Exploring the Interaction of Minor-Groove-Binder Netropsin with DNA Using Optical Tweezers

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Irbazhusain Shaikh

Mentors
Dr. Thayaparan Paramanathan & Dr. Kenneth Adams

Submitted in Partial Completion of the Requirements for Interdisciplinary Honors in Biological Sciences and Physics, Photonics and Optical Engineering

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May 9, 2023
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Dr. Thayaparan Paramanathan, Thesis Advisor Date: 5/9/2023

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# Table of Contents

Acknowledgement ................................................................................................................. 2

Abstract .................................................................................................................................. 5

Introduction ............................................................................................................................... 6

Understanding Cancer as Disease ......................................................................................... 6

DNA – The Heredity Material ................................................................................................. 6

DNA Replication & Transcription ........................................................................................... 9

Targeting DNA with Drugs ..................................................................................................... 11

Netropsin: A Classical Minor Groove Binder ......................................................................... 12

DNA Binding Properties of Netropsin .................................................................................... 13

Optical Tweezers: A Single Molecule Technique ................................................................. 14

Physics of Optical Tweezers ................................................................................................. 15

Trapping and Stretching DNA using Optical Tweezers ....................................................... 19

Studying Netropsin with Optical Tweezers .......................................................................... 21

Materials and Methods ......................................................................................................... 22

Biomaterials ............................................................................................................................ 22

Dual Beam Optical Tweezers Setup ....................................................................................... 24

Flow-cell Creation ................................................................................................................... 27

Pre-Experiment Alignment of the Optical Tweezers ............................................................. 30

Trapping Beads to obtain the Stiffness Curve ....................................................................... 32

Trapping and stretching the DNA Molecule .......................................................................... 35

Experiments with Netropsin ................................................................................................. 37

Results ...................................................................................................................................... 39

Normal Stretch and Release Experiments .......................................................................... 39

Progressive Stretching Experiments ..................................................................................... 41

Time Delay Experiments ....................................................................................................... 44

Discussion ............................................................................................................................... 45

References ............................................................................................................................... 47
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Abstract

Netropsin is an antibiotic that binds in the minor grooves of DNA, which also exhibits anticancer properties. There have been many previous studies that explored the binding of this drug to DNA using traditional methods where an ensemble averaging is used. In this study we explore the interaction of Netropsin with DNA at a single molecule level using dual beam optical tweezers. We trapped and stretched a single DNA molecule using optical tweezers to measure the force experienced by the DNA as a function of extension in the absence and presence of various concentrations of Netropsin. Our results show the binding affinity of Netropsin is in micro-molar range which corroborates with the previous studies. The preliminary data from this study proposes that melting of the DNA facilitates the binding of Netropsin. This outcome is hard to believe because melting of the DNA alters its double helix structure, which should not facilitate any groove binders. Additional experiments would allow us to better understand the binding kinetics of Netropsin and completely characterize it. Furthermore, this can serve as a base model to study drugs with similar binding properties and can assist in the development of potential therapeutics.
Introduction

Understanding Cancer as Disease

Cancer, the most feared disease in the world; is a disease that plagues our society and is at the forefront of scientific research around the globe. There are many types of cancer that affect human beings. Cancer can strike at any age, and it can occur among people of both sexes. Despite decades of scientific research and clinical trials of promising new medicines, cancer is the second leading cause of morbidity and mortality in the US. In 2020, there were over 600,000 cancer-caused deaths reported which accounts around 18% of all deaths in America [1].

But what exactly is cancer? Our body is made up of trillions of cells and it is the fundamental unit of life. All the organs in our body consist of these cells which carry out different tasks. For the proper functioning of organs, the worn-out cells must be eliminated by our immune system [2]. Sometimes these worn-out cells manage to dodge the destruction and form a shape of an irregular lump called tumor. The cell divides uncontrollably and further infiltrates other normal cells and destroys them causing cancer. Cancer can occur anywhere in the body. The root cause of most cancer originates due to the DNA mutation inside the cell [3].

DNA – The Heredity Material

Deoxyribonucleic acid (DNA) is the heredity material which exists inside the nucleus of human cells and in all other living organisms. And it is the molecule of life which determines all our
physical characteristics. DNA was first isolated in 1869 by the Swiss chemist named Friedrich Miescher [4] after Gregor Mendel initiated the basic principles of heredity, through breeding experiments with peas in 1865 [5]. Following Mendel’s principle in 1928, a British biologist named Frederick Griffith displayed the transformation of non-pathogenic to pathogenic strain though the “transforming principle” in bacterium [6]. This was later confirmed in study conducted by Avery, McCarty, Mcleod in 1944 that the DNA is the genetic material of the cell [7].

However, a Russian born American biochemist Phoebus Levene had laid the groundwork to study the structure of DNA even prior to the finding of DNA as a carrier for genetic information. He proposed a study of chemical composition of DNA, in which he discovered that the DNA is abundant with 3 components: five carbon sugar (pentose), a phosphate group and four nitrogen bases- adenine (A), cytosine (C), guanine (G) and thymine (T) (Figure 1).

![Chemical structure representation of four nucleotide bases of DNA.](image)

Levene proposed that these nucleotide are linked together through the phosphate groups forming a tetranucleotide structure [8]. Subsequently, in 1949, Erwin Chargaff expanded Levene’s work by revealing the details of DNA composition. He reported, the amount of adenine was same as thymine and the amount of cytosine was same as guanine. But the amount of A and
T not necessarily the same as G and C, which strongly disapproved Levene’s tetranucleotide hypothesis. These equal amount of ratio in nucleotides propounded the concept of base pairing in which adenine must pair with thymine and guanine must pair with cytosine which is known as Chargaff’s rule [9].

The crucial discovery of the double helix DNA structure was not acknowledged until 1953, when James Watson and Francis Crick postulated the theory [10] using X-Ray crystallography images obtained by Rosalind Franklin and Maurice Wilkins [11]. They showed a 3-dimensional twisted ladder like structure of DNA (Figure 2) [12]. This structure comprised of two strands (green and brown) interwind each other also known as backbone, composed of sugar phosphate and are linked through the nitrogenous bases [13]. The DNA strands run from 5’ end to 3’ end in which the phosphate is at 5’ end and the sugar is at 3’. This arrangement creates two grooves in the DNA double helix structure known as major and minor groove. Based on Chargaff’s rule Watson and Crick demonstrated the base pairing of the nitrogenous bases. In which A shares two hydrogen bonds with T and G shares three hydrogen bonds with C. The space between these base pairs is 0.34 nm and the average diameter of the DNA is about 2 nm [14]. Two out of the three processes that
are associated with the central dogma of biology, replication and transcription, are based on DNA. Understanding these processes is essential to determine how DNA is involved with cancer.

**DNA Replication & Transcription**

DNA replication is one of the fundamental processes that enables cells to maintain the integrity of their genetic material and support their essential functions. Through replication, a double-stranded DNA (dsDNA) molecule is replicated to produce two identical copies of dsDNA. This process ensures that each new cell receives a complete set of genetic material during replication (Figure 3). The dsDNA is unzipped and opened creating a replication fork by an enzyme called helicase making it two single stranded DNA (ssDNA).

![Figure 3: Schematic of DNA replication process showing the “replication fork”. Helicase (green) unwinding and unzipping the dsDNA (blue) into 2 ssDNA, and DNA polymerase (purple) adding the complementary bases to form the new strand (red).](image)

There are many proteins that help in creating this replication fork and synthesizing the DNA further. The most important one among the synthesizing proteins is the DNA polymerase (DNA
The DNA Pol. synthesizes the DNA by adding complementary nucleotides to the template strand of the DNA. DNA polymerase adds new strand of DNA by extending 3' end of the current nucleotide chain. Meaning, it reads from 3' end to 5' of the strand to replicate the DNA.

Transcription is another important process that is part of the central dogma of biology, where DNA segments are transcribed to create the messenger RNA (ribonucleic acid) by RNA polymerase (Figure 4) [15].

RNA molecules are single stranded unlike the dsDNA molecules and one of the nitrogenous bases associated with DNA thymine (T) is replaced with uracil (U) in the RNA. In addition to producing mRNA for translation into protein, transcription is also involved in other RNA-mediated processes, such as the production of ribosomal RNA (rRNA) and transfer RNA (tRNA). These types of RNA molecules are critical components of the cellular machinery responsible for protein synthesis [16]. Transcription is also subject to regulation by various cellular factors, including transcription factors [17], chromatin modifiers [18], and non-coding RNAs [19]. These regulators

Figure 4: Schematic representing the transcription process. RNA Polymerase (yellow) binds to the promoter region of the DNA, opens the strands of the dsDNA entering from the right (pink) and starts the transcription from left to right using template DNA strand (on the bottom) creating an RNA-DNA hybrid (light green and pink). As the process goes forward the dsDNA is reformed (green) and mRNA (light green) is created.
can alter the accessibility of DNA to RNA polymerase, as well as the rate and accuracy of transcription. Dysregulation of transcription can lead to various diseases, including cancer.

**Targeting DNA with Drugs**

One way to control the proliferation of cancer cells is by targeting them with the small-molecule drugs, that could potentially bind to the DNA and stop the replication or transcription process [20]. These small-molecule drugs interact with DNA via covalent or non-covalent bonding. Covalent bonding is irreversible whereas non-covalent bonding of drug-DNA is reversible. Since we study reversible nature of drugs in our lab we will mainly focus on the non-covalent interaction between drug and DNA. There are three major ways drugs bind non-covalently to the DNA; intercalation, single stranded binding and groove binding [21] (Figure 5). The focus of our study in this project is within the groove binders, specifically the minor groove binders.

Groove binding occurs within the grooves of the DNA. As mentioned earlier the dsDNA consists of major groove and minor groove that are unequal in width. Relatively bigger bulky compounds bind into the major groove of the DNA due to the larger space in that groove are known as major groove binders. Smaller molecules that bind to the minor groove are characterized as minor groove binders. Minor groove binders are predominantly crescent shaped molecules that non-covalently binds to the DNA with specific sequences by a combination of directed hydrogen

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Figure 5: Major three reversible binding modes of small molecules (green) to the DNA.
bonding to base pair edges and Vander Waals interaction with the minor groove [22]. Minor groove binder once bound to the DNA act as a roadblock for the helicase. This inhibits DNA replication, which ultimately leads to the death of cancer cells [23].

**Netropsin: A Classical Minor Groove Binder**

Netropsin (Figure 6) is an antibiotic and also possess anticancer properties that was isolated in 1951, from a bacterium called *streptomyces netropsis* [24]. Netropsin binds non-covalently to the minor groove of the DNA and hence preventing DNA synthesis. It is commonly used as a one of the basic framework in the development of potential groove binding drugs [25]. Netropsin consists of unfused aromatic rings with basic group on its terminal allows it to connect with the DNA. It is a crescent shaped molecule with 2-methylpyrrole rings connected by an amide bond.

*Figure 6: Chemical structure of Netropsin*
Using X-ray crystallography the chemical composition of this molecule showed that it binds strongly deep into the minor groove via hydrogen bonding stabilized by van der Waals and electrostatic interaction primarily at the A-T rich region of the dsDNA [26] (Figure 7).

The A-T rich region in the minor groove has narrowness which forces this molecule to fit well in the center of the groove, with each of its pyrrole rings approximately parallel to the walls of the groove in its own region (Figure 7).

**DNA Binding Properties of Netropsin**

It has been reported that Netropsin binds to dsDNA in micromolar range in general and to some specific sequences at nanomolar range [27]. Netropsin has both ends positively charged, which creates an electrostatic attraction towards the negatively charged phosphate groups on the DNA strands. It is shown to have higher affinity to 4-5 repeated AT sequences [28]. Netropsin molecule has an intrinsic twist that helps its insertion into the groove [29]. The insertion of Netropsin to DNA groove interferes and inhibits the enzymes that are required for DNA synthesis. By doing so it obstructs the growth of human breast cancer cells causing programmed cell death [30]. It is
also believed that Netropsin inhibits cell cycle progression and induces apoptosis in cancer cells by targeting DNA replication and altering gene expression by binding strongly to DNA. Alternatively, a research study discovered a very interesting result showing that Netropsin targets the human tumor virus known as Epstein Barr Virus (EBV) in the lymphoma cells [31].

DNA binding of Netropsin has been extensively studied previously using various bulk methods. Several studies have suggested the binding affinity of Netropsin to the DNA in the range of micromolar to nanomolar range. A research using circular dichroism (CD) spectroscopy along with thermal melting and sedimentation analysis reports the binding affinity of Netropsin to be $2.9 \times 10^5 \text{ M}^{-1}$ with 6.0 nucleotides per binding site.[32]. On the other hand, Netropsin studied using different techniques like isothermal titration calorimetry (ITC), differential scanning calorimetry (DSC) and biosensor-surface plasma resonance (SPR) revealed the binding affinity to DNA to be in the nanomolar range. These studies reported binding affinities in the range from $1.7 \times 10^8 \text{ M}^{-1}$ to $2.9 \times 10^8 \text{ M}^{-1}$ [27].

Many of these techniques involves bulk experiments to quantify the properties of the drug [33]. In this project, our goal is to use optical tweezers, a single molecule technique to understand the binding mechanism at the molecular level.

**Optical Tweezers: A Single Molecule Technique**

Optical tweezer is a very powerful tool that is used to study a wide range of biological systems on a molecular level by isolating a single molecule and exploring the interactions with that
molecule. The idea of optical tweezers was first pioneered by Dr. Arthur Ashkin in 1970s by developing the theory of trapping a micron sized particle using light [34]. Later in 1986, Steven Chu a scientist at AT&T bell labs developed the first optical trap to cool and trap atoms [35], he was awarded the Nobel Prize in Physics for this in 1997. The following year in 1987, Dr. Ashkin and his team were successfully able to trap the first biological material without damaging it. Initially it was difficult to trap the biological sample when they used a green argon laser which had 515nm in wavelength that killed the bacterium, that is because the water molecules absorbing the energy from the radiation of light would heat up the sample eventually destroying the sample. Dr. Ashkin overcame this hurdle by switching it to an infrared laser with wavelength of 1064nm, in which the energy absorption by water was minimal [36]. Following Dr. Ashkin’s discovery, many researchers conducted successful experiments using optical tweezers to study the biological materials like, a single cell [37], red blood cells [38], RNA [39], DNA [40], etc. and ever since optical tweezers has been widely adapted technique to study biological materials at the single molecule level.

**Physics of Optical Tweezers**

Dr. Ashkin’s study reveals that a strong laser beam focused to a single point can trap and hold objects ranging from micrometer to nanometer in size [41]. One might be wondering how light traps such tiny objects.

To understand the theory behind light trapping the micron sized particles, we need to understand the force that is produced by the light. In our case, we use light to trap micron sized polystyrene
beads. Particles that are orders of magnitude bigger than the wavelength of the laser, just like the beads we use in our experiments, act as objects of refraction. The beam hitting the bead can be accounted as the collection of rays. The forces acting on the bead can be explained using ray optics.

To better grasp the concept of refraction, imagine a pencil inserted in a glass half filled with water, you’ll see that the pencil appears to be bent at the interface. That is because light travels at different velocities based on the medium it passes through, causing the light to bend when it transmitted via the interface causing refraction.

Snell’s law expresses the phenomena of light refraction mathematically; \( n_1 \sin(\theta_1) = n_2 \sin(\theta_2) \), where, \( n_1 \) is the refractive index of the first medium and \( n_2 \) is the refractive index of the second medium and the angles \( \theta_1, \theta_2 \) are the angles that the rays make with the normal in first and second medium respectfully (Figure 8).

In our situation, let’s consider a bead along the optical axis of the objective and symmetric rays that enter the bead after focused by the objective (Figure 9)[42]. The rays will be refracted twice once entering the bead and again while leaving the bead. During the first refraction, the refractive index of bead (\( n_2 \)) is larger than the refractive index of its surrounding (\( n_1 \)). Therefore, \( \theta_2 \) will be smaller than \( \theta_1 \) according to Snell’s law. The ray bends in towards the normal. After passing
through the bead, the ray is refracted again, and this time away from normal. The same refraction occurs for a symmetric ray on the opposite side.

If we consider light as collection of particles (photons), this change in the direction of light results in a momentum change of photons. The momentum change of photons determines the direction in which these photons experience the force (Insets in Figure 9). And by adding these forces produced by the rays we can get the net force that photons experience in shifting the rays [42,
The force on the photons is equal or opposite to that on the bead. Hence this is the principle of the force that pushes the bead towards the focal point (scattering force).

Alternatively, the gradient force carry through if the bead experience the displacement or it is not centered axis of objective [42, 43]. Gaussian’s profile of beams makes a radially symmetrical diffusion of the laser intensity. High intensity rays comes from the center of the gaussian’s profile, rays with low intensity comes from the side of the gaussian’s profile (Figure 10)[43].

The bending of high intensity ray pushes the bead towards the center of axis and the bending of less intense ray pushes the bead away from its axis (Insets in Figure 10). Since the higher intensity beam will have greater momentum change compared to the less intensity beam, net force on the bead will have two components as shown in the inset. The force pushing the bead towards the

---

*Figure 10: Schematic illustrating how the gaussian profile of laser helps trapping the bead. The ray in the center of the beam (maroon) has higher intensity compared to the ray from the side (pink), therefore create a greater net momentum change as it is refracted through the bead. This will result in a greater force by the bending of maroon ray as shown in the insets. As a result, the net force on the bead will have a component that pushes the bead towards the optical axis (gradient force) in addition to the component that pushes towards the focal point (scattering force).*
optical axis is known as the gradient force and the force pushing the bead towards the focal point is known as the scattering force.

In a dual beam optical tweezers setup, the scattering force is eliminated because the two laser beams travel from opposite directions. This creates a more stable trap that can be used to manipulate trapped objects with higher forces compared to single beam tweezers. In our single molecule biophysics lab at BSU we use dual beam optical tweezers to trap micron sized polystyrene beads and tether a single DNA molecule between two beads.

**Trapping and Stretching DNA using Optical Tweezers**

Optical tweezers revolutionized the study of biomaterials using force spectroscopy. The force measurement in the experiments of optical tweezers is in the order of piconewtons (pN) which match the order of the forces experienced in many dynamic biological systems.

The first isolation of single DNA molecule to study the tension on it while stretching was conducted using magnetic tweezers in 1990s [44]. They were able to measure up to 30 pN force in these experiments. Later the idea of trapping and manipulating DNA molecules with optical tweezers was pioneered by Steven Block and his team in 1997. In their study they applied a force reaching up to around 50pN [45]. Now with dual beam optical tweezers we can stretch DNA and measure up to 150 pN of force.

In our lab we use dual beam optical tweezers that have two counter propagating beams that are finely focused on a single focal point that traps micron sized polystyrene beads within a plexiglass
chamber called a flow-cell. All our experiments take place inside this flow-cell by tethering the DNA between two beads. This allows us to stretch the DNA to obtain the standard stretching curve in which the force is measured as a function of DNA extension (Figure 11).

The standard stretching curve of the DNA is shown in the above figure, in which it has 4 regimes. Initially, the dsDNA starts to uncoil with a small change in force at a low extension (known as entropic regime), once the dsDNA is taught, the force increases rapidly as DNA acts like elastic (elastic regime). Around 65 pN of force the dsDNA base pairs start to break-up and as a result the extension is almost doubled (overstretching transition). At the end of the transition, it is believed that most of the dsDNA is melted into two ssDNA, therefore this transition is also referred to as Force Induced Melting transition [46]. Finally, the force again changes rapidly with extension as the two ssDNA strands are stretched (ssDNA regime). After obtaining this curve we then add the drug of interest to obtain the curve again in the presence of drug and compare the results.
**Studying Netropsin with Optical Tweezers**

There are not many research studies conducted with Netropsin at the single molecule level. In 2003, an experiment conducted using optical tweezers at a lower force of under 25 pN showed no effect of Netropsin binding to DNA (Figure 12) [47].

To our knowledge this drug has not been studied at a higher force using optical tweezers, which would provide us with more insight of the drug binding properties that could be used to characterize the drug. Hence, we use dual beam optical tweezers that have much higher force range than a single beam optical tweezers, we can study the drug through the full spectrum of force. This will allow us to better quantify Netropsin binding at molecular level also understand its binding kinetics. In addition to that this study can serve as a template to study minor groove binders with optical tweezers as no minor groove binders have been completely characterized using this technique.

*Figure 12: Stretching dsDNA in the presence of 10µM Netropsin using optical tweezer study at low force by Tessmer, I., et al. did not show any effect.*
Materials and Methods

Biomaterials

We use four biomaterials which are essential for our experiments: buffer, polystyrene beads, DNA and Netropsin. The pivotal biomaterial in our laboratory is the buffer, which serves as the foundation for all our other biomaterials. Within the SMB lab, the buffer formulation is made to mimic the physiological conditions with a pH of 8.0 and salt concentration of around 100 mM. We formulate the tris buffer solution by adding 5.885 g of sodium chloride (Sigma-Aldrich, S3014) to the 990 ml of Milli-Q water along with the 10 ml of 10 mM Tris (Ambion, AM9856). After mixing it all together thoroughly using pipette, it is then filtered using the Corning™ filter system (Fisher Scientific, 430515) and is tested to make the buffer solution has a pH of 8.0.

Polystyrene beads (Spherotech, SVP-30-5) we use for the experiments are about 3.0 µm in diameter which are chemically coated with streptavidin which allows the binding between the beads and the DNA.

The DNA we use in our lab is from lambda phage (bacterial virus), a commonly used DNA for invitro studies. Lambda phage DNA with overhangs at both ends (Sigma-Aldrich, 10745782001) are polymerized with biotinylated nucleotides A and C (Thermofisher Scientific, 19518018, 19524016) and unlabeled nucleotides (Thermofisher Scientific, R0181) at both ends of the DNA such that the 3’ ends have the biotin label. We then utilized a gel extraction kit (Qiagen 20021) to extract DNA fragments with the aim of isolating the biotin labeled lambda DNA. The chemical
substances, biotin and streptavidin, possess a robust binding affinity towards each other. This binding affinity enables the biotin located at the ends of the DNA to attach to the streptavidin coating present on the beads. Consequently, this enables us to tether a single DNA molecule between two beads for our research purposes.

Another important biomaterial is our prospective drug, Netropsin. It was purchased (Sigma-Aldrich, N9653) in solid form (5 mg). For preparing stock solution, we first added 500 µl of buffer to the 5 mg drug bottle and vortexed before transferring it into a vial. We then added another 500 µl of buffer, vortexed it again to procure all the remaining drug residue which could have been stuck on the bottle or the cap and transferred to the vial. In total we added 1 ml of buffer to 5 mg Netropsin to produce stock solution of 9.93 mM. The vial was mixed thoroughly with the pipette to ensure the solution has uniform concentration. We then transferred the stock solution into multiple Eppendorf tubes with each 100 µl of 9.93 mM Netropsin and stored in a freezer at -20 °C. During experiments one of the Eppendorf tubes with drug would be thawed at room temperature for at least an hour before and vortexed it before flowing the drug. During thawing of the drug, the tubes are wrapped with aluminum foil to avoid exposure to light because Netropsin is sensitive to light.

The DNA and beads are stored in a temperature-controlled refrigerator at a temperature of 4 °C. Proper maintenance and handling of these biomaterials are very important to carry out experiments.
Dual Beam Optical Tweezers Setup

The experiments for this research project were conducted in the Single Molecule Biophysics (SMB) lab at Bridgewater State University (BSU) using dual beam optical tweezers (Figure 13). In this set-up we use a single laser beam that splits into two separate counter-propagating laser beams that are finely focused by microscopic objectives to single point inside a flow chamber to trap micron-sized small particles. The dual beam setup allows us to reach relatively higher forces ~ 150 piconewtons (pN) when conducting experiments. The entire setup of our optical tweezers is constructed on an optical table that is levitated by compressed air to seclude from any vibrations from the surrounding.

The laser used for the setup is housed within a Compact Laser Diode Driver with temperature control which provides the necessary current to operate the laser and keeps the laser at working
temperature of 25°C (Thorlabs, CLD1015). We use a custom-built butterfly diode laser source (Lumics, LU0786M250) that has a wavelength of 785 nm with a power of 275mW. This laser is coupled to a fiber port through a polarization maintaining fiber and is set to be linearly polarized perpendicular to the optical table. The optical components of the set is provided in Figure 14.

As shown in Figure 14, the initial laser beam is split into two with equal power by a 50/50 beam splitting cube. These beams are then guided by laser line mirrors to polarizing beam splitting cubes on both sides, which consist of two prisms with dielectric coatings between their hypotenuse planes. These cubes reflect linearly polarized light that is parallel to the plane with the dielectric coating, while allowing the other polarization to pass through. On both beam
routes, the first set of polarizing beams splitting cubes are positioned so that the vertically polarized light is reflected towards the direction of the objectives. The beams then pass through another set of polarizing cubes that are oriented in way to transmit the beam through them. Then they pass through quarter wave plates, which modify their polarization by altering one component of the polarization by a quarter wave with respect to the other component. This changes the polarization of the light from linear to circular. The circularly polarized beams then enter the microscopic objectives. These objectives have 60X magnification and a 2 mm working distance and focus the laser inside a flow cell (a plexiglass chamber in which we conduct all the experiments) to create an optical trap.

After the beams emerge from the flow cell on the other side they go through the second objective, then they go through another round of polarization adjustment by passing through the quarter wave plate on the other side. This time, the quarter wave lagging of the component makes the circularly polarized light to horizontally polarized light. The polarizing beam splitting cubes positioned adjacent to these wave plates are aligned to reflect the horizontally polarized light towards the position sensing detectors (PSD). This light is focused with the help of a lens into the PSD and used to measure the deflection of the laser that is used to estimate the force created by the trap.

The cubes possess imperfections that permit a small fraction of laser to pass through, which then traverses through a succeeding arrangement and subsequently, projected onto a camera with the help of a beam splitter mirror. This allows the laser spot and optical trap to be visualized on a computer screen that is connected to the camera. Finally, a blue LED source is used to illuminate
the flow cell from either side, and the light passes through all the optical components since it is randomly polarized and projected into the camera.

**Flow-cell Creation**

As mentioned in the previous section, all our experiments are conducted in a custom made airtight plexi glass chamber called a flow-cell. The flow-cells are manually assembled by procuring the plexiglass spacer (Figure 15) which is a 22X30 mm in size from the machine shop at BSU, which is pre-equipped with three strategically placed holes designed to accommodate the micropipette tip, waste tube, and four inlet tubes, as well as a central hollow channel.

![Figure 15: Image of the plexiglass spacer (before crafting a complete flow-cell)](image)

The subsequent step in the fabrication process entails thorough cleaning of the flow-cell using a solution of dish soap, followed by drying. Additionally, the flow-cell undergoes an extensive purification process utilizing methanol, aimed at meticulously removing any residual oils or contaminants resulting from the milling process.
An evenly distributed layer of optical adhesive (Thorlabs, NOA68) is applied to one side of the plexiglass spacer using microscope cover glass slide making sure no adhesive is getting into the any hole or the channel. A new microscope cover-glass slide with dimensions 22 x 30 x 0.13 mm (Fisher Scientific, 12-545A) is then placed on the adhesive with precision. The assembly is then subjected to UV light for a period of 45 minutes to allow for the glass slide to cure onto the plexiglass spacer. This sequence of steps is repeated on the opposite side to produce a chamber. This creates a chamber in the middle of the flow cell.

Next step involves the insertion of a borosilicate glass micropipette (WPI, TIP1TW1) into the flow-cell. Careful manipulation is required as the tip of the micropipette is extremely fragile and breaks easily upon contact. A microscope at 100 times magnification is utilized for insertion of glass micropipette tip through the top canal of the flow-cell until it reaches a point a little less than halfway into the chamber. Once the micropipette is in place, optical adhesive is applied to seal the pipette at the start of the top canal and then cured using a UV lamp for about 10 minutes. A diamond scribe is then used for the purpose of fracturing the micropipette tip, leaving approximately a half-inch extension from the flow-cell. Following this procedure, a 15 cm span of Tygon Microbore Tubing with an internal diameter of 0.050" and an external diameter of 0.090" (Cole Parmer, EW-06419-01), is excised and affixed to the shortened end of the micropipette. The tubing is then adhesively secured to the flow cell in order to ensure optimal functionality.

Following this, four BD Intramedic Polyethylene tubing with an inner diameter of 0.011” and an outer diameter of 0.024” (VWR, PE10) are inserted as inlets through one side of the chamber. The length of these tubes is selected to be around 17 cm, and carefully inserted to the edge of
the hole to avoid having too much of the tubing inside the chamber. Having too much length inside the chamber may cause issue during flowing the biomaterials. Optical adhesive and UV light are used to seal the tubing once it is properly placed. The four inlet tubes are used to flow the four types of biomaterials required for the experiment: buffer, polystyrene beads, Λ-DNA, and the drug of interest.

As the final step, a single BD Intramedic Polyethylene tubing with an inner diameter of 0.045” and an outer diameter of 0.062” (VWR, PE 160) is inserted into the third hole of the flow-cell as the outlet tube (waste tube). The length of this tubing is selected to be nearly 12 inches, and it is placed carefully in the remaining side hole of the flow-cell chamber. After placement, the adhesive and UV light procedure are repeated to seal the outlet tube. Once all the tubing is secured, the flow-cell is now ready to be used (Figure 16).
Pre-Experiment Alignment of the Optical Tweezers

In order to perform experiments, we do the minor alignment of the laser on a daily basis during experiment days. To do this the four inlet tubing of the flow-cell are connected to the corresponding reservoir tubes, the outlet is connected to the waste container and tubing from the glass micropipette tip is connected with a syringe (Figure 17).

Then by allowing the buffer flow through each inlet tubing flow-cell is cleaned and checked for any leakage. After the cleaning the flow cell and the tubing are filled with buffer making sure that there are no air bubbles in the channel and the liquid is flowing smoothly through all four inlet tubes flow. Once we have a working flow-cell, it is placed carefully on the stage that houses it between the two objectives.

Then TEC on the control box is turned on and waited until it reaches 25°C and then by pressing the laser is turned on. The flow cell is viewed from both sides using the camera software on the
The LED source that illuminates the flow cell is turned on along with the corresponding camera application to view the image. Water is squirited between the flow-cell and objectives to create a continuous medium for the light to pass through and form the image. Once this is done the laser dot can be viewed as a bright spot on the camera projection on the computer screen.

Once the flow cell image is projected on the screen, the flow cell is moved slowly by hand to find the shadow of micropipette tip. Using the shadow as our reference point, the X, Y, Z knobs of the flow cell stage are used to get a clearly focused image of the micropipette tip just above the laser spot (Figure 18).

Then we switch the view to the other camera and by using the piezo controller that controls the stage that houses one of the objectives we align the laser spot to be just under the tip similar to

*Figure 18: Image of the camera screen with laser dot and micropipette tip just above the laser dot.*
the image obtained in the other camera. This process is repeated by switching the cameras back and forth until they have the same image where the laser spot is directly under the tip.

**Trapping Beads to obtain the Stiffness Curve**

The stiffness curve is a very essential part to calibrate the force measurements in our experiments. As a first step, the green and blue dots on the computer interface representing the laser spots on the detector are zeroed to be at the center by adjusting the stages that house the position sensing diode (PSD) detectors (Figure 19).

To obtain the stiffness curve a polystyrene bead has to be trapped. The bead solution stored in the refrigerator is vortexed to mix the two layers of pellet beads and supernatant. The bead reservoir tube is filled with 2 ml of buffer then 2 µl of polystyrene bead solution is added to the tube and mixed with the pipet to have even distribution.
The beads are flown until the first bead is trapped by the laser. The micropipette tip is then maneuvered to be just above the bead and the bead is attached to it by suction with the help of the syringe attached to the end of the micropipette (Figure 20). The rest of the beads are flushed out by flowing in approximately 1 ml of buffer.

![Figure 20: Image of the camera screen displaying the laser dot and a polystyrene bead attached to the micropipette tip inside the flow cell.](image)

After catching the first bead, once again the green and blue dots are aligned to be overlapped but now by adjusting piezo controlled stage that houses one of the objectives.

The bead attached to the micropipette tip can be moved across the laser dot to obtain the trap stiffness curve (Figure 21). This is done by a computer program which moves the piezoelectric stage that houses the flow-cell by a step of 100 nm, such that the bead passes across the laser completely and returns back to the original starting position.
The trap stiffness is a result of the bead experiencing laser deflection that is collected by the positioning sensing diode (PSD) when it passes through the laser dot from right to left and returns to its original position. The laser displacement is recorded on the graph, starting at the zero position before the bead entered the trap and becoming zero again when the bead returned to its original position. The blue and green dots in Figure 21 represents the two detectors on both sides. The solid dots represent the bead moving from the original position, and the hollow dots represent the bead returning to its original position. These hollow dots follow the same curve path as the solid dots when the laser is aligned correctly.
Trapping and stretching the DNA Molecule

Once we have obtained the stiffness curve with the first bead (Figure 22a), a second bead is trapped with the laser (Figure 22b). The stray beads are then rinsed out by flowing in 1 ml of buffer. The bead on the micropipette tip is brought closer to the second bead trapped by the laser and tapped to make sure both the beads are on the same plane. If they are not in the same plane the piezo stage that houses one of the objectives is adjusted to bring them to the same plane.

![Figure 22: Schematic summary of catching beads and trapping of a single DNA molecule. (a) a bead trapped by laser. (b) the first bead attached to micropipette tip and a second bead is trapped by the laser (c) one end of a DNA molecule attached to the bead in the trap and the other end is floating with the flow (d) after attaching the other end of the DNA to the bead on the micropipette tip.]

Then 2µl of DNA is added to 2ml of buffer in the DNA reservoir tube gently and mixed slowly with a 1000 µl pipette for about 15 times. Allowing the flow from the DNA reservoir tube, one end of the dsDNA biotin coated will randomly attach to the bead in the laser trap (Figure 22c). The strong chemical bond between biotin at the ends of DNA and streptavidin coating on the polystyrene beads enables this attachment. Using the flow cell stage controls manually moving the bead attached to the micropipette tip through a “fly fishing” motion allows us to attach the other end of the DNA to the bead held the micropipette bead. When the bead in the micropipette tip is
moved to the left; the bead in the laser trap also moves to the left, this indicates that the single DNA molecule is been tethered between both the beads (Figure 22d). If multiple DNA molecules get tethered, it will show up with higher melting force when we analyze. In this case we need to let the beads and DNA go and start over again by trapping new beads.

Following the catching of the DNA, it is stretched to assess whether the DNA is useful to conduct our experiments or not. We use the same computer program to stretch the DNA which is used to obtain the stiffness curve. This program is run a few times by gradually increasing the number of steps until we acquire all the regimes of the standard DNA stretching curve (as mentioned in the introduction). Good DNA is characterized by overlapping stretch and release without significant hysteresis, and the melting transition length around 10 microns. Once we have a good DNA the data is saved and analyzed using the DNA analysis program developed by Northeastern University (Figure 23).

Figure 23: Screenshot of the program used to analyze the DNA for our experiments. The left window of the program displays a typical DNA stretching curve being analyzed.
As soon as the program is loaded the number of base pairs is set to 48500 and salt concentration to 100 mM. Afterwards, the saved stiffness curve file and saved DNA stretching file is loaded in that order. The DNA data is then compared to the polymer fitting model known as Worm Like Chain (WLC) model, that describes the dsDNA to assess its suitability for our experiments.

**Experiments with Netropsin**

Next step is to stretch the DNA in the presence of Netropsin at desired concentrations by diluting the drug stock solution with buffer. For our 10 µM concentration experiments we added 2 µl of 9.93 mM Netropsin to 1998 µl of buffer; for 5 µM concentration experiments we added 1 µl of Netropsin to 1999 µl buffer and for 1 µM concentration, 0.2 µl of 9.93 mM Netropsin was added to 1999.8 µl buffer.

Once the desired amount of Netropsin is added to the drug designated reservoir it is then mixed well by the 1000 µl pipette to get uniform concentration. About 1 ml of drug is flown into the flow-cell and the DNA is stretched again in the presence of Netropsin. The DNA stretching curve
in the presence of drug is obtained by analyzing the data and compared with the DNA stretching obtained in the absence of the drug by plotting it on to excel sheet.

For our experiments, we used a new DNA molecule every time we collected data. Initially we conducted normal stretching and releasing experiments where the number of steps (Figure 24) were set to completely traverse the melting transition. Then we performed progressive stretching experiments in which we changed the number of steps to cover only parts of melting transition.

In addition to that we conducted the time delayed experiments. In time delayed experiments we allowed more time, by slowly stretching the DNA in the presence of Netropsin. The time delayed experiments were conducted by changing the step delay in the computer program. This is the time of pause after each step.
Results

Normal Stretch and Release Experiments

As mentioned in the methods, initially we conducted normal stretch and release experiments of the DNA in the presence of Netropsin. We used 1 μM, 5 μM and 10 μM concentration of Netropsin (Figure 25) in these experiments. We repeated all experiments at least 3 times with each concentration using a new single DNA molecule.

Figure 25: Stretching curves of the DNA in presence of various concentrations of Netropsin (purple, green and red) and in the absence of Netropsin (black).

Figure 25 represents the DNA without the drug displaying the overstretching regime around 65 pN as mentioned in the introduction, where most of the base-pairs melt and hence also known as the melting transition. DNA stretching curves in the presence of Netropsin indicate that the force required to melt the dsDNA (open the base pairs) increases with Netropsin concentration.
Melting transition with 1 µM and 5 µM Netropsin reach just right above the 65pN, whereas the melting transition in the presence of 10 µM Netropsin reaches about 75 pN.

In addition to that, the stretching curves in the presence of Netropsin exhibit lengthening of the dsDNA (elastic regime corresponding to the dsDNA shifting to the right). From our initial findings it can be stated that Netropsin stabilizes and lengthens the dsDNA.

Once we observed 10 µM Netropsin showing a higher force melting transition compared to 1 µM and 5 µM we decided to conduct our further experiment with 10 µM Netropsin, stretching it twice using the same DNA. Figure 26 represents the stretch and release curves compared between DNA without Netropsin and with 10 µM Netropsin stretched twice.

For this experiment we stretched the DNA with Netropsin into melting transition and upon releasing (red broken line) it exhibits hysteresis. The release curve shows more lengthening of
the DNA indicating more drugs bound. As we stretch the same DNA furthermore into melting transition and released it, the release curve (dark red broken line) extended even further.

This indicates that there is more drug binding upon stretching the same DNA more into melting transition. The possible explanation for this is either the melting is somehow facilitating the binding of Netropsin, or it is a slow binder. The first explanation is an unexpected result because groove binders are not known to facilitated by the melting. As the melting of the DNA alters its structure which would make it difficult for our minor groove binder to bind. After conducting our initial experiments with normal stretch and release of DNA with Netropsin, we were intrigued about discovering whether the melting of the DNA in fact facilitates the binding or is Netropsin indeed a slow binder? In order to examine these questions, we performed multiple progressive stretching experiments and then also conducted multiple time delayed experiments.

**Progressive Stretching Experiments**

During progressive stretching experiments, we progressively stretched the DNA into melting transition with 10 µM Netropsin. At first the DNA was stretched to barely into the start of melting transition and is released in the presence of Netropsin, then the same DNA was stretched almost halfway through the melting transition and released. That was followed gradually going more and more into the transition finally all the way to the end of the transition.
Figure 27 displays the DNA stretching curve in the presence of 10 µM Netropsin (blue) and the DNA stretching curve without Netropsin (black). It is visible that the blue curve is stretched until the beginning of the melting transition and released. Noticeably, the release curve (blue broken curve) is overlapping the stretching curve (blue solid curve), indicating that there is no additional extension observed in the release curve suggesting no additional binding of the drug.

![Figure 27: The stretching curve of DNA without Netropsin (black) and DNA stretched to the beginning of the melting transition in the presence of 10 µM Netropsin (blue). The stretch (blue solid) and release (blue broken) curves overlap in the presence of Netropsin.](image)

We then continued stretching the same DNA further into the melting transition. In Figure 28 the arrows display the extent to which the DNA was stretched and released in the presence of 10 µM Netropsin. Blue arrow highlights the stretch (blue solid curve) and release curve (blue broken curve) of the DNA till the beginning of the melting transition both overlapping. The green arrow indicates the stretching (green solid curve) reaching nearly 50% into the melting transition. Upon releasing it (green broken curve), a little extension can be seen shifting the release curve to the right. Suggesting additional drug binding after the melting. The yellow arrow highlights the
stretching (yellow solid curve) nearly 75% into the melting transition. The corresponding release curve (yellow broken curve) extends more than blue and green release curves, suggesting even more drug binding. The red arrow highlights stretching (red solid curve) all way into the end of the melting transition and upon releasing (red broken curve) it extends a lot more. This experiment clearly indicates that melting more DNA facilitates the binding of minor groove binder Netropsin.

![Graph showing DNA stretching and release curves](image)

Even though the progressive stretching experiments suggest that melting is facilitating the binding of Netropsin, it is hard to believe that the disrupted double helix structure without proper grooves will support the binding of Netropsin. Is it possible that the additional time that we provide to stretch further is facilitating the binding? To answer this question, we incorporated this time into step delay and stretched the DNA slowly in the presence of the drug.
Time Delay Experiments

In time delayed experiments the DNA was slowly stretched in the presence of 10 µM Netropsin at different pulling rates. This was achieved by adding step delay in the computer program after each step, anticipating this would allow more time for the drug to bind. In Figure 29 we can see that the curves in green, red and blue almost overlapping each other until they reach the melting transition, which suggests that upon allowing additional time it does not help the binding of the drug in elastic regime of DNA. Experiments with 300ms and 600ms step delays almost overlap each other during release curve. However, the release curve with 1000ms step delay shows that spending more time in the melting transition allows more drug to bind.

Figure 29: Time delayed experiment data for DNA with 10 µM Netropsin slowly stretched (solid curves) and released (broken curves) at different pulling rates. By adding step delays of 300ms (green curve), 600ms (red curve) and 1000ms (blue curve); DNA stretching curve without Netropsin in solid black.
Discussion

Netropsin is an anticancer and antibacterial drug that inhibits DNA replication which can potentially stop the cancer cells from proliferating. Its crescent shape chemical structure allows it to strongly bind to the A-T rich regions into the minor groove of the dsDNA [48]. We conducted a research study of interaction between Netropsin and DNA at single molecule level using dual beam Optical Tweezers.

The results from this study suggest that Netropsin binds to the dsDNA at micromolar range, which corroborates the previous studies that suggest it’s binding affinity to the generic dsDNA in micromolar range as well [32]. We initially collected our data with three different concentrations of Netropsin (1 µM, 5 µM and 10 µM) by stretching a single new DNA molecule each time. In these normal stretch and release experiments we observed stabilizing of dsDNA in the presence of Netropsin. The hysteresis observed during these experiments suggested that Netropsin may be a slow binder, or the DNA melting facilitated the binding. To test whether the DNA melting facilitates the binding, we conducted experiment by progressively stretching the DNA into the melting transition in the presence of 10 µM Netropsin. From our progressive stretching experiment, we observed more binding of Netropsin when more DNA melted. Furthermore, by conducting time delayed experiment showed no additional binding during stretching indicating Netropsin is not a slow binder. But providing more time in the melting transition allows more drug to bind, which suggest that melting facilitates the binding.

Our experimental results are not expected because melting of the DNA should not facilitate the binding of the groove binders. As the shape of the DNA is altered during the melting which
supposedly would make it difficult for the drug to bind in the groove. However, our research data from multiple experiments suggests otherwise. This reveals more questions to be answered by doing further experiments. It can be achieved by performing constant force measurement experiments with Netropsin, in which we would stretch the DNA in the presence of Netropsin to a certain force and hold it at a constant force until the drug binding reaches equilibrium. These experiments measure extension as a function time. This will allow us to learn more in depth about the kinetics of Netropsin binding. Since this is the first attempt to our knowledge to characterize minor groove binders with optical tweezers, a detailed study of Netropsin can serve as a template to study other minor groove binders. It would also be very helpful in the development of prospective drugs that share similar properties.
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