup.

It can be concluded that the 5kb-ssDNa can be used for mimicking the DNA structure in HR to repair DSBs, which provides suitable DNA substrates for the future research of the interaction between DNA and protein Rad51 in single molecule experiments.

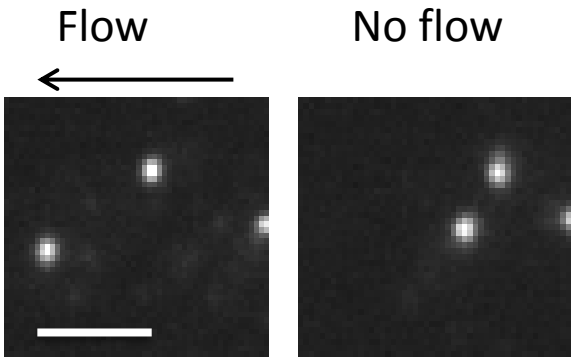

Fig 4.7: Pictures from fluorescence microscopy is shown. The lambda-DNA with the 5kb-ssDNA and its binding to RPA-GFP is shown. Scale bar is 10 μm.

Chapter V Conclusion and Future Work

In this project, the dsDNA of 5kbp was successfully amplified by PCR using suitable primers. After optimization of the lambda-exonuclease treatment, long ssDNA pieces with and without biotin were produced, and the appropriate reaction conditions were found. For future work longer dsDNA pieces by PCR, such as 10kbp or 15kbp, even 20kbp could be produced. Through optimization of the relationship between the concentration of reagents and the whole reaction volume in solution reaction, ssDNA can be produced in higher concentrations using lambda-exonuclease.

The DNA structures, lambda-DNA with ssDNA overhang at different lengths, were engineered, and confirmed by binding to RPA in microfluidic devices, using fluorescence microscopy. From the results of the microscopy, it can be concluded that the desired DNA structures were attached to the lipid surface. However, in the case of the short ssDNA overhangs, it was hard to distinguish between the ssDNA parts and the dsDNA part by RPA binding, since the RPA bind to certain sites on dsDNA as well. When using the long ssDNA overhangs, the difference between the two DNA structures is clearly shown. The conclusion is that the 5kb ssDNA bound to lambda-DNA can be used as a DNA-repair mimicking substrate for future research.

Chapter VI Acknowledgments

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Appendix

1X Lambda Exonuclease Reaction Buffer:
67mM Glycine-KOH
2.5mM $MgCl_2$
50ug/ml BSA
pH9.4 @25°C

1X T4 Polynucleotide Kinase Reaction Buffer:
70mM Tris-HCl
10mM $MgCl_2$
5mM Dithiothreitol
pH7.6 @ 25°C

1X DNA Ligase Buffer:
30mM Tris-HCl
10mM $MgCl_2$
10mM DTT
10mM ATP
pH7.8 @ 25°C