Universität Bielefeld

Faculty of Physics
Experimental Biophysics

Testing the Cutting-Functionality of Cobalt-, Copper- and Nickel-Complexes with Magnetic Tweezers

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Master Thesis

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Erklärung

Hiermit versichere ich, Ying Wang, dass ich die vorliegende Arbeit eigenständig verfasst und nur die angegebenen Hilfsmittel und Quellen benutzt habe.

28.02.2013, Bielefeld
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Gutachter:
Prof. Dr. Dario Anselmetti
Dr. Andy Sischka

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Contents

1 Introduction

Genes are the DNA segments carrying the genetic information. All this genetic information is saved in the form of a DNA. DNA with double helix structure are macromolecules, consisting of two polymers of some nucleotides. They are made of four nucleobases which are connected by phosphodiester bonds. The bonds are very stable to ensure the saving of genetic informations.

Three new metal complexes, which are supposed to be able to break these phosphodiester bonds, have been synthesized in 2011 by Thomas Jany, from faculty of chemistry Bielefeld Universitity. They are cobalt-, nickel- and copper-complexes.

This thesis investigates the cutting functionality of these three metal complexes. With this functionality the metal complexes might be bound with molecules recognizing a target sequence, leading to a cut of the DNA at a prescribed sequence. This concept would be applied e.g. on cancer cells. These cells could then be inactivated by reducing or stopping the replication of DNA, which needs an unbroken structure. It could also be a possible cure method for many genetic diseases.

In the past years many tools have been developed to manipulate single DNA molecules and to investigate their mechanical properties. By most of those tools, one end of the DNA molecule is attached and held to a surface and the other end is pulled by a force sensor (AFMs, cantilever) or a bead (optical tweezers or magnetic tweezers). Forces are put directly on DNA (AFM), by a laser-trapped bead (optical tweezers) or by a magnetically trapped bead (magnetic

tweezers). The measurement results provide mechanical information such as the relation between external forces and DNA extensions.

Since magnetic tweezers perform well by stretching and coiling a single DNA molecule, they have become a widely used single-molecule research technique. Magnetic tweezers have produced a large amount of information on the elastic properties of DNA (e.g. coiling) and provided new insights into the dynamic activity of DNA-processing enzymes (e.g. topoisomerase). In recent years, many technological developments and advances have been introduced into magnetic tweezers, which will enable more and previously inaccessible information to be extracted from biophysical systems.[1]

Compared to other methods, magnetic tweezers do not have a long history, but as a modern method they have their special advantages. To study DNA, the most frequently used instruments previously are optical tweezers and AFMs. But these two instruments can only stretch the DNA molecules, not twist them, unlike magnetic tweezers can. Moreover, one laser focus of an optical tweezers can manipulate only one bead, which corresponds to only one single molecule. Magnetic tweezers can trap and take the measurements of several beads at once. Due to the low trap stiffness, magnetic tweezers cannot apply a large range of forces. Instead, they have a force resolution of typically 10fN. Magnetic tweezers manipulate DNA molecules per generating a magnetic field, which creates external forces to move and rotate magnetic beads that bind on one end of DNA molecules.

This work uses the magnetic tweezers as the main experiment method. The principal aim of the experiments is to test whether those three new metal complexes can cut the DNA strand. If the DNA has a break on the strand, it cannot be twisted anymore. Thus the cuts can be detected by taking rotation curves. Besides, the process of releasement of DNA supercoiling with topoisomerase has also been experimentalized.

2 Background

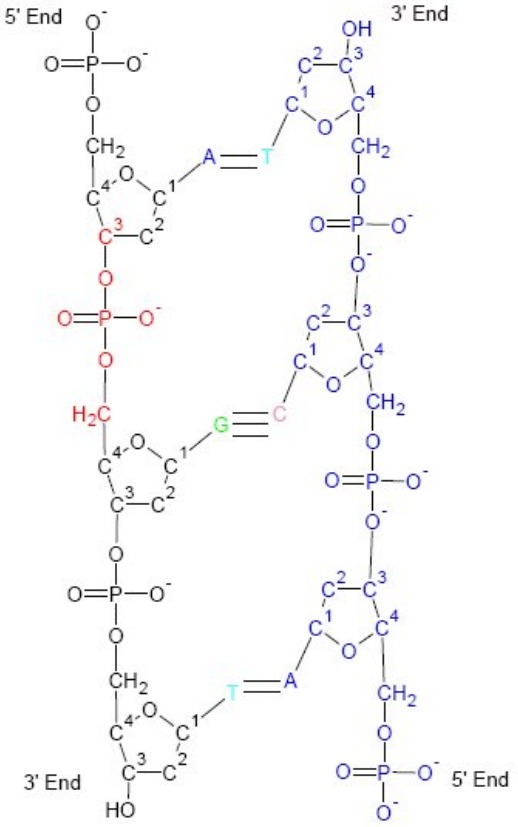


Figure 2.1: The schematic chemical structure of DNA strand.

In this chapter we will give necessary information on the background of our experiments. We start with general information on structure and function of DNA, then we focus on the new metal complexes. We close with an introduction to function and calibration of the magnetic tweezers.

2.1 DNA

Deoxyribonucleic acid (DNA) is composed by two long polymers, called polynucleotides. Each of these strands carries the same information, encoded in a sequence made from four nucleobases, adenine(A), thymine(T), guanine(G) and cytosine(C). Each base of one strand binds to a complementary strand, (A with T and C with G), so the sequence of bases corresponds in both strands. The strands have a backbone consisting of phosphate groups and sugar molecules. To each sugar molecule one nucleobase is bound. When two nucleobases bind together, they are named a base pair. Base pair (bp) is also a unit length of DNA molecules. The number of

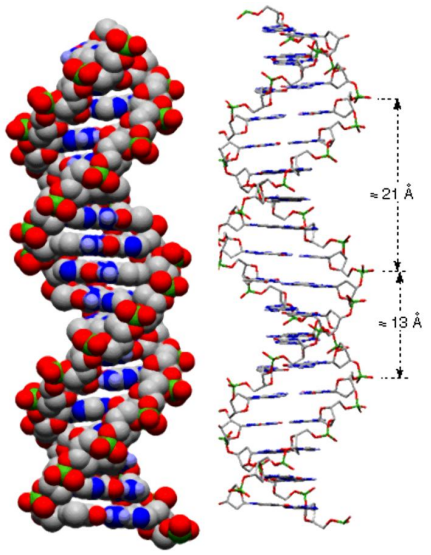


Figure 2.2: DNA structure, double helix

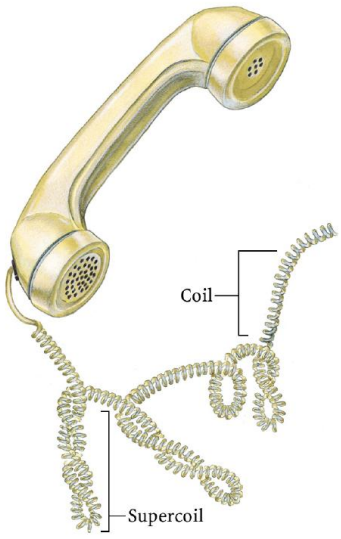


Figure 2.3: Supercoiling like a tangled telephone cord

base pairs determines the length of DNA molecules. For example, a λ-DNA molecule has 48502 base pairs, in other words it is 48502 bp long.

The structure of the DNA is a double helix which is formed by the two strands running in opposite directions to each other. These two long strands wind with each other, thereby DNA has a twistable structure, just like a telephone cable (Fig.2.2). With this structure DNA can be twisted into two directions, clockwise and counter-clockwise, which will be from now on called "in right" and "in left". The natural direction of the DNA twisting in the counter-clockwise direction.

§ Replication and Transcription

As the basis of biological inheritance, DNA is needed to be copied in all living cells. This process is called replication. To preserve the original information through many cell divisions, the genetic information must be copied without any errors. To achieve such replication, each strand of the double helix acts as a template to synthesize a new complementary DNA strand. Because strand synthesis goes al-

ways in the so-called 5'-to-3' direction, DNA replication has a special mechanism to adjust the strand running in the opposite direction. After the the strands have started to separate (by an enzyme), a complementary strand is built by the enzyme DNA polymerase onto the strands. Since the sequence of bases on the original strand determines uniquely the sequence of bases on the new strand, this process results in a perfect copy of the DNA. Finally, the two strands of the new double helix enwind around each other.

The information stored in the DNA is only usable by transcribing it into functional proteins. A DNA strand is used as a template for a complementary ribonucleic acid (RNA) strand (U (uracil) instead of T). A protein is synthesized using an mRNA (messenger RNA) template under the help of other types of RNA. This process is termed translation. In such way, genetic information in cells flows from DNA to functional proteins, which is called gene expression. (By viruses, all these processes happen within RNA.)

§ Supercoiling and Topoisomerase

The over- or underwinding of the DNA is called supercoiling. Since DNA has a length which can be thousands of times than that of its cell, supercoiling allows to pack the genetic material into the cell. "The overwound or underwound double helix can assume exotic forms known as plectonemes (like the braided structures of a tangled telephone cord, Fig.2.3) or solenoids (similar to the winding of a magnetic coil)"[2] or a combination of both. Positive helical twists lead to positive supercoiling, while substractive twistings cause negative supercoiling. DNA of most organisms is negatively supercoiled. These tertiary structures have an important effect on the molecules's secondary structure and eventually its function. For instance, both replication and transcription need an unwinded DNA. Since supercoiling may hinder or favour the capacity of the double helix to unwind, it thereby affects the interactions between DNA and other molecules.

In order to facilitate the DNA-replication, transcription and reparation, the supercoiling topology should be changed or released. Thereby topoisomerases can help. As we use type IB of topoisomerase in the experiments, we will focus on this certain type, often abbreviated as TopIB.

Topoisomerase IB forms a protein clamp around the DNA helix and creates a temporary break that permits the removal of supercoils. First, TopIB binds noncovalently on a supercoiled DNA molecule.[2] It then establishes a covalent bond to the DNA and makes a break on a strand of the DNA. The broken strand can spin about the intact one (green arrow), so that the supercoils are released. Then the DNA is religated by the topoisomerase, which terminates the releasement (Fig.2.5). With the removal of the supercoils, the DNA molecule appears longer on a microscope. By taking a curve of the DNA extension in units of time, each activity of removal shows on the curve a step (Fig.2.7[2]).

Topoisomerase does not release all the supercoils at once, but typically in multiple steps. A few years ago, Daniel A. Koster and Vicent Croquette et al. have made several experiments to study how topoisomerase removes the supercoils on DNA strands. They have found, that the number of supercoils removed per step follows an exponential distribution (Fig.2.8).[2] Fig.2.4 shows the schematic explanation of the linking number. The linking number Lk is the key topological property of a circular DNA molecule.[4] Fig.2.8 shows the distribution of ΔLk. Moreover, if the stretching force is increased, the probability of religation per turn decreases.

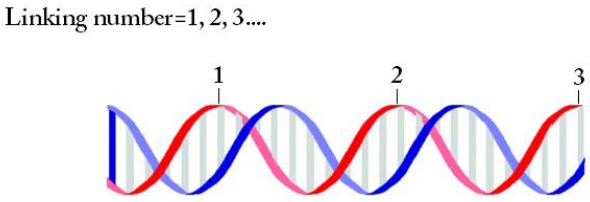


Figure 2.4: Schematic explanation of linking number Lk

Under a larger force, DNA rotates faster, thereby it is more difficult for TopIB to religate the DNA. As Fig.2.6 shows, the larger the force is, the longer the DNA becomes. Briefly speaking, the larger the force is, the more DNA rotates itself and the more supercoils will be released.

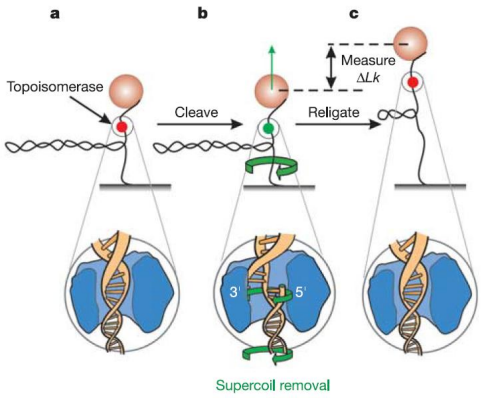


Figure 2.5: Supercoil removal

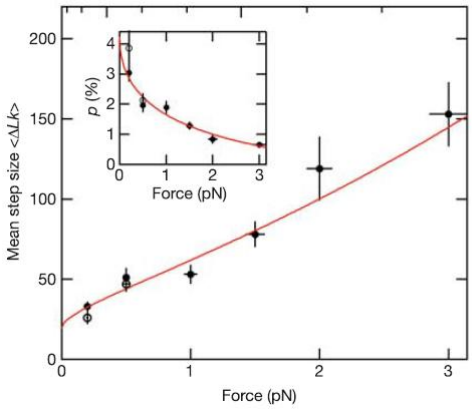


Figure 2.6: Mean step size $\langle \Delta Lk \rangle$ as a function of applied force[2]

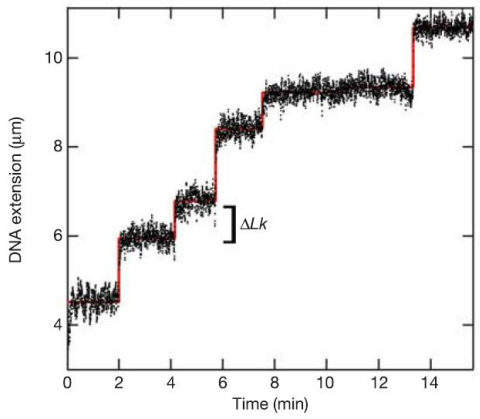


Figure 2.7: Each time TopIB removes supercoils from the DNA, a step is observed in the DNA extension.[2] The change of the DNA extension is the step size ΔLk.

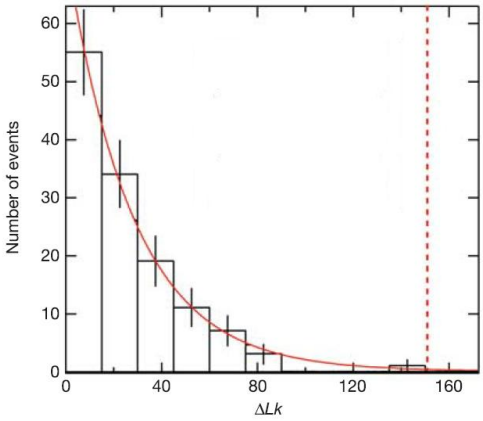


Figure 2.8: Distribution $P(\Delta Lk)$for TopIB in units of ΔLk [2]

2.2 The Worm-Like-Chain Model

Many different biological molecules have been analyzed (e.g. analysis of force extension curves) by using theoretical models. Bustamante et al. have shown that the force extension diagram of a DNA molecule is well described by a worm-like chain (WLC) model.[5]

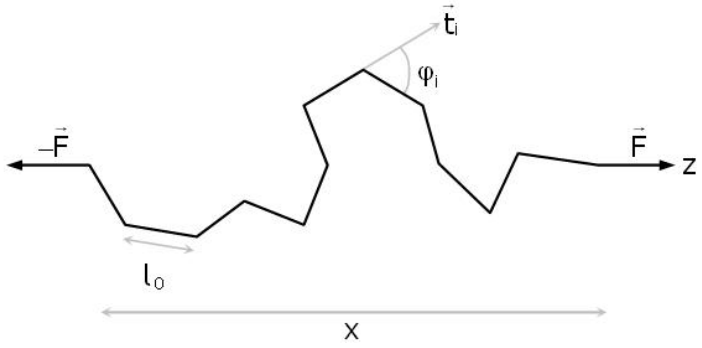


Figure 2.9: As the Kratky-Porod model, DNA can be considered as a chain which consists of N freely rotatable segments with a length of l_0. With the external forces $\vec{F}$ and $-\vec{F}$ in z direction, each segment has an orientation factor $\vec{t_i}$, that results in an angle φ_i from the neighbouring segment. x is the end-to-end distance which means a lot to us in the experiments.

In the WLC model, DNA is treated as if it was an idealized, macroscopic, circularly-symmetric beam element.[6] To introduce this model, we view the DNA as a chain consisting of N freely rotatable line segments, each with a length of l_0. The WLC model corresponds to the case of $l_0 \to 0$, $Nl_0 = L_0$, where L_0 is the contour length of the rod (the contour length of a λ-DNA molecule is about 16.4μm). At the ends of the chain there are external forces $\vec{F}$ and $-\vec{F}$ acting in the z direction. Each line segment has an orientation vector $\vec{t_i}$, yielding an angle φ_i between the two segments. The distance between the two endpoints of the chain is denoted by x, modeling the measurable length of the DNA. It can be shown that the correlations between different orientation vectors decrease exponentially,

$$\langle \vec{t_i}, \vec{t_j} \rangle = e^{-|j-i|l_0/L_p}.$$

The constant $L_p > 0$ is called persistence length and depends on the angle distribution. It is a measure for the stiffness of the chain. If small angles are more likely than larger ones, the persistence length will be larger which requires more segments between segments i and j to have their orientation vectors decorrelated.

In the limit $l_0 \to 0$ one finds according to Marko and Siggia[7] the following relation between the external force F, persistence length L_p, contour length L_0 of the rod and end-to-end distance x:

$$F = \frac{k_\mathrm{B}T}{L_p}\left[\frac{1}{4(1-x/L_0)^2} - \frac{1}{4} + \frac{x}{L_0}\right], \tag{2.1}$$

where k_B is the Boltzmann constant and T is the temperature. This expression describes the solution quite well if either $x \to 0$ or $x \to L_0$. In between it may differ by as much as 10% for $x/L_0 \sim 0.5$.[7]

As a compensation one can substract the Marko-Siggia interpolation formula from the numerical solution using a seventh-order polynomial, which provides all the correction terms. Then the equation is written as[5]:

$$F = \frac{k_\mathrm{B}T}{L_p}\left[\frac{1}{4(1-x/L_0)^2} - \frac{1}{4} + \frac{x}{L_0} + \sum_{i=2}^{7}\alpha_i\left(\frac{x}{L_0}\right)^i\right]. \tag{2.2}$$

Using this interpolation in fitting the results leads to a small overvalue of L_p and the error is of the order 5%.[5]

The force-extension curves in this thesis and the calibration are fitted by the WLC equation and the extended WLC model, so that the contour length L_0 and the persistence length L_p can be confirmed. Since the stretching force lies most of time in a small range (0$\sim$2pN) and our DNA molecules are not long either ($\leq$10μm), the error will not be large enough to disturb the total experiment results. In fact, by using (2.1), it already fits the curves well.

2.3 Metal Complexes

Fig.2.10 displays the idea how the metal complexes are synthesized. There is a distance of 6.5Å between two phosphate groups. Two metal atoms are bound on the phosphate groups (green). In order to ensure that there are exactly 6.5Å between two metal atomes, a stiff backbone (blue) is bound to each two atoms. By making a sterical hindrance (red), the complex is prohibited to bind either with the nucleobases or with the neighbor metal complexes.

It is presumed that the bond between metal complexes and DNA is permanent and in addition creates a cooperative hydrolysis of phosphate esters. This process is called cutting (Fig.2.11). Note that cutting does not mean the DNA to be split into seperated polymers. However, since the permanent bond on the DNA and the cooperative hydrolysis of phosphate esters are assumed to prevent the particular DNA segment from beeing replicated or transcribed, the cutting disables the function of the DNA segment, which is the effect needed in medical and other possible applications.

The cutting functionality works theoretically according to the Lewis-acid-base-theory. The concept of Lewis-acid and -base was first proposed by Gilbert Newton Lewis in 1923. The Lewis-acid is defined as an acid substance “which can employ an electron ion pair from another molecule in completing the stable group of one of its own atoms”[9]. The International Union of Pure and Applied Chemistry (IUPAC) defines it as “a molecular entity (and the corresponding chemical species) that is an electron-pair acceptor and therefore able to react with a Lewis base to form a Lewis adduct, by sharing the electron pair furnished by the Lewis base” [10]. The positively charged metal ion plays the role of the Lewis-acid, it will bind onto the oxygen and can take away the electrons from the negatively charged phosphate backgrad so that the bond between phosphate and sugar can be destroyed. Therefore, theoretically, all three metal complexes should be able to break the DNA strands.

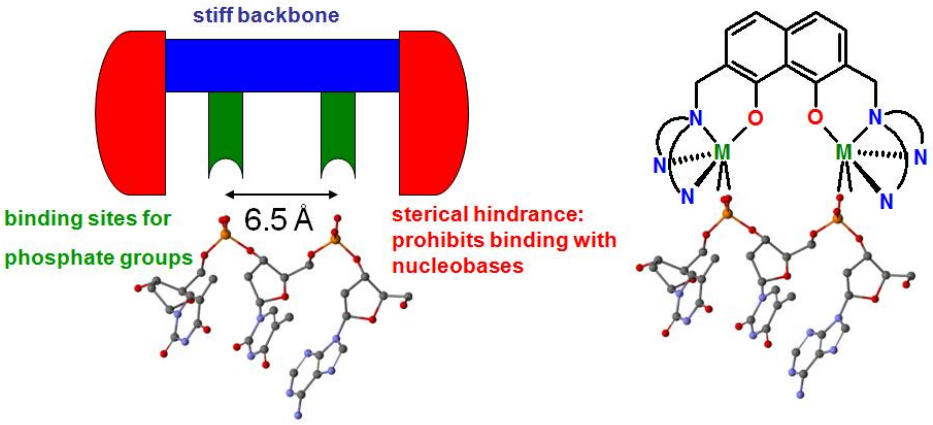


Figure 2.10: Idea of synthesizing metal complexes[8]

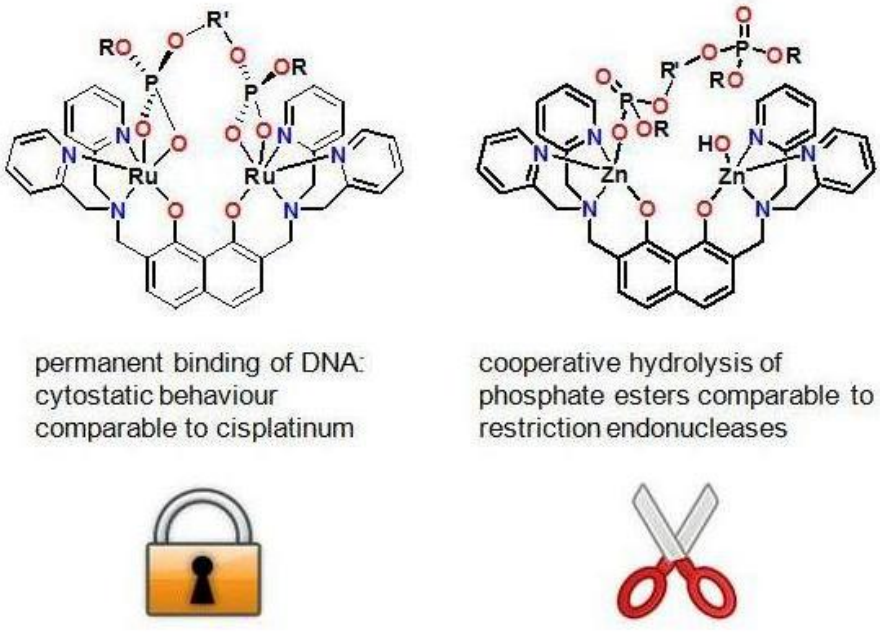


Figure 2.11: Metal complexes should bind on DNA permanently, and in addition create a cooperative hydrolysis of phosphate esters.[8]

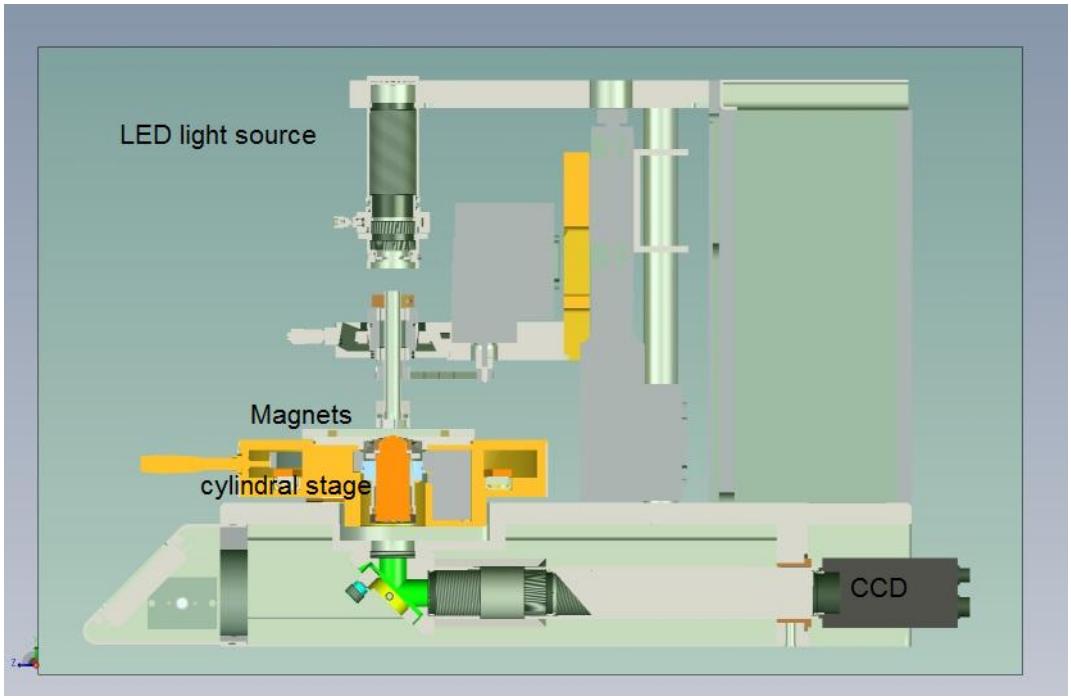


Figure 2.12: Setup of the magnetic tweezers[12]

2.4 Magnetic Tweezers

Fig.2.12 shows the setup of the magnetic tweezers. Magnetic tweezers are essentially microscopes. The LED is used as the light resource. The light goes through the magnets and enters the objective located in center of the cylindrical stage. "The light collected by the objective is reflected at 90° by a mirror and imaged by a lens onto a CCD video camera."[12] The camera is connected to a computer (Fig.2.13, Fig.2.14) with appropriate software where the image can be seen. The focusing of the microscope is achieved by using a piezo-electric stage. The cylindral stage contains the objective in its center and can control the temperature of the sample by using Peltier elements and a sensitive thermistance. This point is also very important for a biological experiment, as a constant settled temperature ensures the activity of the biological sample.

There are magnets at the bottom of a 10mm stainless steel tube (Fig.2.12). The magnets are controlled per a translation and rotation ensemble above the sample stage. These magnets generate a strong field gradient along the optical axis. The typical range of a magnetic influence is 2–3mm below their surface. That means, the sample should be put 2–3mm below the tube in order to get the best effect, which can be realized by swinging the motor ensemble (which determines location and height of the tube).

Since the magnetic tweezers are connected to the computer, control of magnets and temperatures can also be achieved using a software.

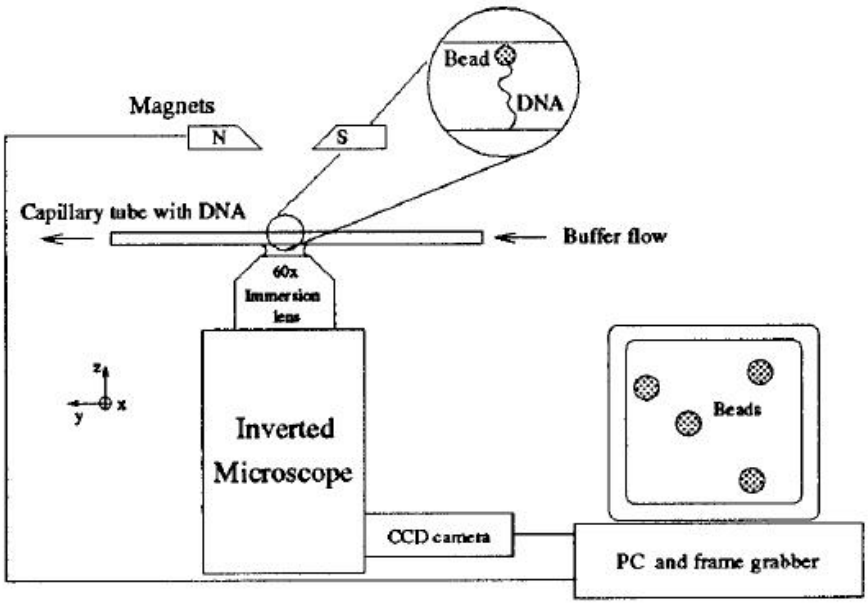


Figure 2.13: The magnetic tweezers are connected to a computer, with the help of software the beads are shown on the monitor.[11]

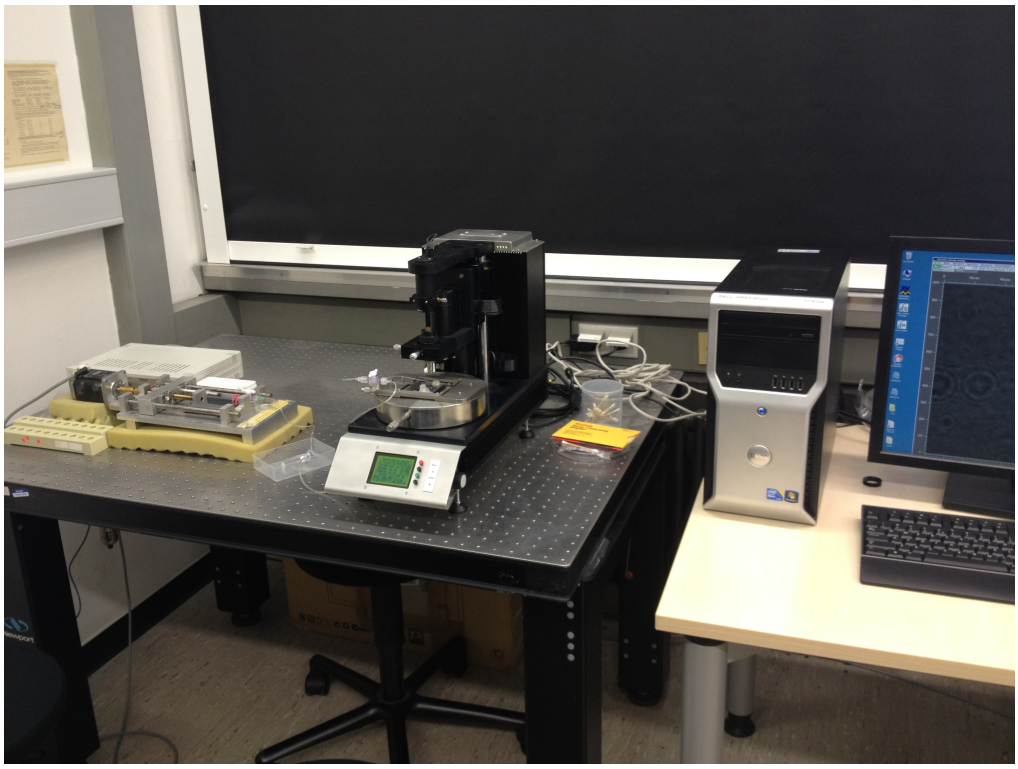


Figure 2.14: Magnetic tweezers connected to a PC and a pump

2.4.1 Working Principle and Calibration

As Fig.2.15(left) shows, the magnets sit above the sample and generate a magnet field. A DNA molecule hangs on a magnetic bead (see **3.2.1**). The bead can be manipulated via the magnet field to be pulled or twisted. Varying the height of the magnets changes the stretching force which works on the bead and pulls it high, whereas turning the magnets allows for rotation of the beads (Fig.2.16).

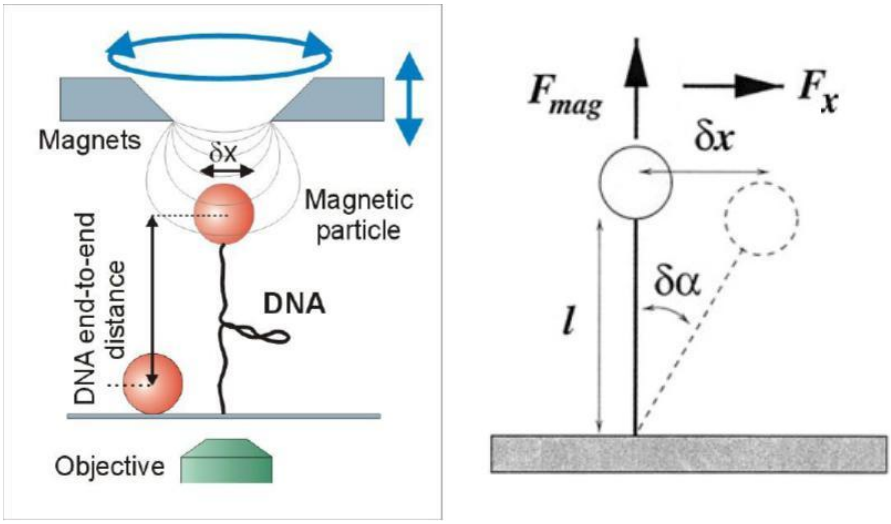


Figure 2.15: The magnets build a field and control the moving of the magnetic bead.

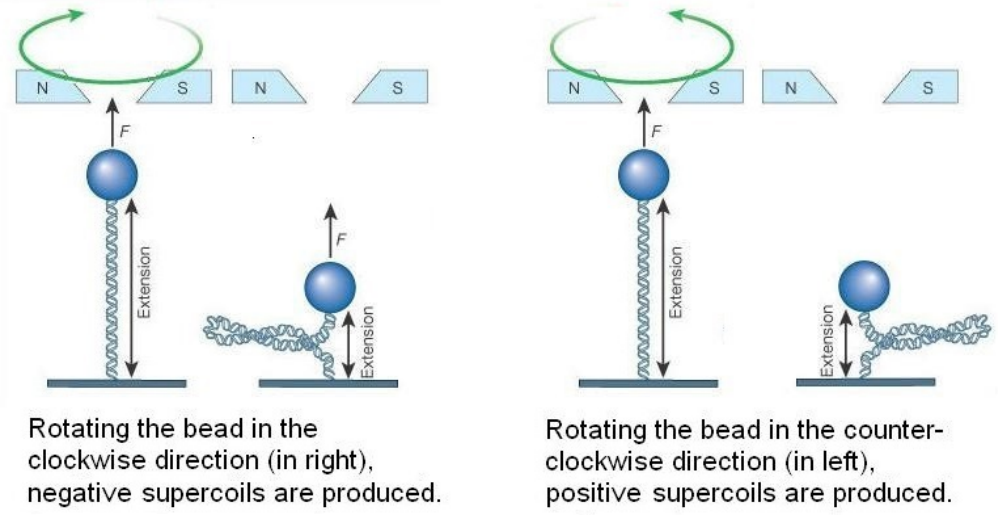


Figure 2.16: The bead can be rotated in both directions to produce positive or negative supercoils.

Since the DNA binds on the bead, the change of the bead-position expresses the extension of DNA, the end-to-end distance, which is set as l here. The particle transverse fluctuation is denoted as δx and the angle as $\delta\alpha$.

Since the beads and DNA molecules as well are surrounded by aqueous solution (buffer solution), they perform Brownian motion. The amount of Brownian motion presents the force F_x acting on the bead.

The general potential energy of such a system is

$$E_{\text{pot}} = -\int_0^{\delta x} F_x \cdot dx = \frac{1}{2} F_x \delta x,$$

$$\frac{F_{\text{mag}}}{F_x} = \frac{l}{\delta x}. \tag{2.3}$$

The average potential energy of this system $\langle E_{\text{pot}} \rangle$ is

$$\langle E_{\text{pot}} \rangle = \frac{1}{2} \cdot \frac{F_{\text{mag}}}{l} \left\langle \delta x^2 \right\rangle. \tag{2.4}$$

According to the equipartition theorem, the oscillator has average energy $\langle E \rangle$ in thermal equilibrium,

$$\langle E \rangle = \langle E_{\text{kin}} \rangle + \langle E_{\text{pot}} \rangle = \frac{1}{2} k_{\text{B}} T + \frac{1}{2} k_{\text{B}} T. \tag{2.5}$$

This means

$$\frac{1}{2} \cdot \frac{F_{\text{mag}}}{l} \left\langle \delta x^2 \right\rangle = \frac{1}{2} k_{\text{B}} T. \tag{2.6}$$

Summarizing, the relation between the stretching force F_{mag} and the bead's flucuations $\langle \delta x^2 \rangle$ can be written as

$$F_{\text{mag}} = \frac{k_{\text{B}} T l}{\langle \delta x^2 \rangle}, \tag{2.7}$$

where F_{mag} is the stretching force applied from the magnets, δx is the bead's transverse fluctuations, k_{B} is the Boltzmann constant, l is the DNA extension and T is the temperature.

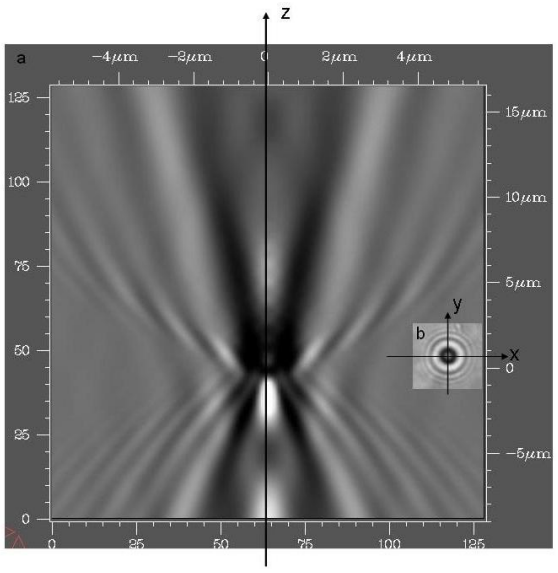


Figure 2.17: Image **a** shows a bead at all the focuses with a sidelooking observation. If the bead is observed from top, it looks as in image **b**.

With this principle, the external magnetic force F_{mag}, acting on the bead, can be calculated by measuring the DNA extension l and the transverse fluctuations δx. The magnetic beads can be seen under the microscope (Fig.2.17). The fluctuations $\langle \delta x^2 \rangle$ can be measured by the software. For the determination of l, a set of calibration images of the bead at different (predefined) focuses has to be taken first. To record these images, an external magnetic force is put on the bead by approaching the magnets to the surface. With a stretching force of 3∼4pN, the DNA molecule attached to a movable bead is pulled straight without the strand structure being broken. This is done in order to mimimize vertical fluctuations which might disturb the measurements. The calibration routine determines the height of the focus, say f_t, at which the bead is sharp. Then the force is decreased to allow the bead to sink to the surface. Now, f_b is determined, the height of the focus at which the bead is sharp now. Using f_t and f_b, the length l can be computed according to the refraction equation

$$\frac{n_{\mathrm{water}}}{n_{\mathrm{oil}}} = \frac{l}{f_{\mathrm{t}} - f_{\mathrm{b}}}, \tag{2.8}$$

where $n_{\mathrm{water}} = 1.33$ is the refractive index of water and $n_{\mathrm{oil}} = 1.52$ is that of the oil which is between the objective and the flow cell.

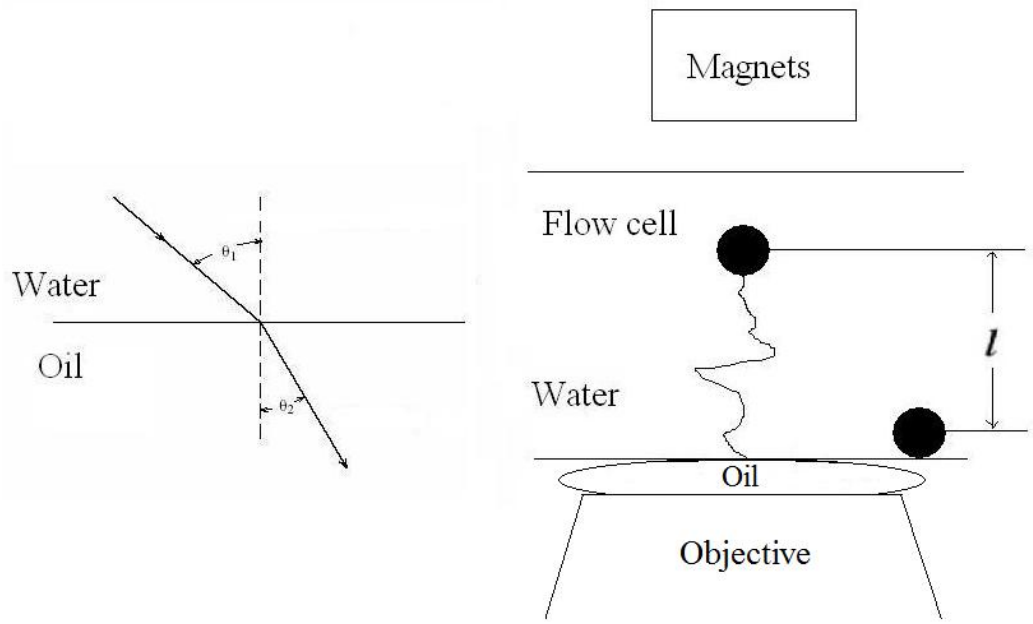


Figure 2.18: According to the refraction equation, it can be estimated how long the DNA molecule is stretched.

In an actual experiment, the software compares the images of the bead in real time with the reference images (stored in calibration). Fig.2.19 shows that as the focus is increased from underfocus to overfocus, the diffraction rings (the circles) of the bead look different, depending on the focal positions. Using the reference images, the distance of the bead to the focus can be determined and using (2.8), l can be identified. The calibration process is very helpful to keep the length of the DNA molecule l at a stable value in the experiments. More details about the calibration are given in Section 4.1.

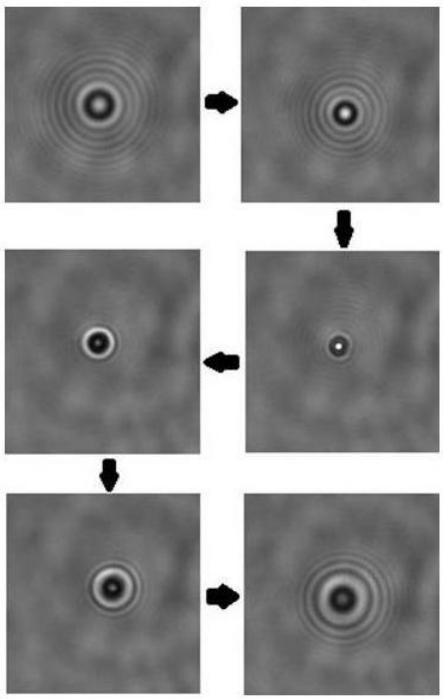


Figure 2.19: The change of the x coordinate of the circle center is δx. The focus increases in array direction (from underfocus to overfocus).

3 Preparation

3.1 Flow Cell and Surface Preparation

The sample cell was constructed to be as thin as possible so that the magnets could be brought as close as possible to the sample to apply the strongest pulling forces. A flow cell was built with a very thin glass coverslip (24∗60mm), having a thickness of 0.14mm.

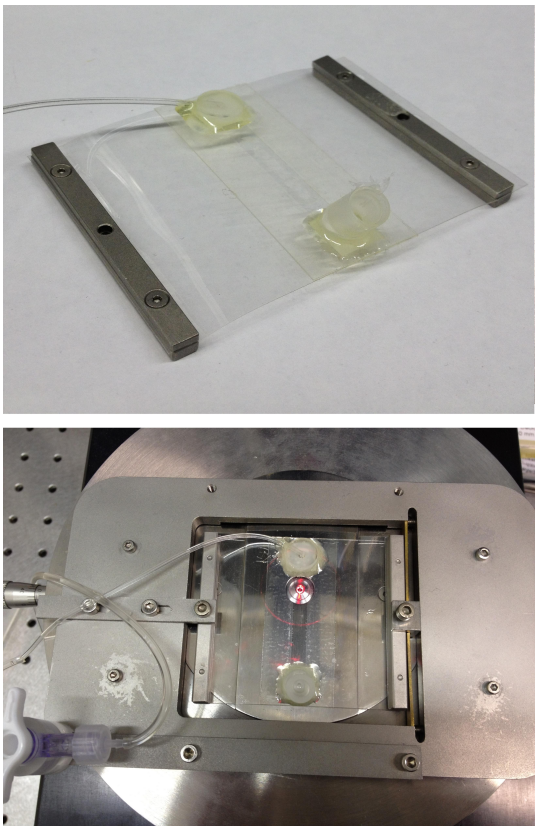


Figure 3.1: Sample holder

Since the glass is very thin and easily breakable, usually more than one glass coverslip has been prepared each time. At first, the glass coverslips have been washed with acetol and DI-water. To ensure that DNA molecules can bind well to anti-dig, the glass surface must be perfectly saturated with anti-dig. This was achieved by using sodium hydroxide. Sodium hydroxide is mildly corrosive to glass, therefore it can make the glass surface "unprotected" and let the sigmacote and anti-dig solution bind well to the glass surface. The glass coverlips have been layed completely in the 2M concentrated sodium hydroxide solution in a PE-Petri plate for several minutes as pretreatment. The longer the

glass coverlips stay in NaOH, the more likely it is that the coverslips become too soft and get small cracks.

Then coverslips have then been washed again and put on a hot plate for half an hour with a temperature of 150°C, in order to totally vaporize the remnant from the pretreatment (Acetol, NaOH or DI-water). Sigmacote (SIGMA) was then given on the surface. It is important that sigmacote can only be pipetted by a glass pipette. If sigmacote solution was given using a plastic pipette, the plastic would partly dissolve, resulting in ruining the entire cell. Standing the coverslip sidewards, the sigmacote was given from the top. As sigmacote flows downwards slowly, this ensures that not too much sigmacote stays in one spot. The coverslips have then been heated again with 150°C for half a hour.

After the vaporizing of the sigmacote's solvent, a cell has been built with the glass coverslip and the other parts together.

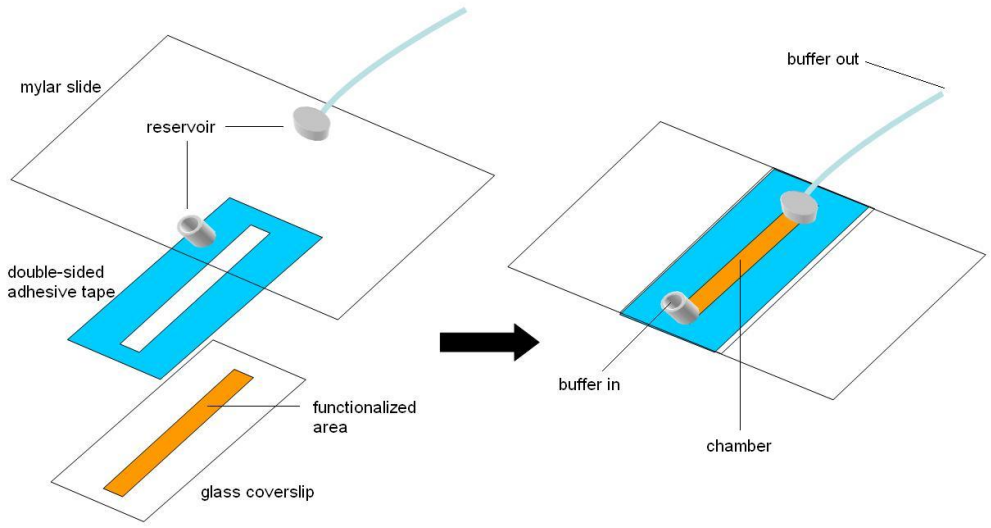


Figure 3.2: Schematical setup of the flow cell

The sample consists of a cell defined by a double sided scotch-tape, sandwiched between a microscope coverslip at the bottom and a stretched mylar sheet at the top (Fig.3.2, Fig.3.1 above). The mylar sheet had two small holes, where reservoirs have been glued on

them. The glass coverslip has been sticked on the mylar, building a chamber with the mylar sheet and reservoirs together. The sample solution has been put into one reservoir, while the other reservoir has been connected with a pump. The pump controls how fast the solution will constantly flow through the chamber.

The mylar sheet was extended on both sides of the coverslip and pinched by two stainless bars. A stainless steel frame has been used to stretch the mylar sheet and move the cell. The glass coverslip was pushed by the mylar sheet on the objective holder which was gently curved in a cylindrical shape. When the sample was moved, the coverslip slid on the objective holder. It is important for the mylar sheet to be under tension since it acts as a spring that pushes on the coverslip and maintains its contact with the objective holder, thus ensuring that the sample remains at a constant vertical position.

70μl Anti-Digoxigenin solution (from Roche, with a concentration of 200μg/ml dissolved in PBS buffer) has been given into the cell and flew through the cell. The solution has stayed in the cell for at least 2 hours at a room temperatur or 37°C. As, in case of success, one of the main applications of the metal complexes would happen inside living human cells, the 37°C seems reasonable. The surface could have certainly been longer incubated with Anti-Dig solution so that more Anti-Dig molecules might bind on it and therefore the DNA molecules might also better bind with them. If the flow cell was not required at once, it was storaged in a refrigerator between 2-8°C.

3.2 Sample Preparation

3.2.1 Bifunctional $\lambda-$DNA

λ-DNA comes from a virus called Phage-Lambda. It is a bacterial virus, or bacteriophage, that infects the bacterial species E-Coli. λ-DNA is 48502 bp long. Since 1 bp is 3.4Å long, it can be estimated that λ-DNA is about 16.4μm long. The λ-DNA used in the follow-

ing experiments has already been cut to a length of about 20000 bp. That means that the measured DNA is about 6-7μm long. As shorter DNA has less supercoils, we expect less errors in the results while winding the DNA molecules.

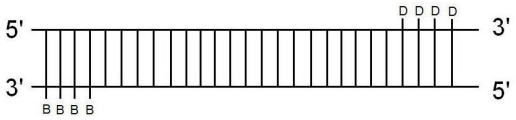


Figure 3.3: bifunctional λ-DNA, binding on one 3'-tailing with multi-biotin and the other with multi-digoxigenin

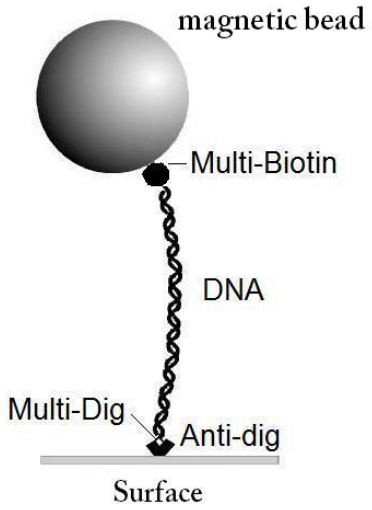


Figure 3.4: schema of DNA-bead-bond

DNA has on each side a 3'- and a 5'-tail. Multi biotin binds on the 3'-tail and multi digoxygenin on the other, which comprise the bifunctional λ-DNA[1]. Multi digoxygenin is supposed to have a strong interaction with anti-dig, which is already prepared on the surface. This ensures that one side of the DNA molecules will stably stick to the surface. Multi biotin will bind with a streptavidin coated bead, so that with the help of a magnet field to regulate the beads, the molecules can be stretched or twisted.

Since equipped with strong magnets, the magnetic tweezers can produce a horizontal magnetic field with a very steep vertical gradient.[12] The stretching force depends on the chosen bead. With a smaller radius of the bead, the noise in the experiment will also decrease.

[1]Preparation see Appendix.

Therefore the Dynabeads® MyOne™ Streptavidin C1 10mg/mL (Invitrogen™ by Life Technologies) with a very small diameter of 1μm have been chosen.

3.2.2 Sample Preparation

First, 5μl of magnetic beads have been washed several times in a tube with 200μl PBS buffer. After each washing the tube has been put on a magnet for about 1 minute and the supernatant of the PBS buffer has been discarded. The beads have then been resuspended in 5μl PBS buffer. 1μl the DNA solution have been dissolved in 200μl PBS buffer. A 1μl drop from the diluted DNA solution has then been deposited at the bottom of a small microfuge tube. 5μl of prepared beads have been pipetted onto the drop of DNA and the reaction has immediately been diluted with 90μl SB buffer. All the depositing in touch of DNA should be very gently and slowly, otherwise many breaks would be created on DNA strands. It takes about 30 minutes for DNA and beads to bind together, hence the tube has been spined softly several times to ensure that the beads did not either sink to the bottom or bind unspecifically with each other. The DNA coated beads have then been washed with SB buffer for 3-4 times. This helped not only the beads from adhering with each other but also helped preventing that more than one DNA molecule binds on a bead.

3.3 Buffers

- **PBS buffer(Invitrogen™ by Life Technologies)**, as basic buffer
- **PBS buffer (1mg/ml BSA)**
- **passivation buffer**
  - PBS
  - 0.2% BSA (SIGMA, $\geq$ 98% (agarose gel electrophoresis), $\leq$ 0.02% Fatty acids)
  - 0.1% Tween® 20 (SIGMA)
  - 5mM EDTA (SIGMA, $\geq$ 99%(titration))
  - 10mM Sodium Azide (MERCK, $\geq$ 99%)
- **SB buffer**
  - PBS buffer
  - 0.1mg/ml BSA (SIGMA, $\geq$ 98% (agarose gel electrophoresis), $\leq$ 0.02% Fatty acids)
  - 0.1% Tween® 20 (SIGMA)

4 Experiments

In this chapter we present settings and results of the conducted experiments. First giving an introduction into the calibration, we then demonstrate the abilities of the magnetic tweezers via stretching and rotating λ-DNA. After a brief investigation of the influence of topoisomerase on negatively supercoiled DNA, we turn to our main experiments on the cutting functionality of the metal complexes.

§ Preconditions for Experiments

The cell has been incubated with passivation buffer for at least one night. The sodium azide in passivation buffer can also prevent bacterial growth.

The next day the mixture solution has been given into one reservoir. Then 50$\sim$100μl SB buffer have been injected into the reservoir which had been connected to the capillary tube. After turning on the pump, 5$\sim$10μl of the mixture have been given slowly into the reservoir, directly into the hole. With the help of the pump, the mixure flew slowly with a constant speed into the cell. The beads could then be seen under the microscope. Then the pump was turned off and the magnetic beads were allowed to sediment. Finally, after incubation for 15 minutes, the majority of beads should be seen to move about on the surface and not appear immobile.

One of the main advantages of the magnetic tweezers is the ability to rotate the DNA molecules. In order to get good experimental results, some preconditions have to be satisfied. First of all, like in the experiments with AFM or optical tweezers, the sample should

consist of single molecules. Furthermore, the molecules should also have an unbroken double helix structure as if a small break occurs on a strand, the molecule can not be supercoiled anymore. Similarly, single strand DNA would be unusable either. However, damage to the DNA structure is unavoidable, since during the preparation more or less strand breaks occur. The only solution to this problem is to carefully deal with the sample, e.g. when DNA solution should be in touch, the pipetting should be as slow as possible and as gentle as possible.

As mentioned, multi biotin and digoxygenin are supposed to bind on the DNA sides. If this is not the case, the binding would be unstable and produce errors in the measurements. Some errors occuring from this event will be shown in the later discussion.

Besides, PBS buffer is used as washing buffer and SB buffer as flowing buffer. The buffers should not affect the interaction of metal complexes. In addition, right buffers can also prevent unspecific bonds, so that the beads will not stick to the surface. The BSA in the buffers is supposed to provide this hindering effect. However, BSA itself might hinder binding either from the cutting molecules on DNA strands or the cutting functionality itself. A possible solution to this problem will be shown later.

4.1 Calibration

Before the actual experiments, the magnetic force must be calibrated. The height of the magnets is denoted by Z_{mag} (the below surface of the mylar slide is considered as origin). Note that for conventional reasons Z_{mag} will be negative, hence the vertical distance of the magnets to the surface of the flow cell is $|Z_{\text{mag}}|$. Clearly the magnetic stretching force is determined by Z_{mag}.

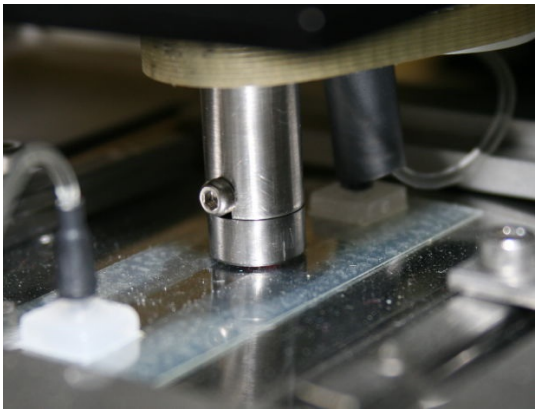


Figure 4.1: By placing the magnets closer to the sample, the stretching force would increase .[12]

As the curve from Fig.4.2 shows, the force decreases nearly exponentially in the distance between the bead and the magnets. If the magnets sit already very near above the sample, each tiny approaching creates a large force increasing. Hence without calibration, the error in the experiment could be unacceptable. Besides, when the magnets are placed already near above the sample, the approaching should be decreased too in order to not overstretch the DNA molecules.

The magnetic tweezers are preset to provide 50pN estimated force at exactly $Z_{\text{mag}} = -0.1$mm. Hence adjusting the magnets to Z_{mag}=-0.1 allows to calibrate a force of 50pN. It is also the largest force that the magnetic tweezers can precisely provide, which is limited from the presetting. It is defined, that the thickness of the sample is 0.1mm. In order not to break the sample, it is such set that the magnets can keep the maximum reachable position to -0.11mm, which can be understood that the magnets just have contact with the mylar slide.

In the calibration, the magnets are moved slowly near to the flow cell

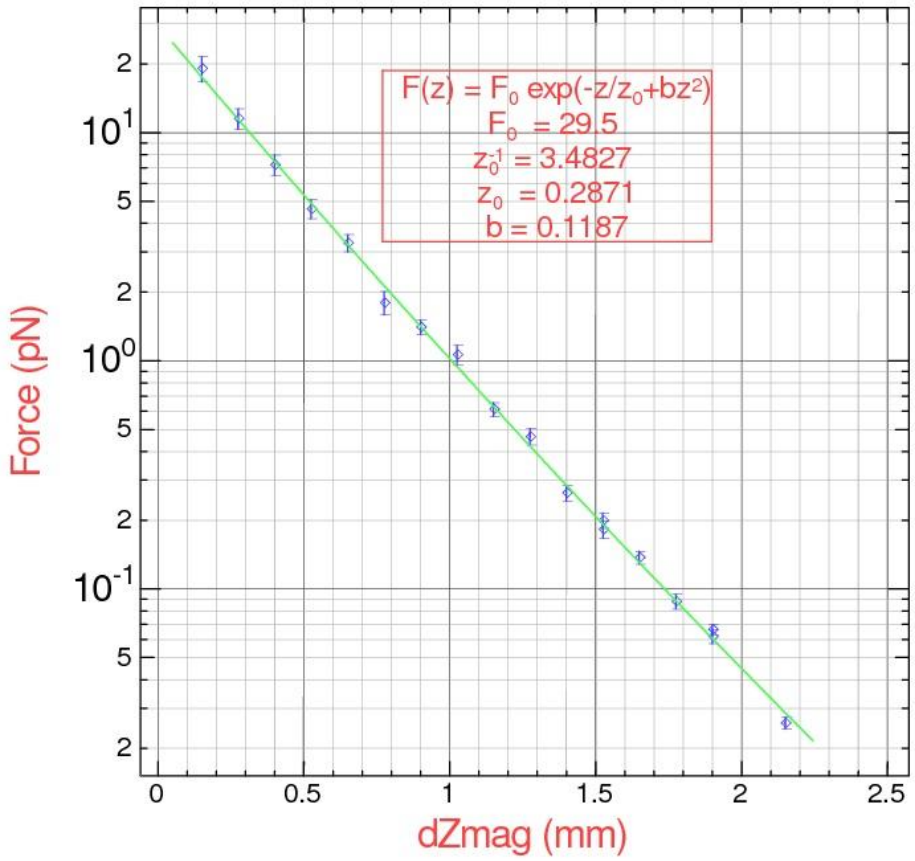


Figure 4.2: The force decreases nearly exponentially in the distance between the bead and the magnets.[12]

(Fig.4.1, Fig.4.3) while watching the computer monitor carefully. If the beads "jump", Z_{mag} is -0.1mm. The jump is due to the contacting of the magnets with the mylar slide.

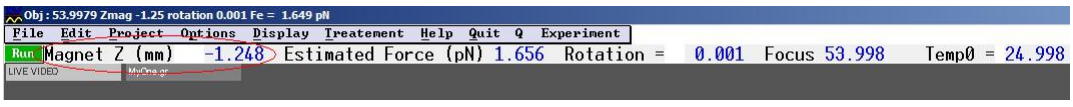


Figure 4.3: By running the magnets near to the surface, a external force will be put on the bead to stretch the DNA molecule long.

Fig.4.4 shows an example of one bead in the calibration process.

Normally several apparently moving beads can be seen once on the monitor. The cross marks the bead for the software. In the example

is $f_t - f_b = 61 - 57 = 4$. Here the result of l is obtained from (2.8) under the force 2.37pN as 3.5μm.

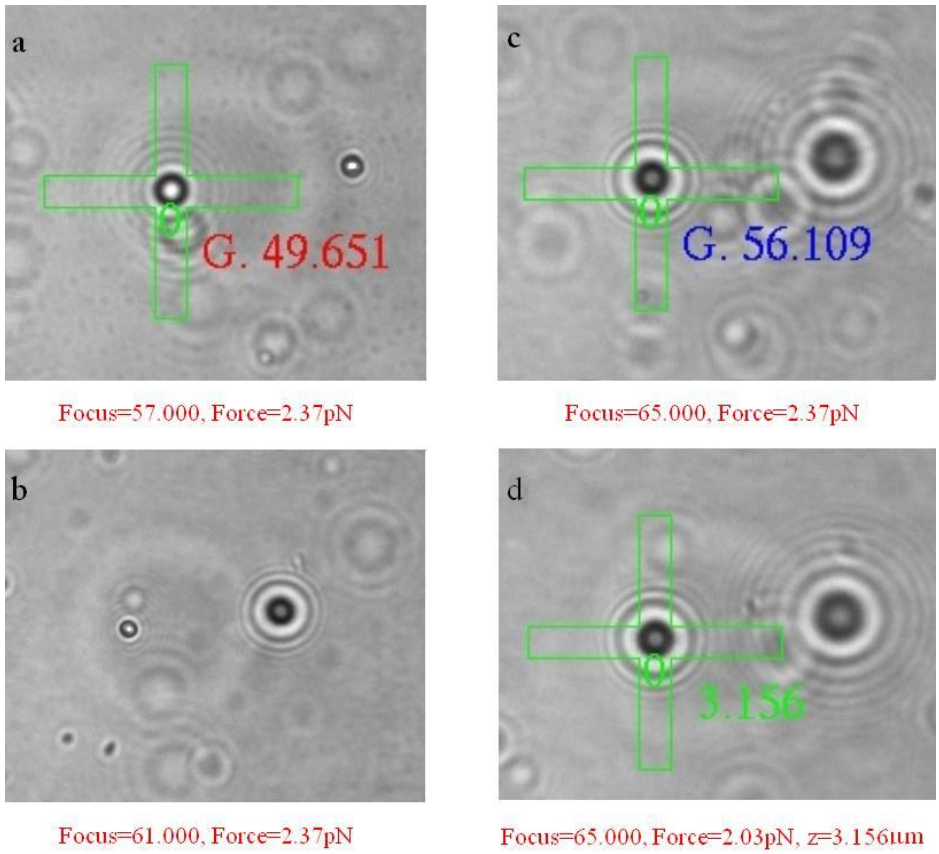


Figure 4.4: Some live photos of the calibration process: **(a)**.First, picking up the bead which is supposed to be observed; **(b)**. In the second step, changing the focus till the bead is sharp; **(c)**.The focus should be further heightened; **(d)**. With the help of the programm, the calibration will be run automatically.

Actually, when the bead is sharp, the cross can not grab the bead well. Therefore lifting the focus a little higher is required (Fig.4.4(c)). The length of the DNA molecule in the example is shown as 3.156μm under 2.03pN. It matches the estimated result quite well. If available, beads sticking to the surface should also been measured for calibration to account for systematic errors like drifts in the cell.

During the calibrations, many sets of images have been taken. As mentioned in Section 2.4.1, these images will be compared with the ones in the experiments to identify the DNA extension l, thereby

with (2.7), the stretching force can be calculated.

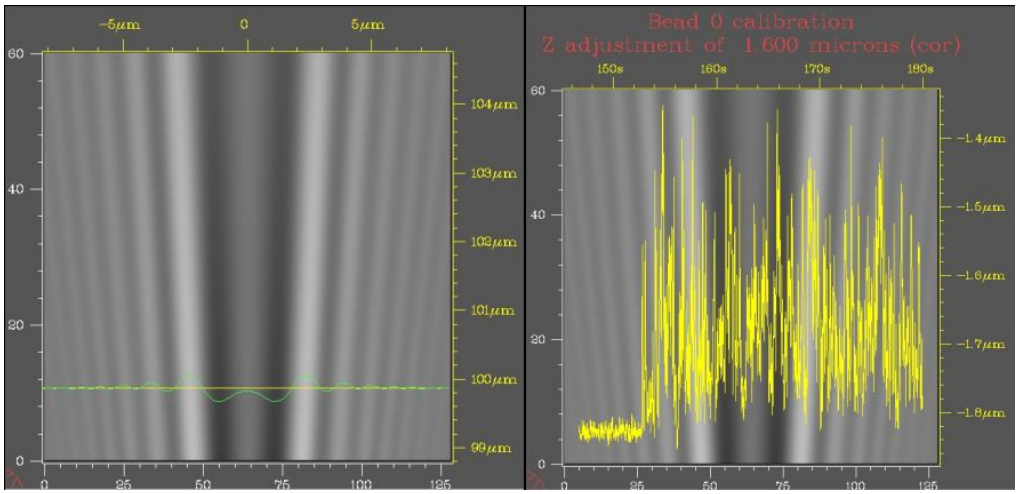


Figure 4.5: The distribution of the bead's position.

The yellow curve (Fig.4.5) shows the distribution of the bead's position. During the calibration, the bead with DNA oscillates all the time, the calibration program records each position in each frame. A diffraction curve is shown in green, describing the intensity distribution about the focus. With this method each bead can be calibrated and then the stretching and rotating experiments can begin.

4.2 Stretching and Rotating

As mentioned, magnetic tweezers can not only strech a DNA molecule but also rotate it. Fig.4.7 shows the process how the DNA molecule removes the supercoils during rotation. The curves are measured from right to left. Under a constant force, the DNA single molecule winds itself first almost together. (Fig.4.7a, III) In this situation many positive supercoils on the DNA strand have already been produced. Then the bead is rotated with a constant speed in clockwise direction.(Fig.4.6)

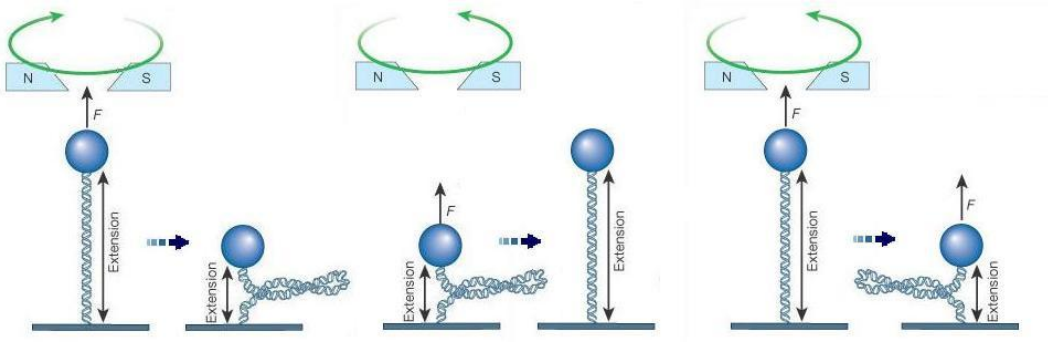


Figure 4.6: The bead is rotated in counter-clockwise direction(in left). The DNA molecule is twisted in left and winds itself almost together with positive supercoils. Then the bead is rotated in clockwise direction (in right), the positive supercoils are released and negative supercoils are produced. [33]

DNA molecules are topologically like a telephone cable. If the DNA is winded, the supercoils are removed and the distance between bead and DNA (the extension of DNA) becomes larger. When all the positive supercoils are removed by rotation, the DNA molecule is longest, having its natural double helix form (Fig.4.7a, II). At position I there seem to exist two different statuses. If the molecule is rotated further in right with a low force, negative supercoils are produced (Fig.4.7a, I). Under a higher force, e.g. 1.0 or 1.2pN, the DNA strands seem not to be winded any more. The reason is the formation of the sugar-phosphate-backbone. The double helix structure is damaged during the further rotation and no negative supercoils are made. Additionally, by loosing the helix structure, no obvious change

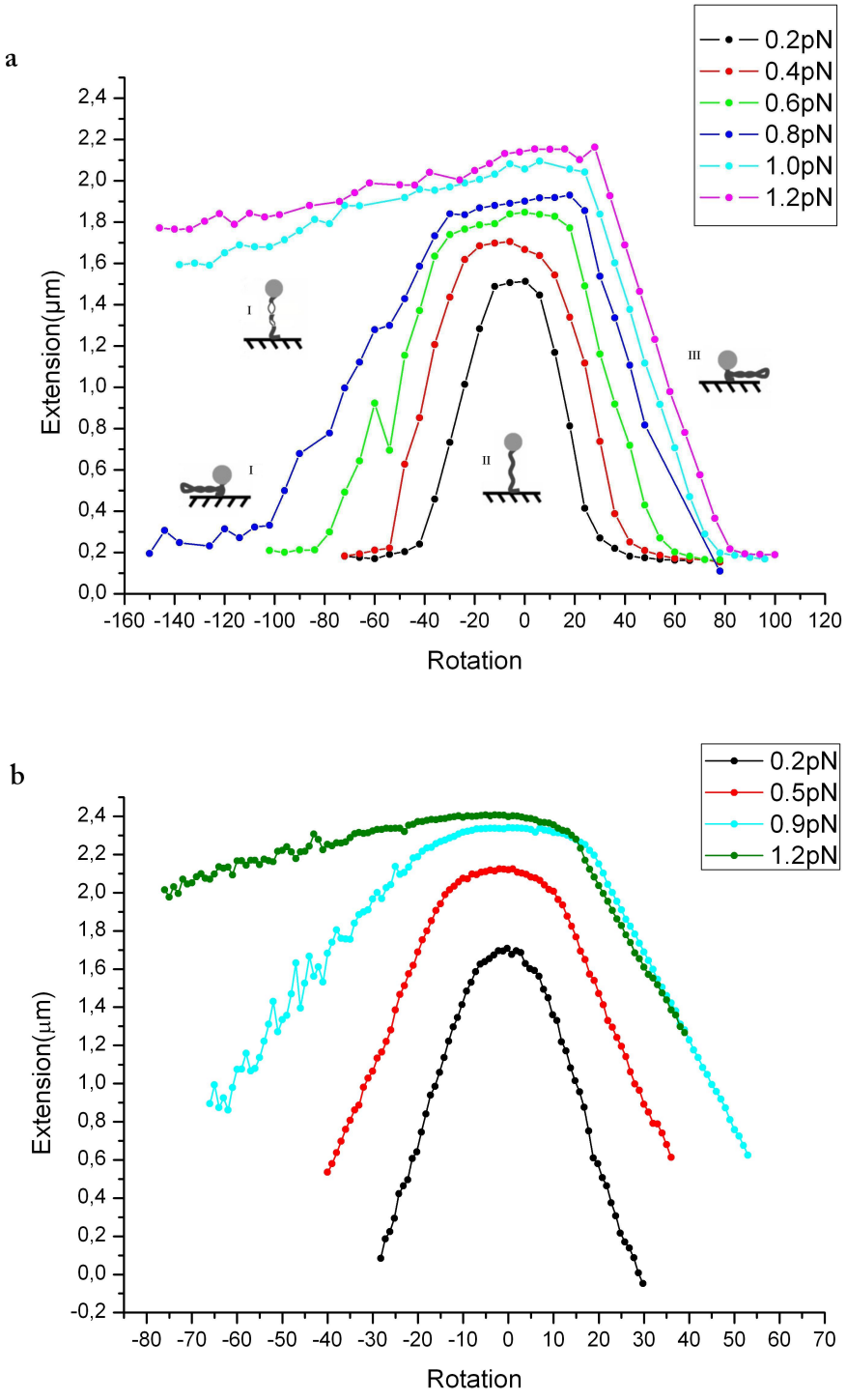


Figure 4.7: Rotation curves with forces from 0.2pN to 1.2pN

of the length can be seen. If the molecule was twisted even further (in the left direction), at a certain point the DNA molecule would have no helix structure anymore, but instead two parallel backbones. Since the DNA molecules under a force larger than 3pN can not be supercoiled anymore (in both directions), we are only interested in the behaviors of DNA molecules under smaller forces ($\leq$ 3pN). The following mentioned "high forces" mean the forces between 1.0pN to 3.0pN.

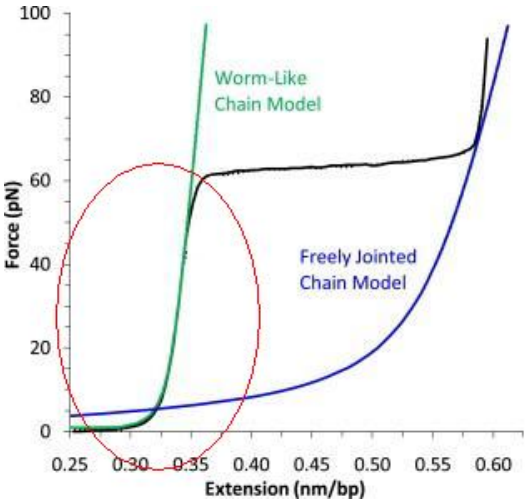


Figure 4.8: DNA stretching curve

Fig.4.9(top) shows the rotation curves of a DNA molecule. In order to get the contour length and persistence length of the molecule, the stretching curves have also been taken. Since our forces are smaller than 50pN (before the overstretching plateau), we have fitted this curve with the WLC model (2.1), as shown in Fig.4.8. (All the following stretching curves of unsupercoiled DNA molecules will be fitted with the WLC model (2.1).)

The stretching curves (Fig.4.9 bottom) have been taken from three rotation positions which are from the three areas as in Fig.4.9 (top): Rot. 0 from area I, Rot. 105 from area II and Rot. 200 from area III, called positions I, II, III, respectively. The orange curve is taken at position I, the blue at position II and the green at position III. It can be seen, that in position II the DNA extension is much larger than in position III, because at the position II the molecule stays longest, not having any supercoils. On the opposite, the molecule stays at position III under small forces fully positively supercoiled, where the DNA is much shorter.

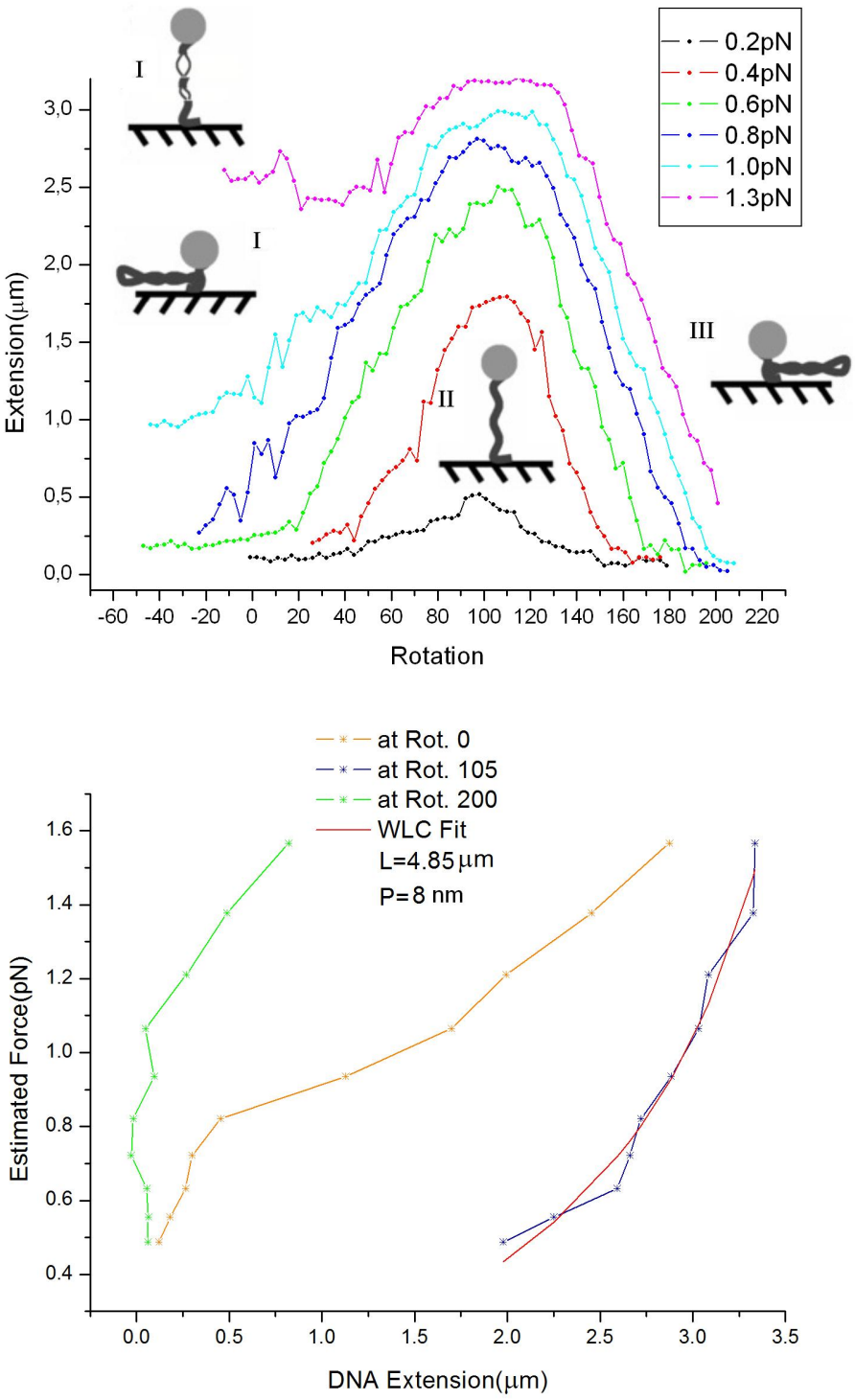


Figure 4.9: Stretching curves (bottom) at different rotation positions I, II, III. (Rot. 0, Rot. 105, Rot. 200, corresponding to the top figure.)

The orange curve is somehow special. It shows, that increasing the force from 0.6pN to 1.0pN on negatively supercoiled DNA results in large increases of the DNA extension. From 1.0-1.2pN, there is no large change of the DNA extension. This resembles the transition in position I from one status to the other. This curve fits the rotation curves very well. Under a high force, the molecule can not be twisted anymore into supercoiled form in the clockwise direction. Without producing supercoils, only a small increase of the length, which is only caused from increased force, can be observed. With the WLC fit, we have got that the contour length of this molecule is 4.85μm. Normally, λ-DNA molecules are 48502bp long (16.4μm) and ours are cut shorter (20000bp, about 6.8μm). Since not very large forces are put on the bead, the length of the DNA molecules is a little shorter than 6.8μm. The persistence length of the molecule is 8nm. Wang et al.[34] have shown their results of the persistence length of the $\lambda-$DNA, which is about 40nm. Our result seems much smaller. Since the curve has been fitted from 0.4pN to 1.5pN, the measurements might not be enough to fit the stretching curve correctly with WLC model.

In the stretching experiment, the DNA extension is related to the estimated force by taking the measurement program "vertical scan" (see also Fig.4.10). This force is in principle, i.e. except for error compensation, calculated from an equation like $F = F_0 \exp(-z/z_0)$. The value of the estimated force is only determined from the position of the magnets Z_{mag}.

We can also take the program "force scan" to obtain the stretching curve with measuring the real stretching force, i.e. the force which actually acts on the bead (Fig.4.11). This force is calculated from equation (2.7), $F_{\mathrm{mag}} = k_{\mathrm{B}}Tl/\langle\delta x^2\rangle$.

Fig.4.12 expresses the relation between the real stretching force and the estimated force by taking force-extension curves with the same bead. It can be seen that the two forces do not differ much at small

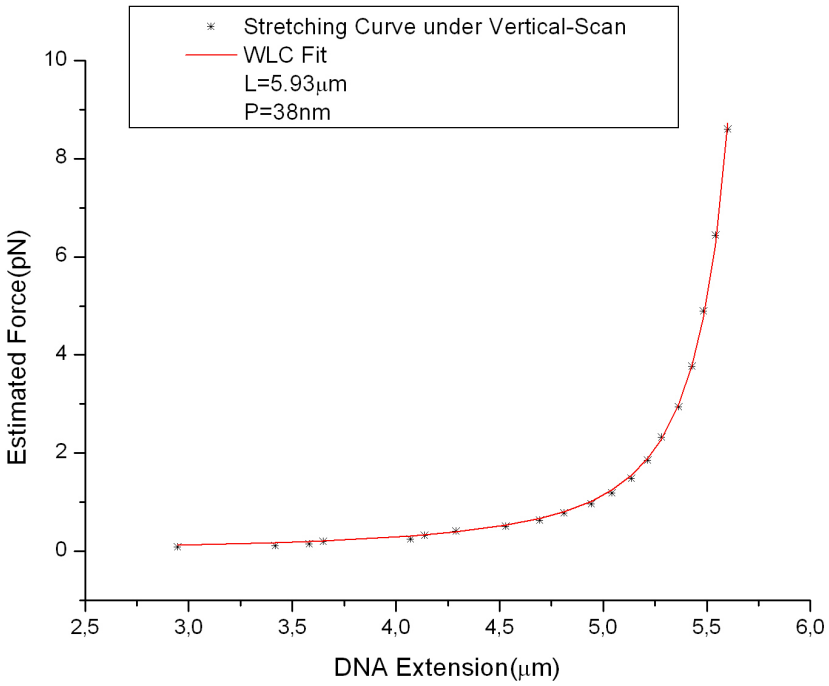


Figure 4.10: Taking the program "vertical scan", the curve represents the relation between the estimated force (which is determined by Z_{mag}) and the DNA extension.

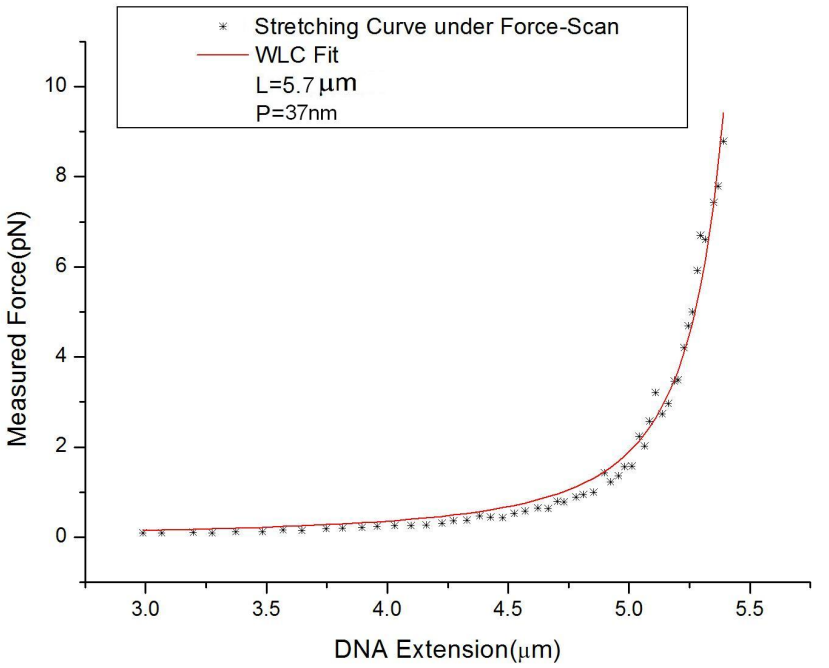


Figure 4.11: Taking the program "force scan", the curve represents the relation between the real stretching force (which actually acts on the bead) and the DNA extension.

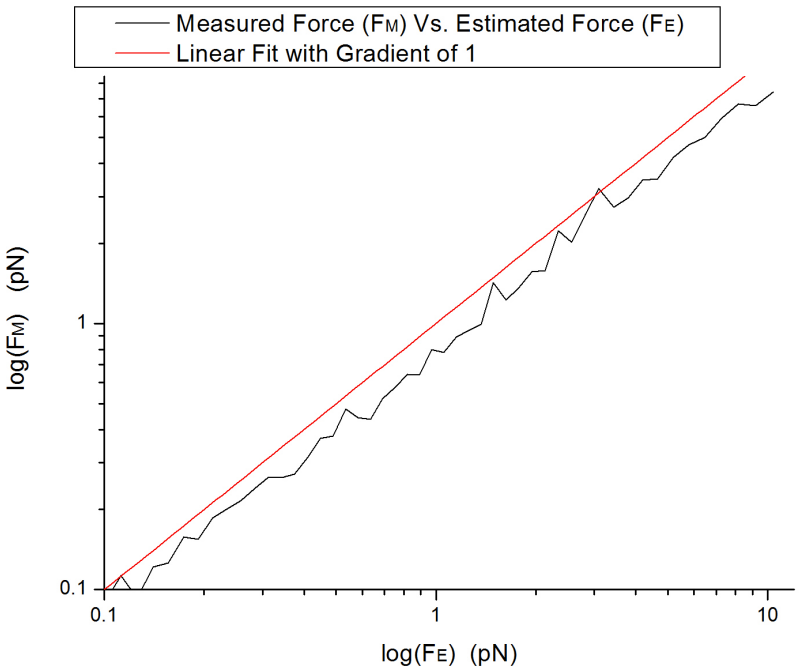


Figure 4.12: The curve represents the relation between the real stretching force and the estimated force.

values ($\leq$ 10pN). Since the stretching force is also correlated with the transverse fluctuations of the bead $\langle \delta x^2 \rangle$, which is influenced from the solution flow, the higher the bead is pulled, the more the force will be affected. Considering this point, the error between two forces is not always the same. Since the experiment duration of taking a curve with "force scan" is much longer than with "vertical scan", we prefer to use the "vertical scan". It takes several hours to take a "force scan" curve, in contrast, a "vertical scan" curve needs only about a few minutes. (For example, we have used both programs to take stretching curves of one DNA molecule. It took 171s with the "vertical scan" to take the curve and 116min with the "force scan".) To find out whether the error will perturb our measurements, we have compared the two force-extension curves (Fig.4.13). It can be observed that the error of the DNA extension at large forces is only about 0.25μm. In the most of our experiments, we are interested in the binding effect of DNMC on the DNA and their cutting effect, some small errors of the DNA extensions will be accepted. Thus in the following experiments, we have just taken the force-extension

curves with "vertical scan". The contour length of the DNA molecule is shown in both curves about 6μm with forces smaller than 10pN, which matches the expected length from the preparation. The persistence length of both curves is about 40nm, that fits the value in reference [34].

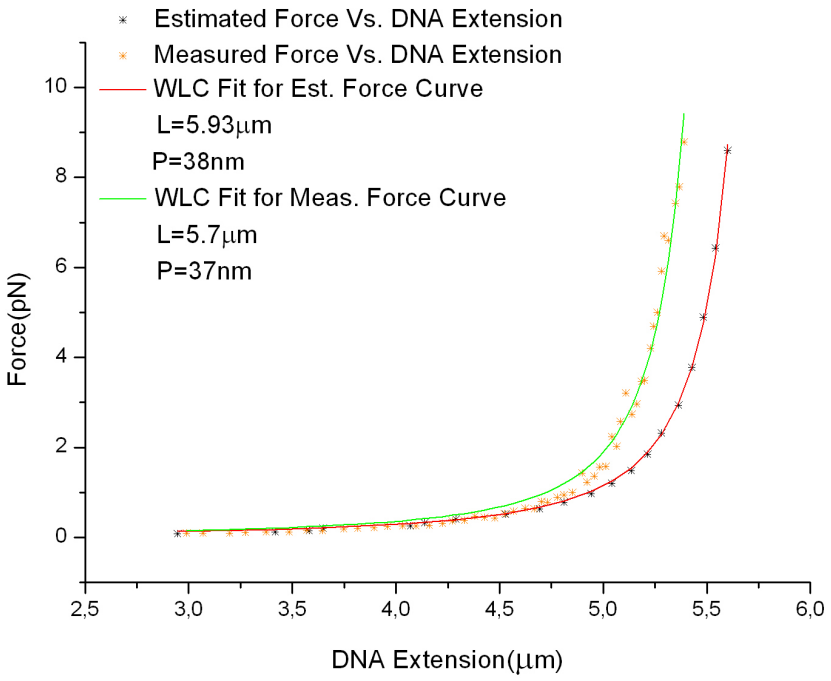


Figure 4.13: Compared with using the program "force scan", the "vertical scan" creates a error of the DNA extension of about 0.25μm.

4.3 Topoisomerase

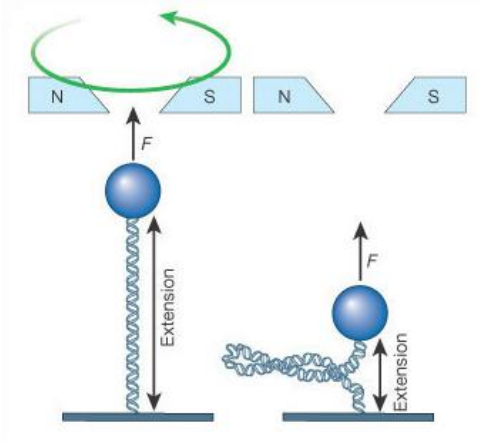


Figure 4.14: The bead is rotated in right and the negative supercoils are produced.

Normally, supercoiling can be unfolded by rotating the DNA molecule. But with topoisomerase the uncoiling could even be observed without rotation. TopIB can release the negative supercoiling. Our λ-DNA is in a natural form, which means that without any external force it stays like a soft stick, without supercoils. In order to observe the removal of supercoiling, some negative supercoiling has to be produced first. Therefore, the bead should be rotated in left.(Fig.4.14) Streching or shortening the DNA by rotating is actually a coiling process, just like with a telephone cable. During this progress supercoiling occurs. It may happen at both sides of the rotation curves (Fig.4.7). When the DNA molecule becomes quite short, the molecule has been already supercoiled. As seen in the previous section, negative supercoiling (produced by twisting the DNA in right) can only be induced using small forces (< 1.0pN).

First, the DNA has been rotated in the left direction using a small force to produce negative supercoils as shown Fig.4.14 and stretched with a force of 0.2pN. The TopIB has then been flowed through the cell. After taking the curve, the flowing has been stopped and the experiment has been repeated with the next higher force. The results of these experiments are given by Fig.4.15[1].

As Fig.2.5 shows, topoisomerase binds onto the DNA strand, cuts it and so releases the supercoiling. The topoisomerase solution is injected into the reservoir, and flows through the cell. The molecules

[1]On the x-axis, 60 Frame=1 Second.

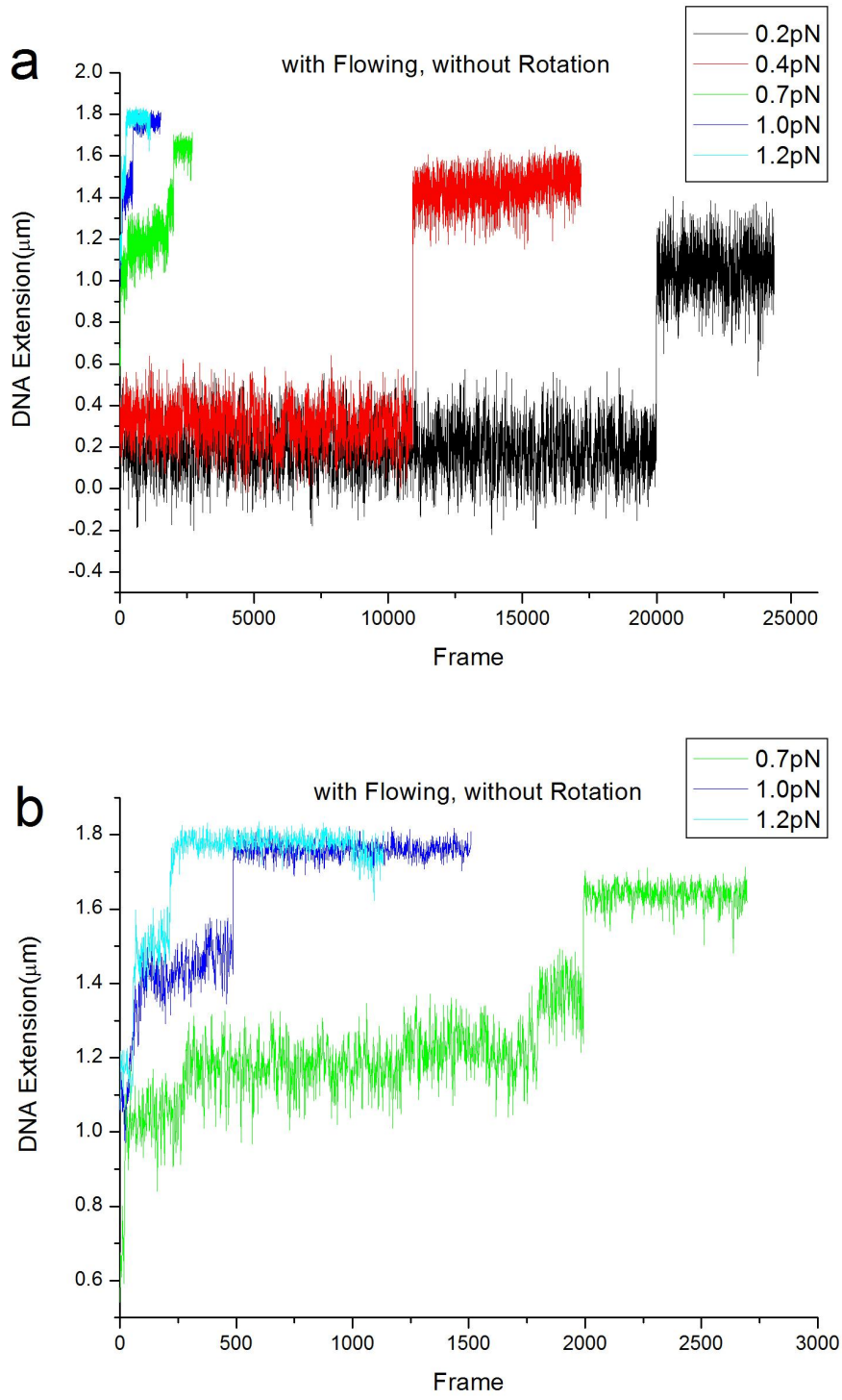


Figure 4.15: The figure **a** shows the extension of the DNA molecule with flowing and without rotation with forces from 0.2pN to 1.2pN. Since the releasement of the supercoils under large forces (0.7-1.2pN) happens much faster than under low forces, the respective curves are enlarged in figure **b**.

coil themselves during flowing into a ravel. When topoisomerase releases the supercoils, the curve exhibits a step. At a normal λ-DNA molecule with a length of 16.4μm many steps may be found on the curve, just like Fig.2.7 indicates. As the used DNA molecules are short (about 6.8μm), in the experiments with the magnetic tweezers the curves cannot have many steps. Actually, under small forces such as 0.2pN or 0.4pN it has only one step, but the size is relatively large, over 1μm. The size of the steps under the forces of 1.0pN and 1.2pN is small, only 0.2$\sim$0.4μm, but instead several steps occur in the same time period. As mentioned in Section 2.1, the larger the force is, the more supercoils are supposed to be released at one step. That means, the step size ΔLk is also supposed to be larger. But the graphic Fig.4.15 shows large steps at small forces (0.2pN and 0.4pN) and small steps at large forces (0.7pN, 1.0pN and 1.2pN). An explanation of this phenomenon might be that our DNA molecules are relatively short. When a high force is applied, the DNA molecule is pulled almost instantly into its full length. It does not have many supercoils to be released.

Furthermore, as Fig.4.15 shows, the removal of supercoils occurs earlier at the higher forces. A reason for this might be the growing concentration of the TopIB molecules during the ongoing experiment. When TopIB binds onto a DNA strand, the cutting will happen immediately. The measurements have been started from 0.2pN. It can be imagined that as only a little TopIB flew through at the begining, it took a relatively long time until TopIB bound onto the DNA molecule. But as more and more TopIB came gradually through, it became more likely for TopIB binding on the observed molecule. Thus the waiting time for the releasement was expected to be shorter. This may also explain, why under larger forces in each curve more than one step was observed. Here we would like to mention that after an interaction of TopIB with the molecule, the enzyme may have been removed from the DNA by the constant flow through the cell. So, the next steps might be due to new bonds from other TopIB molecules.

4.4 Cutting Tests of Co-, Cu- and Ni-Complexes

Thomas Jany, from the faculty of chemistry at the university of Bielefeld, has synthesized the following three dinuclear metal complexes (DNMC)[2]:

- $[(\text{tom}^{\text{6-Me}})\{\text{Co(OAc)}_2\}] \cdot \text{nH}_2\text{O}$
- $[(\text{tom}^{\text{6-Me}})\{\text{Cu(OAc)}_2\}] \cdot 8.5\text{H}_2\text{O}$
- $[(\text{tom}^{\text{6-Me}})\{\text{Ni(OAc)}_2\}] \cdot 13\text{H}_2\text{O}$

The functionalities of these complexes have been tested and will be presented in the following text.

Some experiments with DNMC have been already made using AFM and the optical tweezers. It could be seen that the metal complexes attract each other and finally interact. Therefore some knots of DNA molecules had been built. (Fig.4.16 light points, Fig.4.17) The DNA molecule had become shorter, details will be presented in the following parts.

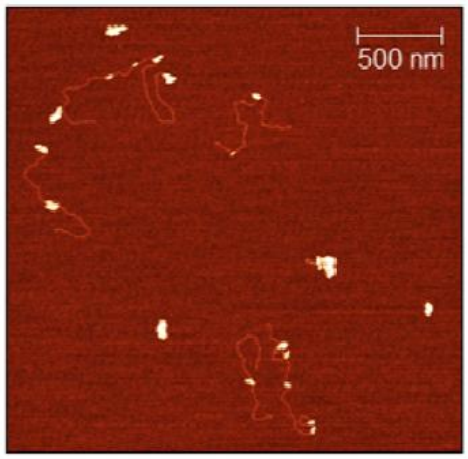


Figure 4.16: DNA gets some knots after the binding of metal complexes.[8]

By using the optical tweezers, the interaction between cobalt complex and DNA could be observed clearly. The optical tweezers can fix one end of the DNA via fixing an attached optical bead in an optical trap and pull on the other end via another attached bead. The following Fig.4.18 shows the behavior of the DNA molecule before and after injecting the solution of the metal complexes (the curve includes a stretching and relaxing cycle). The black curve shows the behavior of DNA without the influence of

[2]More informations see Bib.[8]: Molekulare Erkennung von Biomolekülen mit maßgeschneiderten Metallkomplexen, Thomas Jany.

metal complexes and the red one its behavior with metal complexes. It can be seen, that the DNA molecule became shorter. At some places a larger force had to be given in order to pull the knot apart. In the graphic it can be seen that knots could be unloosened by a force of about 20∼50pN. More details will be given later. In fact, not all the knots could be pulled apart.

In the measurements with the optical tweezers, we can always see the peaks on the stretching cycle. But with magnetic tweezers the peaks could not be discovered. The magnetic force is increased very gently by the magnetic tweezers, which might be a reason why those peaks would not appear on our stretching curves.

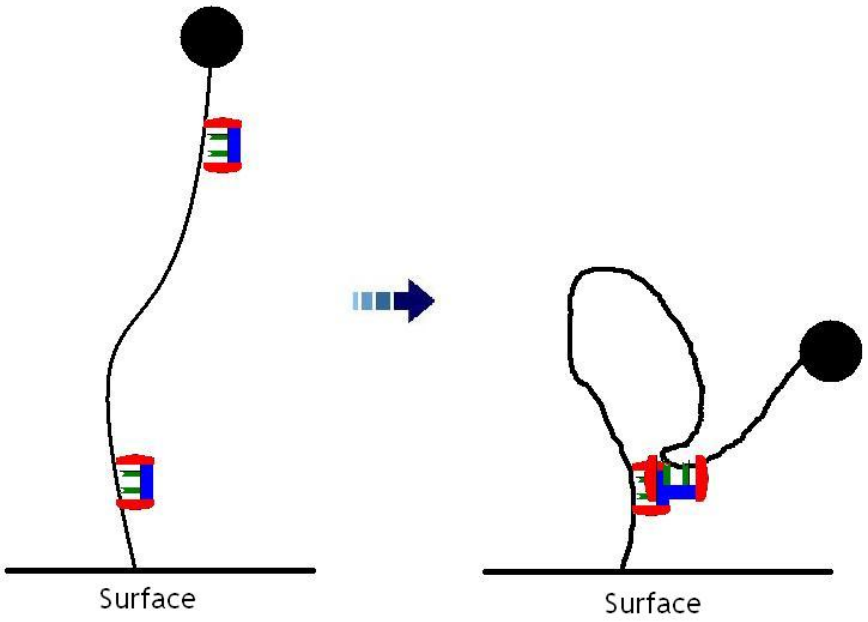


Figure 4.17: The conception of interaction between two metal complexes, thereby the knotting happens.

From the three new metal complexes, the cobalt complex has been chosen first to be tested with the magnetic tweezers, since in AFM experiments at the university of Bielefeld, some splits on an DNA molecule have been found. However, as these results could not been reproduced reliably, the cutting functionality could not be confirmed.

It is assumed that the cobalt complex may work under some special conditions.[8]

DNMC crystals can be dissolved in DI-water, MT-buffers (buffers especially for the magnetic tweezers, can be passivation buffer or standard buffer) or phosphate buffer. The solution has then been diluted with a MT-buffer, which must not disturb the bond between DNA and DNMC and besides can hinder the unspecific binding.

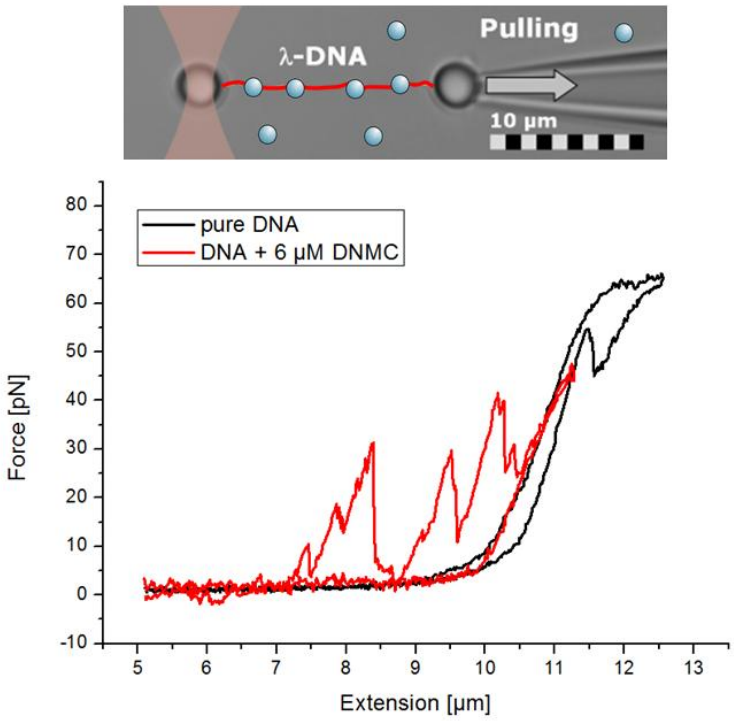


Figure 4.18: The curve shows the behavior of DNA molecule before and after injecting the solution of metal complexes.[8]

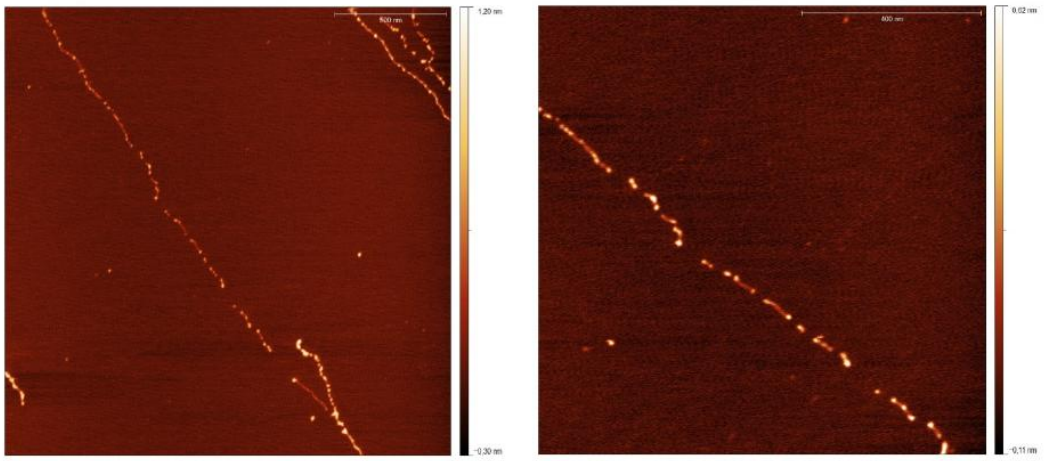


Figure 4.19: The "cutting" experiment with the cobalt complex using AFM.Some broken positions on the DNA strands can be discovered. It is suspected that the cobalt complex has cut the DNA molecule.[8]

4.4.1 Cutting Tests with Cobalt Complex

The following table shows the chemical character of the cobalt complex.

DNMC	Co
Chem. Fomula	$C_{44}H_{46}N_6Co_2O_6$
Molar Mass	872.74 g/mol(not dissolved in solution)
Elem. Analysis	$[(tom^{6\text{-}Me})\{Co(OAc)_2\}]\cdot nH_2O$

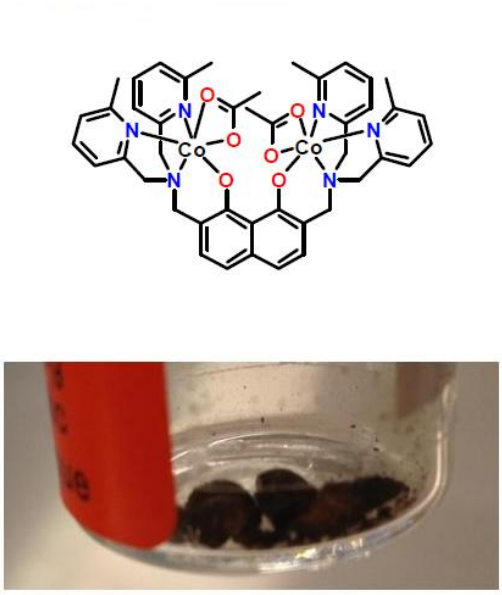


Figure 4.20: The crystal of cobalt complex looks coal-black, the upper picture shows the molecule structure of cobalt complex $[(tom^{6\text{-}Me})\{Co(OAc)_2\}]\cdot nH_2O$.

In order to avoid that DNA molecules stick on the surface after adding the cutting solution, the experiments begin first with a relative low DNMC concentration of 10μM. The interaction should happen under a low force or even without stretching. The DNA molecules should be relaxed as a ravel, so that the metal complexes can bind better on DNA strands and hence better interact with the DNA molecules. It is believed that the metal complexes need a long time to create a hydrolysis of phosphate esters. Therefore it is important to let the metal solution incubate at least one night in the chamber.

The black curve (black points) in Fig.4.21 is the original DNA stretching curve. The force extension curve with injecting 10μM cobalt complex has been shown in orange. It can be seen that obviously the DNA-DNMC complex has got about 2μm shorter.

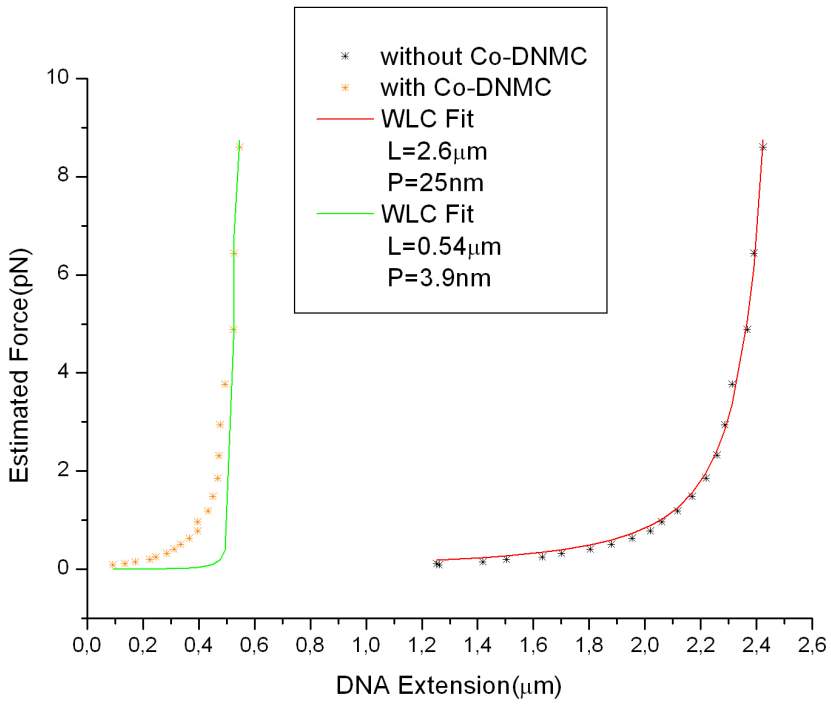


Figure 4.21: Force-extension curve before and after injecting 10μM cobalt complex.

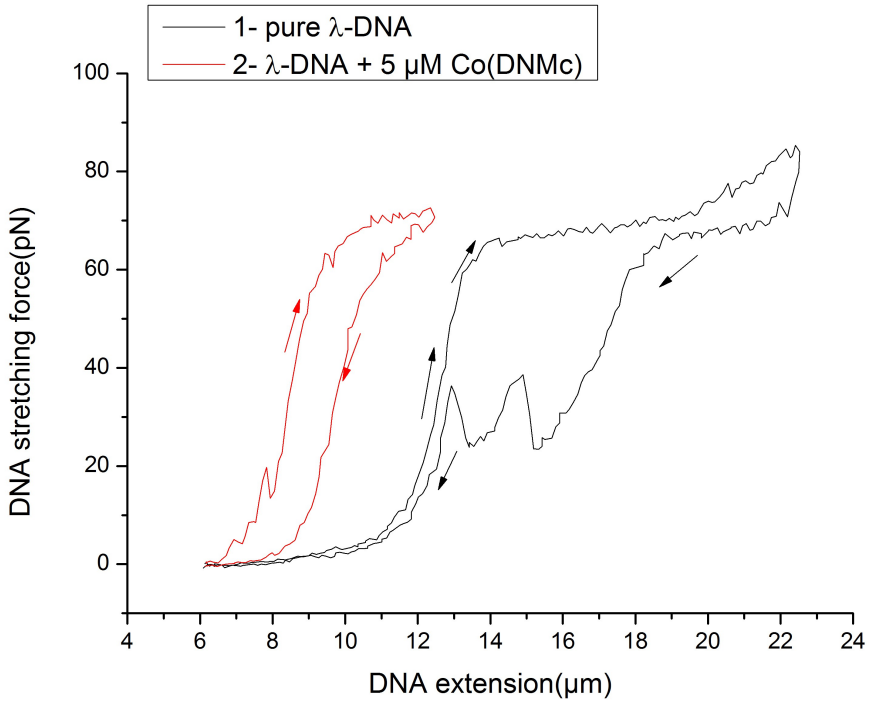


Figure 4.22: The curve has been taken using the optical tweezers with and without 5μM cobalt solution.[13]

The same result has also been observed with the optical tweezers. (Fig.4.22) The DNA molecule has also become shorter. Certainly, since the contour length of DNA molecules used for the optical tweezers is much longer than the one for the magnetic tweezers, much more knots could be built. Therefore the molecules in the experiments with the optical tweezers can become much shorter. There are several small peaks on the curves that show the unloosening of the knots. The effect of DNMC may be so strong that not all of the knots can be pulled apart. This might be an explanation of why there are only few peaks although the length of the DNA molecule decreased strongly.

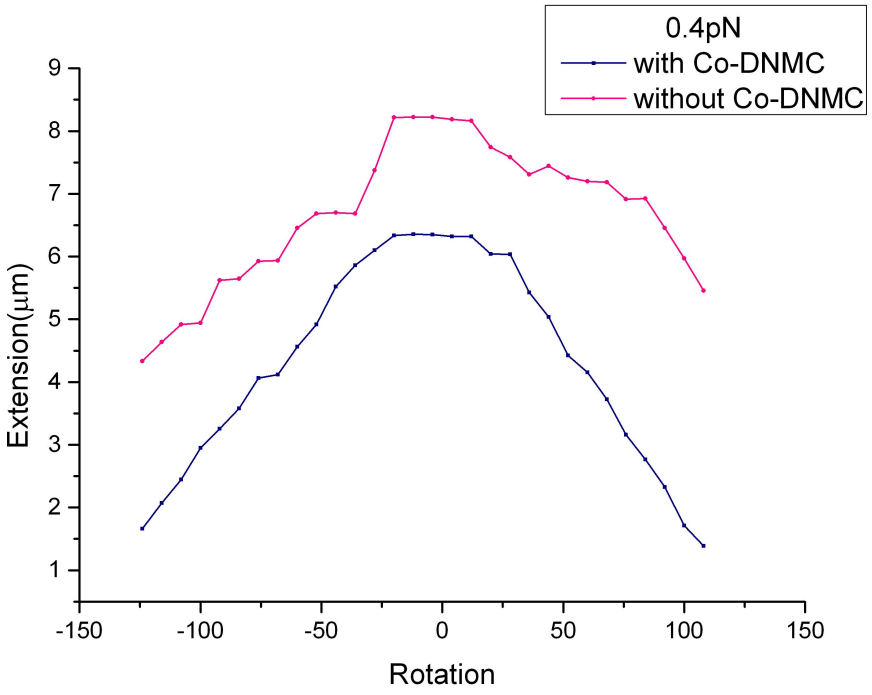


Figure 4.23: With the effect of Co-DNMC, the molecule gets shorter but can still be twisted. A cutting has not happened.

Since strand breaks of DNA can not be prevented, we have not found many quite useable molecules (twisting feasible) to test whether Co-DNMC can cut DNA molecules. However, we have found a twistable DNA molecule which has accidentally retained a length of over 10μm. Fig.4.23 shows its rotation curve. The molecule has got a shortening

of 2μm because of the effect of Co-DNMC, but it can still be twisted. The means that the cutting functionality has not worked for this molecule. We will try to make the test experiments in many other ways in the future, e.g. with different concentrations of DNMC. We are also interested in results under different temperatures.

4.4.2 Cutting Tests with Copper Complex

DNMC	Cu
Chem. Fomula	$C_{44}H_{63}N_6Cu_2O_14.5$
Molar Mass	1035.12 g/mol(1106.46 g/mol with $8.5H_2O$)
Elem. Analysis	$[(tom^{6\text{-Me}})\{Cu(OAc)_2\}]\cdot 8.5H_2O$

The table above shows some chemical characters of the copper complex. The crystal has a typical blue copper colour. In the experiments we have firstly, just like with Co-DNMC, used a 5μM concentrated cu solution. Fig.4.28 represents the DNA stretching curves with and without DNMC solution.

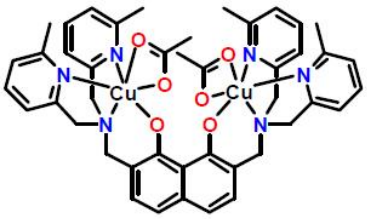


Figure 4.24: The crystal of the copper complex has a typical colour from copper salt: blue. The upper picture shows the molecule structure of copper complexes $[(tom^{6\text{-Me}})\{Cu(OAc)_2\}]\cdot 8.5H_2O$.

Interesting is that compared to the situation with Co-DNMC, the DNA molecule has only got a shortening of 0.5μm. Considering that our DNA molecules are rather short, many knots might probably not have occured. Moreover, 0.5μm is not long enough to be the result of 2 interacting complexes (Fig.4.25). With this length, two DNMC are not supposed to be able to bind with each

other. The DNA molecule is pulled with a force, it needs much energy to bend the strand as shown in Fig.4.25 (top). An explanation

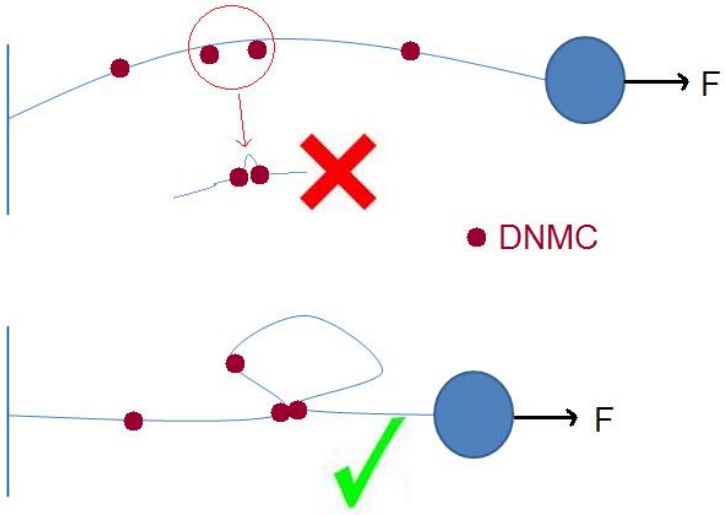


Figure 4.25: Two DNMC molecules, which are near to each other, are not supposed to be able to bind together, since such a binding needs too much energy.

here would be, that the unspecific bond between complex and surface leads to the change of length (Fig.4.26). Besides, the stretching cycle of the DNA molecule with the optical tweezers shows that the knot building ability of copper solution is also weaker than that of the cobalt solution.

From the same molecule, the rotation curves have been taken. Fig.4.29 shows that the molecule can still be twisted, hence no cutting has occured. The distance between two peaks is 0.5μm, which matches the stretching curves. Fig.4.31 shows the results of another molecule. Many measurements have been made with Cu-DNMC solution of a concentration of 5μM. 50μM concentrated solution has also been tested. Both temperatures of 25°C and 37°C have been on trial. Here we can say that Cu-DNMC with concentration lower than 50μM does not seem to be able to break the DNA strand. The experiments with higher concentrations will take place in the future.

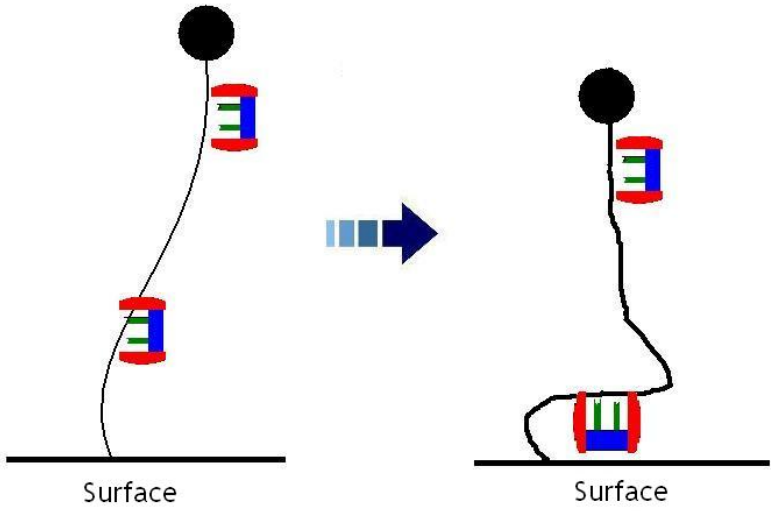


Figure 4.26: The DNA molecule with Cu-DNMC has become only about 0.5μm shorter. An explanation could be the unspecific bond between DNMC and the surface.

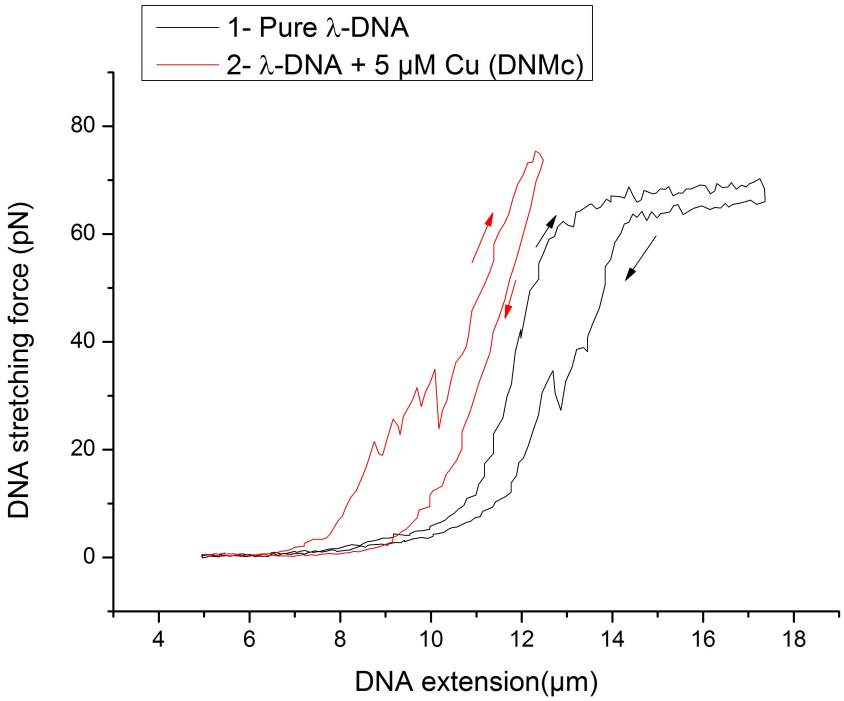


Figure 4.27: Stretching cycle of DNA molecule before and after injecting Cu-DNMC with optical tweezers.[13]

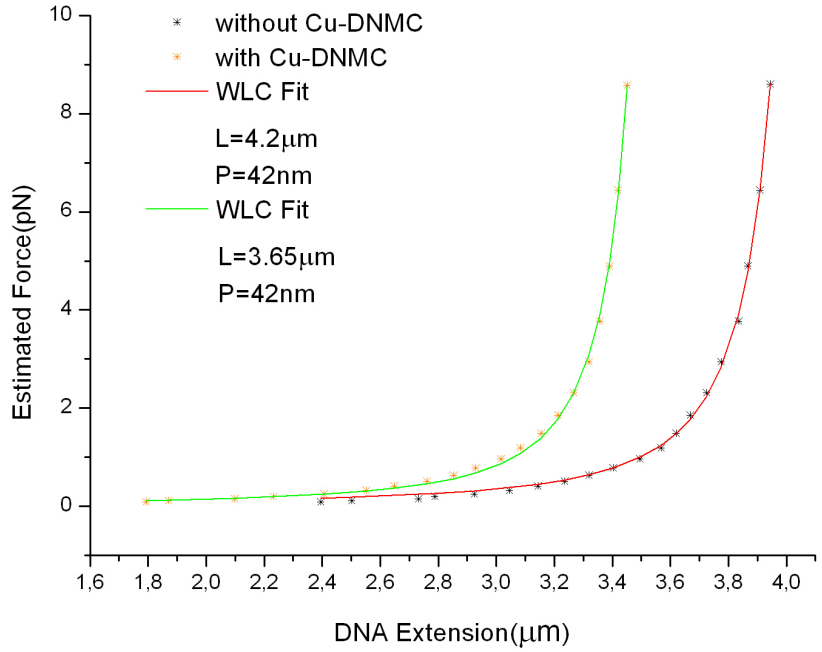


Figure 4.28: The stretching curves with and without the effect of Cu-DNMC, the molecule became 0.5μm shorter.

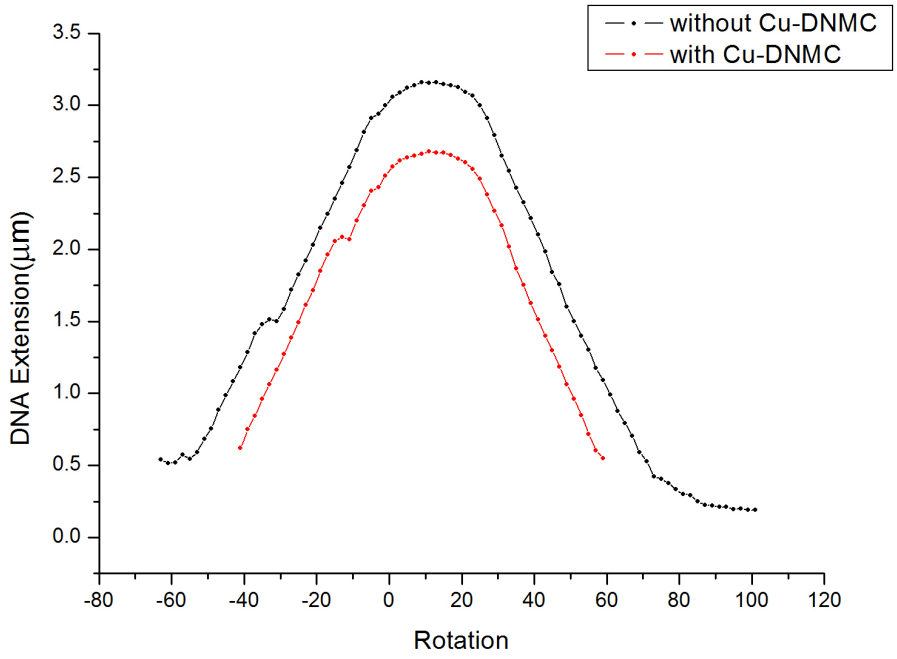


Figure 4.29: The difference between two rotation curves (under 0.4pN) shows the effect of Cu-DNMC.

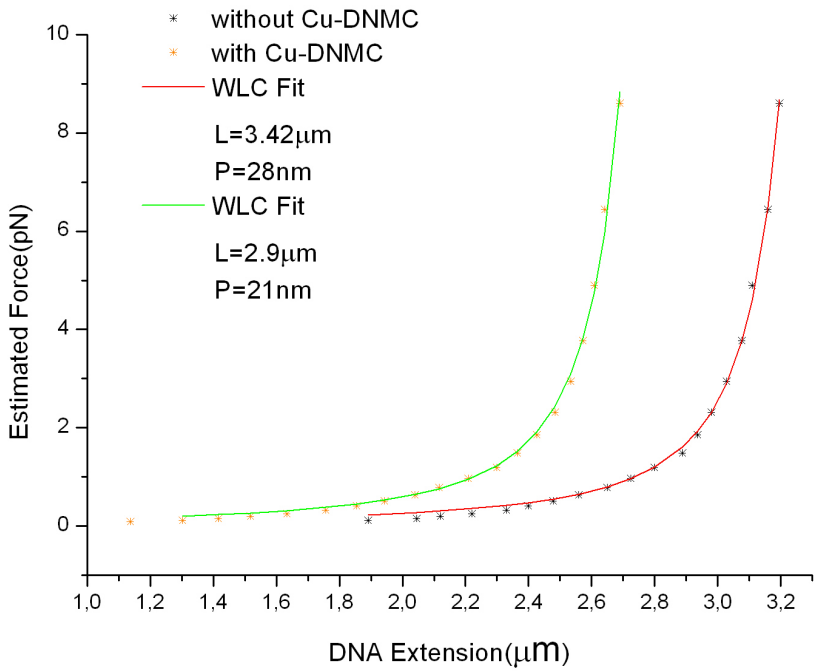


Figure 4.30: Stretching curves with and without Cu-DNMC.

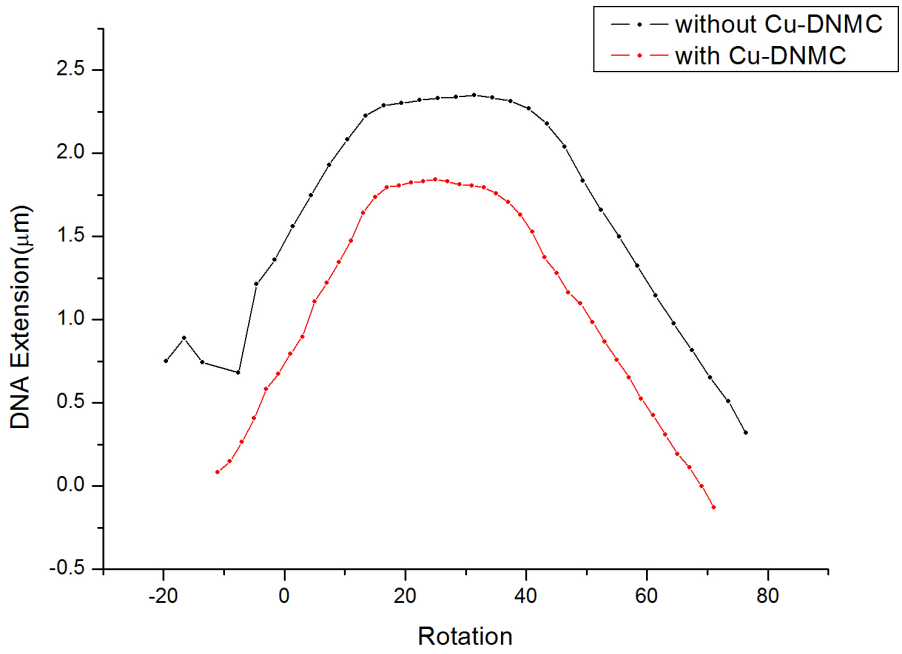
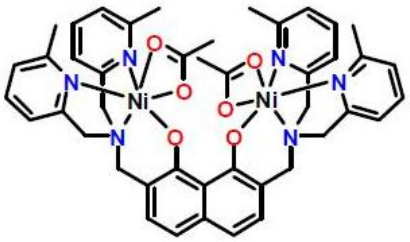


Figure 4.31: Rotation curves from the molecule (under 0.4pN) with and without Cu-DNMC.

4.4.3 Cutting Tests with Nickel Complex

DNMC	Ni
Chem. Fomula	$C_{44}H_{72}N_6Cu_2O_19$
Molar Mass	1106.46 g/mol(1106.46 g/mol with $13H_2O$)
Elem. Analysis	$[(tom^{6\text{-}Me})\{Ni(OAc)_2\}]\cdot 13H_2O$



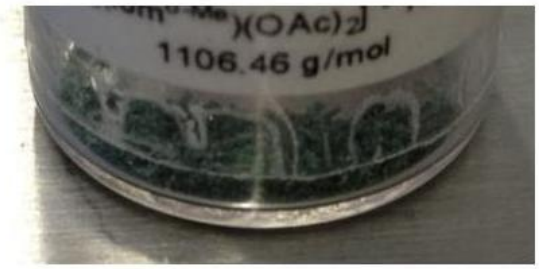


Figure 4.32: The crystal of nickel complex looks green. The upper picture shows the molecule structure of nickel compexes $[(tom^{6\text{-}Me})\{Ni(OAc)_2\}]\cdot 13H_2O$.

The table above shows some chemical characters of the nickel complex. Since in the above experiments we have got no success with low concentration, the experiments with nickel complex have been started directly with a higher concentration of 50μM under 37°C (in experiments with Cu-DNMC we have found that a concentration of 50μM is not so high that the molecules stick on the surface). Fig.4.33 represents a stretching cycle of DNA with the optical tweezers. With 50μM Ni-DNMC we can see many peaks on the curve after injecting the nickel solution.

Analogous experiments have been done with the magnetic tweezers. The stretching curves on Fig.4.35 are the results we have got. A DNA-bead system has been found (Fig.4.34), for which good rotation curves under various forces could be drawn. The stretching curves have been taken before and after injecting the complex solution.

The DNA is about 1μm shorter. Obviously, nickel complexes have already built at least one knot. Fig.4.36 represents the rotation curves from the molecule with and without DNMC. It can be seen that

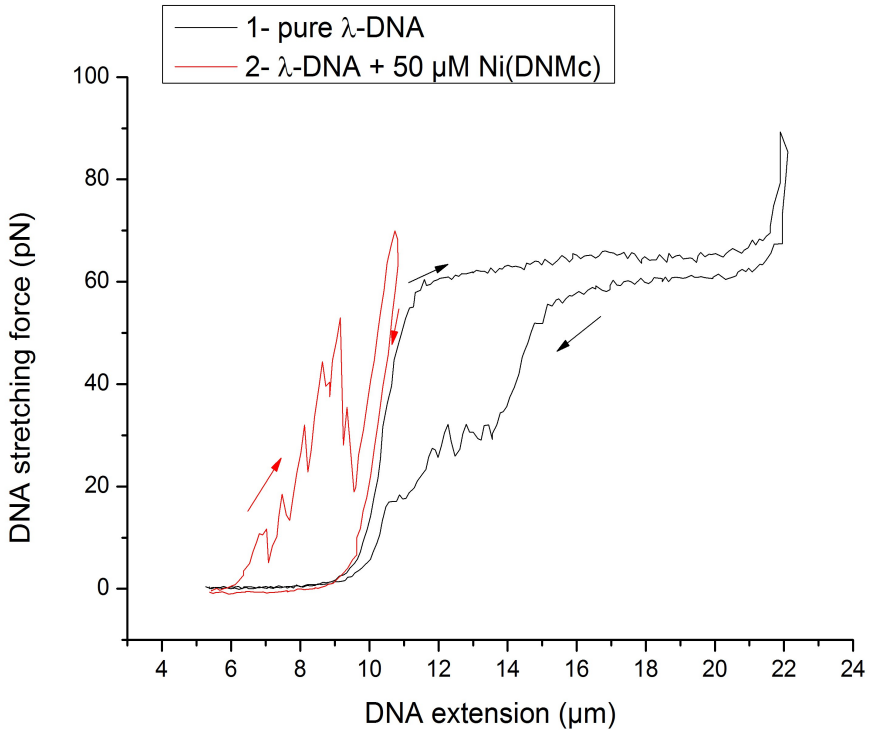


Figure 4.33: The curves represent the effect of Ni-DNMC by taking the stretching cycle of the DNA molecule with the optical tweezers.

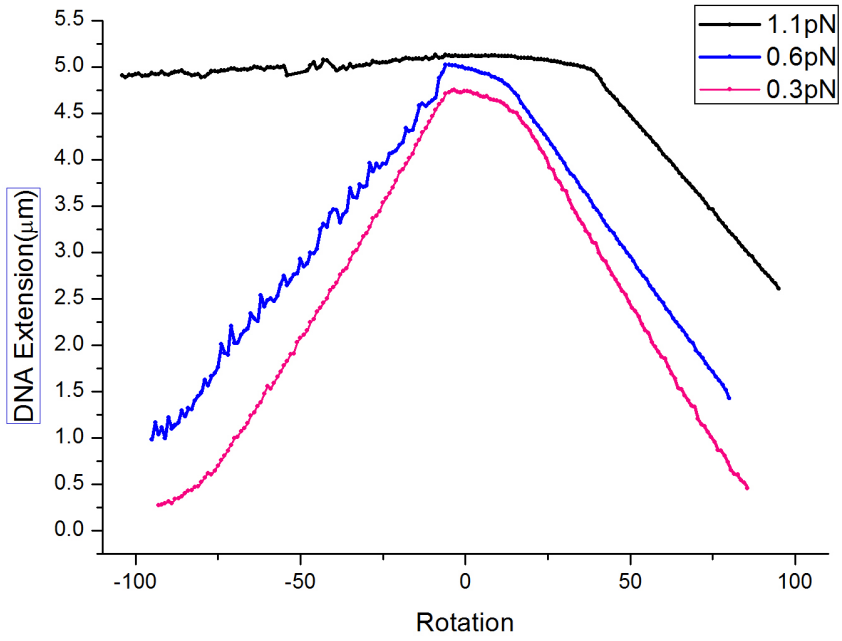


Figure 4.34: The DNA single molecule has been represented with three rotation curves under various forces.

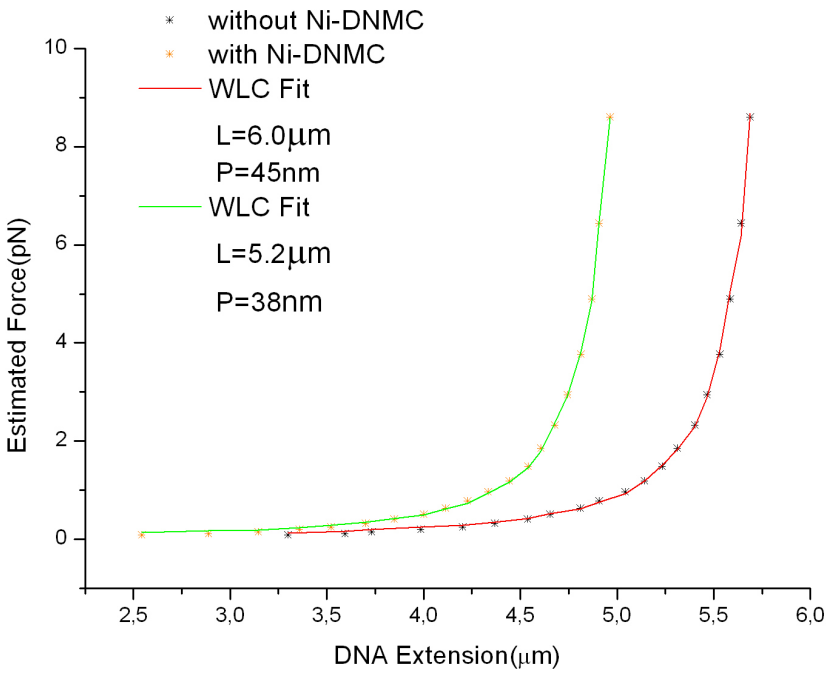


Figure 4.35: Stretching curves have been taken before and after injecting the complex solution.

after injecting Ni-DNMC, the DNA molecule could not be twisted anymore. The length stays constant. It has been found in the experiments with the optical tweezers that the DNMC binds on DNA strands so strongly that they cannot be rinsed away. This is exactly the idea of a "permanent binding".

Since the "permanent binding" can not be destroyed with the optical tweezers, a try with magnetic tweezers has been made. The idea is, since DNA can be twisted and the backbone of the metal complex is supposed to be stiff, it may be possible to break the bond between the strand and the DNMC while twisting DNA molecules a lot (may be over thousands of rotations). Then the phosphate ester would be free again and was able to bind with sugar. The DNA strand is supposed to be repaired and the molecule could be twisted again. With this concept, per rotating the bead we have twisted the molecules in both directions over thousand turns. But the molecule shows no change of its length. (Fig.4.37) That means, the bond between the

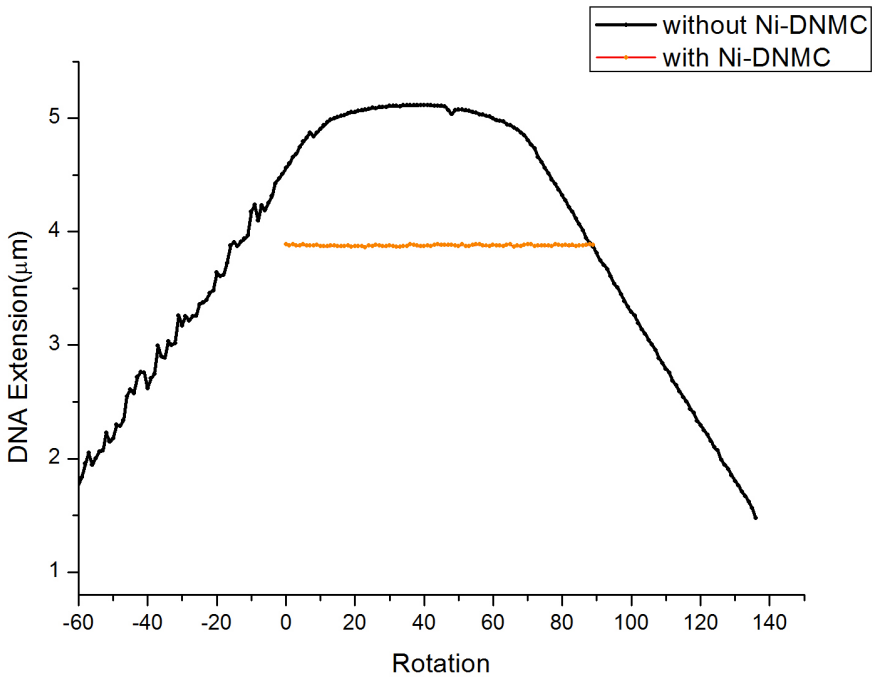


Figure 4.36: Rotation curves are taken under 0.6pN. After injecting Ni-DNMC solution, the DNA molecule can not be twisted anymore.

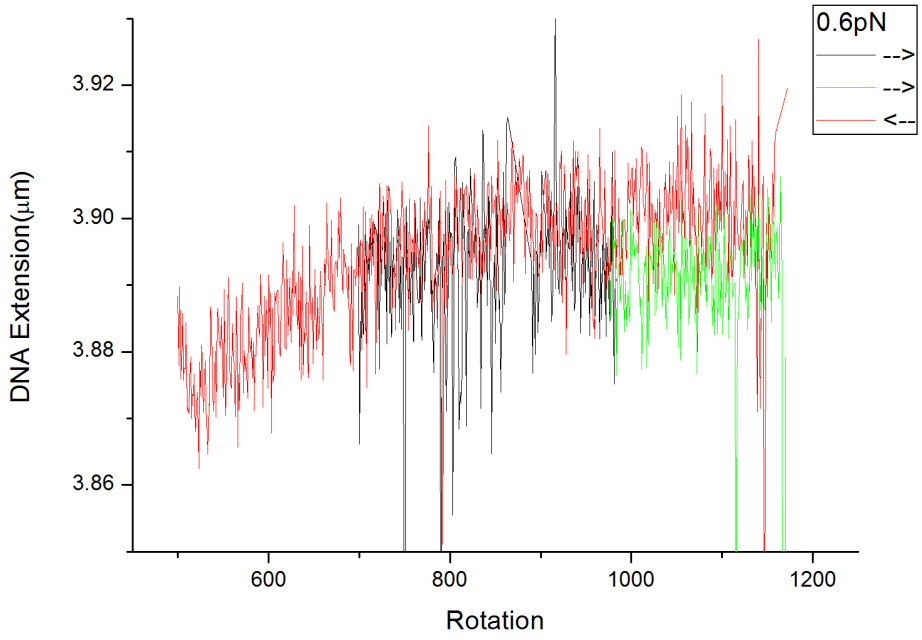


Figure 4.37: Trying to break the permenant binding per twisting DNA a lot, it does not seem to work.

DNMC molecules and the DNA strand was not broken. The magnetic tweezers were not able to destroy this "permanent binding".

Actually, some other molecules have also been found, which can still be twisted although they seemed to have reacted with Ni-DNMC as the lengths have become all shorter. Until now, the cutting phenomenon has not shown itself again. Another possible explanation would be that the structure of the molecule had been damaged for other reasons and therefore was not twistable anymore. Thus we need to make more experiments to make sure if the results have proved what we wanted to test.

4.5 Discussion: Difficulties in the Experiments

In the following we discuss several problems which influenced the outcome of our experiments.

4.5.1 Unstable Bond between Multi-dig and the Surface

The first problem and also the worst of all is from the DNA molecules. Because of the complicated process of DNA preparation, it cannot be ensured that the DNA molecules have no strand breaks.

Our DNA molecules are bifuncional. One side of a molecule is bound with multi-biotin and the other with multi-digoxigenin. "Multi" is here very important, it ensures that a DNA molecule can bind stably onto the surface and with a bead, thereby the DNA molecule can not be freely rotated by itself. Certainly, because of the indeterminancy from the preparation process, it cannot be known whether more than one biotin or digoxigenin molecule bind on the DNA and whether the bond is stable. But in the experiment this can be tested right away. Because the bond between multi-biotin and streptavidin is normally relative stable, the problem is imputed to the bond between multi-digoxigenin and anti-dig. This problem may happen in three different situations.

Fig.4.38 shows the difference between two rotation curves. Both curves are taken under 0.4pN. The blue curve is taken by a DNA molecule whose dig-side binds well on the surface. The curve looks just like other rotation curves under 0.4pN. If the bead is rotated in right, the molecule will firstly be stretched longer and at the meantime releases the supercoils. After the peak, because of the further rotation, the DNA strands create negative supercoils and the length is also shortened. The pink curve shows exactly the problem of a DNA molecule whose dig-side binds unstably on the anti-dig. The further rotation after the peak is an unnatural winding. Each further rotation faces large resistance force. When the bond between

multi-dig and anti-dig is not stable enough to contend against the resistance force, the bond would be broken. Negative supercoils cannot be created anymore, and the molecule stays at its length without supercoils. The pink curve shows the situation that multi-dig binds unstably on the surface.

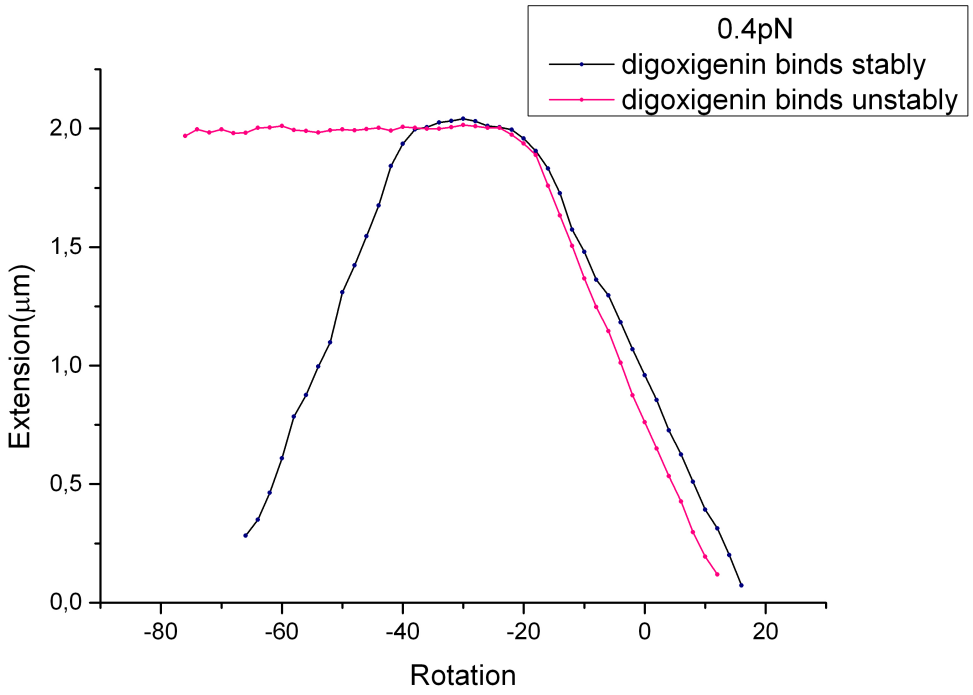


Figure 4.38: The graphic shows the difference between two rotation curves. One is taken by a molecule whose dig-side binds not stably on the surface and the other whose dig-side binds well on the surface.

We have obtained some measurements from these unstably bound molecules. If the molecules can hold the positive supercoils from being removed without rotation, they can be still used to test whether DNMC works. In the cutting-experiments with Cu-DNMC, we have obtained the following curves. (Fig. 4.39) The peaks from the rotation curves have moved. In the top figure, the rotation curve without DNMC has been taken first. The molecule has been twisted in counter-clockwise direction, thereby positive supercoils have been produced. Due to the unstable binding between the dig side of the molecule and the cell surface, the molecule has rotated itself without

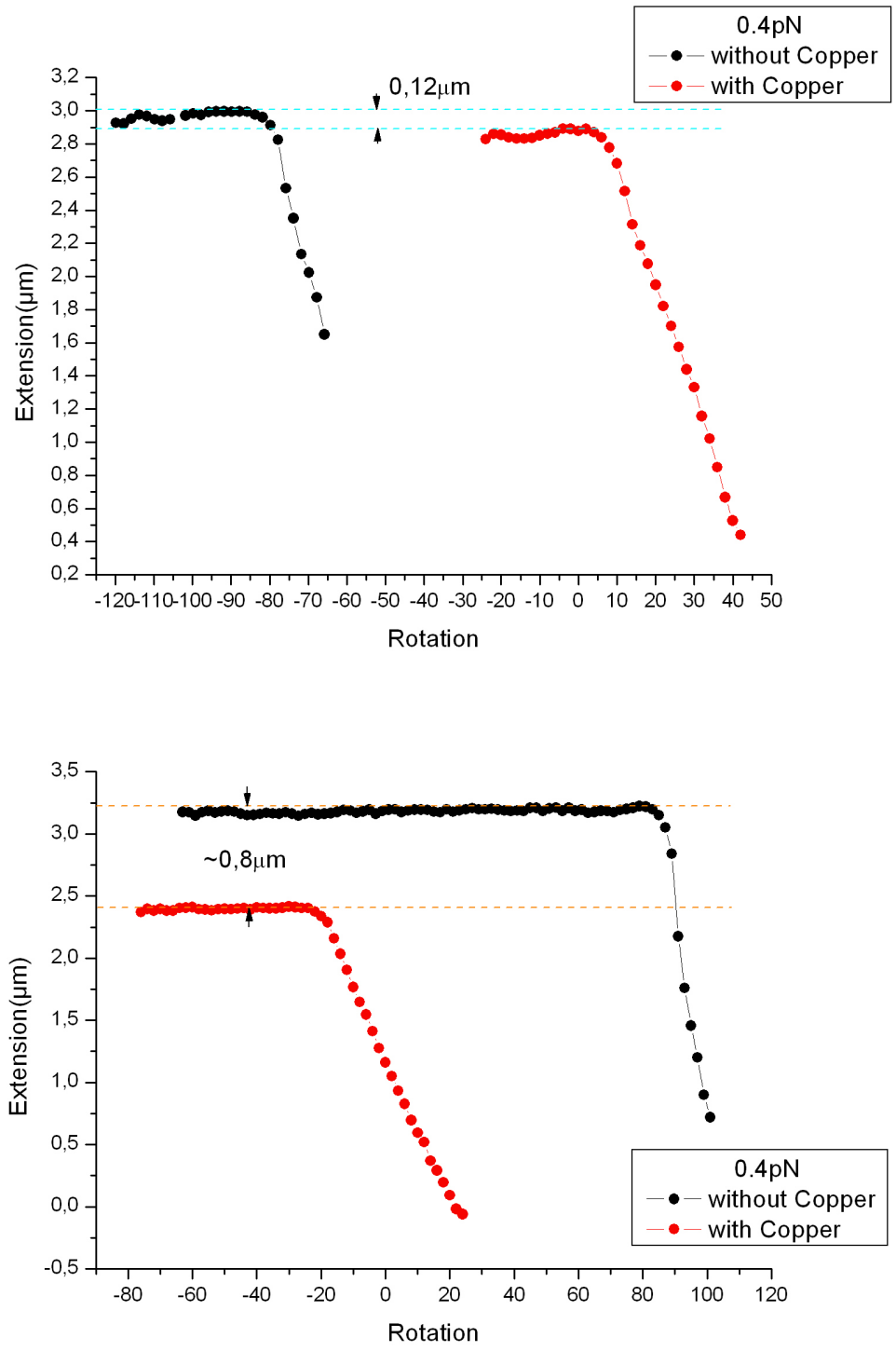


Figure 4.39: In the experiments to test the cutting-functionality of Cu-DNMC, we obtained these results from the molecules which do not bind stably on the surface.

the bead rotated by the magnets (the dig side has been loosed from the surface). This process has happened for several times, thereforce the peak of the rotation curve has slid to the right. In the bottom figure, the positively supercoiled molecule has been twisted in the right direction. Because of the unstable binding of the dig side, the double helix has not been twisted into two parallel strands, the winded structure has been well maintained. When we rotated the bead in left, it did not take many rotations to produce the positive supercoils since the molecule has actually kept an good, unsupercoiled structure as an untwisted one. Hence, the peak has slid to the left.

It seems that Fig.4.40 and Fig.4.41 show another possibility of weak binding. In the measurement which Fig.4.40 shows, it is possible to take a quite good rotation curve under 0.4pN if the magnets rotate not too slow. The rotation was held where circled in red and ran a time curve (Fig.4.40). It is found that the DNA molecule got gradually longer, although the stretching force had not been changed at all. Another molecule has been found, that could build negative and positive supercoils under continual rotation. Its time curve has been taken under rotation Fig.4.41. The molecule can be pressed shorter, but if the rotation is held unchanged at the lowest place of the curve, the molecule will extend slowly, that means the supercoils will be released automatically. This situation is even worse than the first one mentioned. The molecule cannot even hold the positive supercoils.

Anyway, in either of these three situations, both DNA and the surface should be checked until this problem disappears.

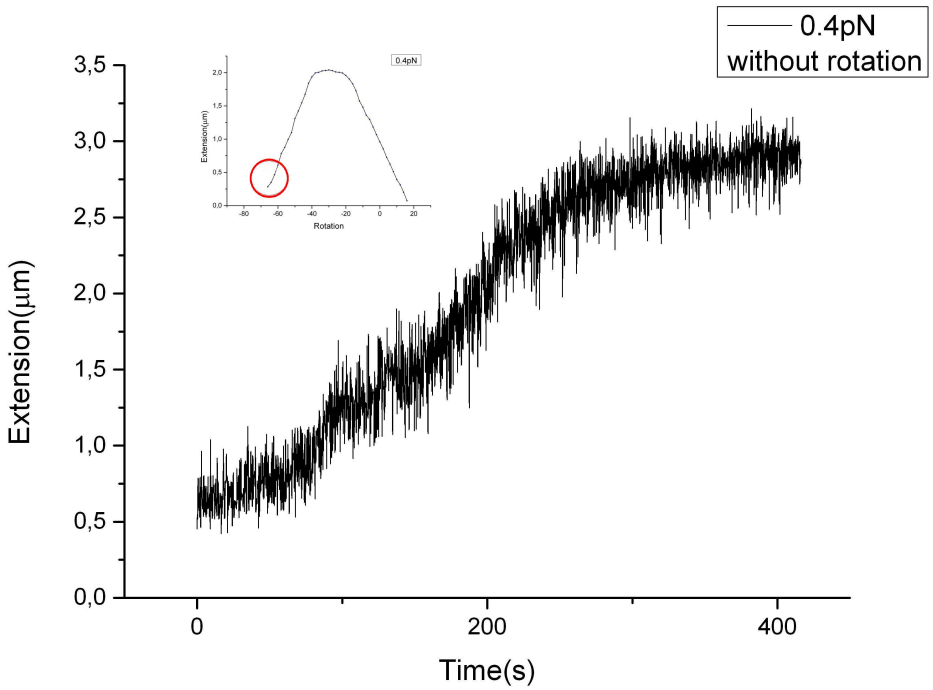


Figure 4.40: For this molecule, it is possible to take a quite good rotation curve under 0.4pN if the magnets rotate not too slow. The rotation position was held where is circled in red and ran a time curve.

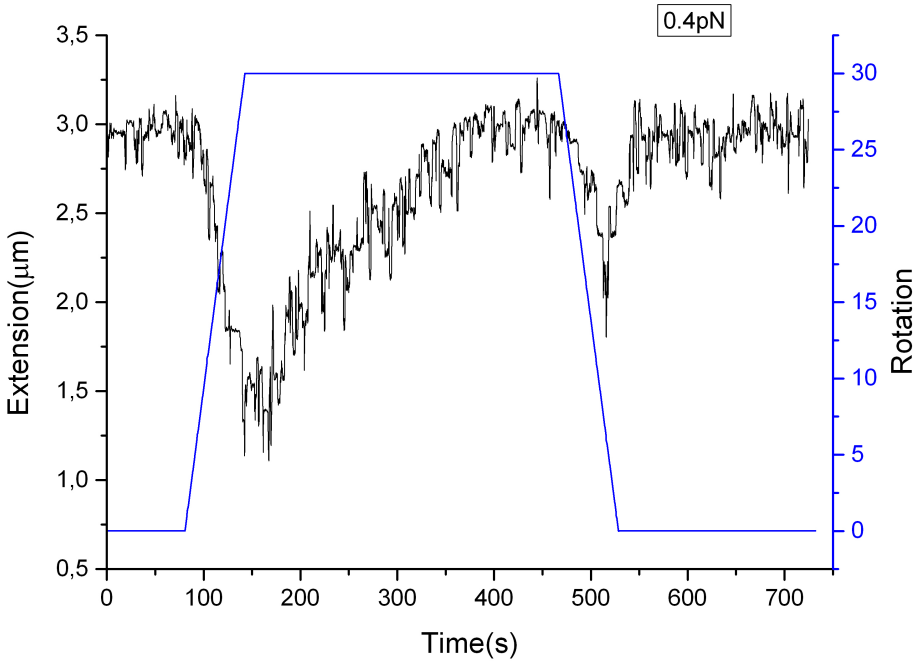


Figure 4.41: The molecule can be pressed shorter, but if the rotation is held unchanged at the lowest place of the curve, the molecule will extend slowly.

4.5.2 Double Molecules instead of Single Molecules

Our aim is to test whether metal molecules can really cut DNA. This can only work with single molecules. The metal molecules bind onto a strand of DNA and damage the bond between phosphate and sugar. The stucture of DNA molecules would be partly broken such that they could not replicate themselves anymore. The cutting functionality would be seen by rotating the beads. Assume that two DNA molecules are bound to one bead, as Fig.4.42 shows. Suppose that DNMC molecules make a few strand breaks on both DNA molecules. By rotating the bead the both DNA molecules (with strand breaks) wind with each other, and we can still get a rotation curve. Since we can not see the cutting process by our unaided eye, we can not be sure whether the cutting has worked. Only if two DNMC bind on both strands of one DNA molecule at the same position, as shown in Fig.4.43, the peak of rotation curve (Fig.4.44) will disappear, since now only one single molecules hangs on the bead. This situation may occur, but it is supposed to be very improbable. Therefore in the experiments, only single molecules are applicative.

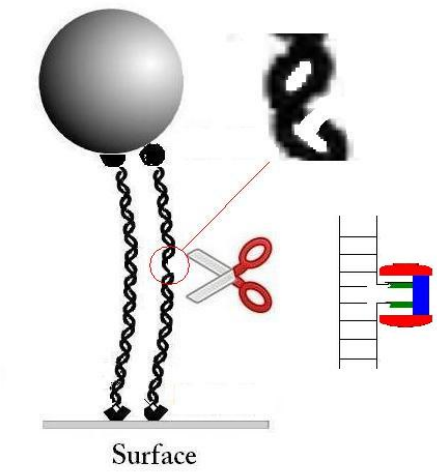


Figure 4.42: DNMC makes a strand break on the DNA strand. The bead has still two hanging molecules.

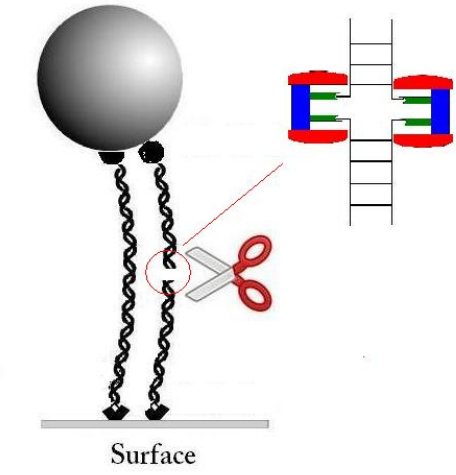


Figure 4.43: Two DNMC bind on both strands of one DNA molecule at the same position, one DNA molecule is cut through. Now only one single molecules hangs on the bead.

In the following figure we will show how a double molecule (more than two molecules hanging on a bead is also possible) performs in a rotation curve. Non-single molecules show themselves either with an obvious peak or as a symmetric curve under a high force over 1pN, which we can compare with Fig.4.7.

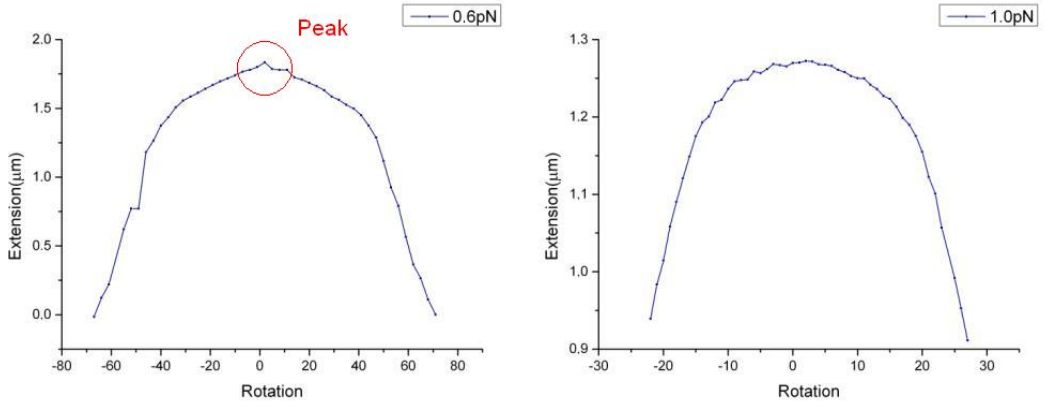


Figure 4.44: Non-single molecules perform in the curves either with an obvious peak or as a symmetric curve under a high force over 1pN.

Apart from this, whether the surface is well incubated and all the ingredients in buffers are still active is also very important. But this would be easy to find out. The appearence of some negative factors would result in a surface on that nothing sticks or everything sticks (e.g. beads without DNA on them).

4.5.3 Another Problem

In the experiments above, we have seen that the metal complexes have bound on the DNA strands, but the expected cutting effect has in most cases not happened. It is assumed that the BSA in SB buffer might have hindered the cutting functionality of DNMC. We have thought of a possible solution. In this idea, PBS buffer without BSA would take the place of SB buffer. Since the beads will bind unspecifically on the surface without BSA, the external force would be always put onto the sample and stretch the DNA molecules a little straight while flowing the DNMC solution into the cell. The DNA molecules have then no chance to be relaxed as a ravel. It might perturb the DNMC to bind with each other and create DNA knots on the strands, but instead it might increase the possibility that DNMC breaks the DNA strand. This idea still has to be tested.

5 Conclusion

In our experiments so far, we have got only one result of nickel complexes which may prove the cutting functionality of DNMC. Since the concentrations of the other two DNMC solutions were relatively low, we have causes to believe that with higher concentrations the cutting performence might be caught. Actually, in complementary experiments with electrophoresis, it has been found out that with 200μM Cu-DNMC, the cutting has occured several times. That should also be a good hint, how we should do the next experiments. In addition, all three metal complexes are supposed to be able to create a hydrolysis of phosphate esters. The problem is under which conditions this functionality can be activated.

The cutting functionality of DNMC is supposed to be applied in medicine science. With binding firstly on recognizing molecules, they could reduce or even stop the replication of DNA, so that cells can not be proliferated. This could be widly used in therapy and the medicinic area, cure of cancer would be a good example. The functionality could be applied to fight against all pathogenic cells. Our experiments are just the beginning of this research. The coming experiments will bring us more.

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Appendix: Preparation of Bifunctional DNA[1]

- Materials:
  - native λ-DNA(250μg/500μl konz: 465μg/ml, Promega)
  - Klenow Fragment exo-(NEB, 5000units/ml)
  - Biotin-11-dCTP(Metabion, 5mM)
  - dGTP, dATP, dTTP(Metabion dNTP set, 4×25μmol in H_2O)
  - Amicon-filter / cut-off 50,000/cut-off 10,000
  - Restriction Enzym ApaI(NEB, 50000units/ml)
  - Oligo 1 with Oligo 2(Metabion)
  - T4 DNA Ligase(NEB, 400,000 cohesive end units/ml)
  - Digoxigenin-11-dUTP, alkali stable(Roche,1mM)
  - NaCl-Tris Buffer(150mM NaCl+10mM Tris pH8.0)

- Methods:
  - Mark both sticky ends of the native λ-DNA with biotin.
    - Melt 102.4μl λ-DNA in PCR-tube at 50°C, in thermocycler for 5 minutes.
    - During this moment pipette the following components together.
      ATTENTION: WORK ON ICE!
      For 1120μl solution:
      100μl 10×NEBuffer 2
      900μl H_2O (steri. Milli-Q)
      10μl Klenow fragment exo-
      dATP, dGTP, dTTP each 1μl
      62.5μl Biotin-11-dCTP

[1] from Helene Schellenberg, referred to the protocol of Niklas Bosaeus & Steve Smith: How To Make 1/3-Lambda DNA for the Minitweezers. 19.11.2009

* Portion the mixure into 10×0.2ml Eppendorf tubes, each with 112μl. Pipette 10.24μl melted λ-DNA into each tube, divide the solution into 4 portions 4×280μl.
* In thermocycler with the program/Bio/37°C for 2 hours.
* Clean the biotinylated λ-DNA from rest of the enzymes etc. by using Amicon Ultra 50K devices.
* Use an Amicon-filter for each 280μl solution.
* Pipette the complete mixure into an Amicon-filter and centrifugalize it in centrifuge at 14000g for 10 minutes. Discard the supernatant.
* Wash the mixure with NaCl-Tris buffer 4-5 times, in centrifuge again at 14000g for 10 minutes. Discard the supernatant.
* Put Amicon-filter in a new tube and centrifugalize the solution at 1000g for 3 minutes.
* Pipette 160μl sterile Milli-Q H_2O+40μl NEBuffer 4 into each 280μl biotinylated λ-DNA.
* There is about 220μl biotinylated $\lambda-$DNA left in eluate(about 11-12μg/220μl).
* The biotinylated $\lambda-$DNA can now be storaged at -20°C or be used for the next experiment.

– Cut the biotinylated $\lambda-$DNA with restriction enzyme ApaI at 10068bp.
  * **ATTENTION: WORK ON ICE!**
  * For 220μl solution
    200μl biotinylated $\lambda-$DNA
    8μl restriction enzyme ApaI
    4μl 100× BSA
  * In thermocycler/Program/Restriction/25°C for 2 hours, 65°C for 20 minutes.
  * The cut biotinylated $\lambda-$DNA (termed as Fragment 1)

can now be storaged at -20°C or be used for the next experiment.

- Hybridize oligo 1 with oligo 2.
  For 50μl solution
  Pipette 25μl Oligo 1 and 25μl Oligo 2 into a PCR-tube.
  In thermocycler/Program/Primerhybridation/80°C − 30°C

- Hybridize fragment 1 with oligo1+2.
  * Pipette 25μl oligo1+2 to 50μl fragment 1 and hybridize at room temperatur for 30 minutes.
  * **ATTENTION: NOW WORK ON ICE!**
    For 20μl solution
    1μl T4 DNA ligase
    2μl 10×T4 DNA-ligase buffer
    17μl fragment 1 + oligo1+2
    Pipette them all together in one PCR-tube
  * In thermocycler/Program/Ligation/16°C for 30 minutes 65°C for 10 minutes.
  * Seperate the T4 DNA ligase from fragment 1 + oligo1+2 with Amicon Ultra 10K devices.
  * Pipette the complete mixure into an Amicon-filter and centrifugalize in the centrifuge at 14000g for 10 to 15 minutes. Wash with 400μl NaCl-Tris buffer 2 times.
  * Discard the supernatant, and put the Amicon-filter into a new tube. Centrifugalize at 1000g for 2 minutes.
  * There is now about 35μl sample(fragment 1 + oligo1+2) and 15μl NaCl-Tris buffer in the eluate.

- Mark the 3' end with digoxigenin-11-dUTP.
  ATTENTION: WORK ON ICE!
  For 254μl solution
  50μl fragment 1 + oligo1+2

20μl 10×NEBuffer 2
180μl H_2O (steril. Milli-Q)
2μl Klenow fragment exo-
1μl dGTP
1μl Digoxigenin-11-dUTP

- Divide the mixure into 2 PCR-tubes with each 127μl.
- In thermocycler/Program/Bio/37°C for 2 hours.
- Clean the fragment 1 + oligo1+2 + digoxigenin from the rest enzyme etc. with Amicon Ultra 10K.
- Pipette the mixure into an Amicon-filter and centrifugalize in the centrifuge at 1000g for 2 minutes.
- Discard the supernatant. Put the Amicon-filter into a new tube and centrifugalize at 1000g for 2 minutes.
- There is now about 22μl sample (fragment 1 + oligo1+2 +Digoxigenin) and 28μl NaCl-Tris-buffer in the eluate.
- Storage at 4°C (for short time) and -20°C (for long time).