Abstract

Since the famous discovery of double helix structure in 1953, many other confirmations of deoxyribonucleic acid (DNA) followed. One of such structures is the G-quadruplex that may form at guanine rich sequences of single stranded DNA. Using single molecular methods of magnetic tweezers, we can perturb the structural integrity of the G-quadruplex by applying an external force to determine its stability. We found the force needed to unfold our targeted G-quadruplex sequence, Bcl-2 is at \((23.5 \pm 0.6)\) pN. Following that we tested changes in its stability when a G-quadruplex specific drug, Phen DC3 was added to its medium. The bind state of Bcl-2 with the drug ligand is found to have a higher unfolding force at \((49 \pm 2)\) pN with 50 nM concentration of Phen DC3. These results could serve as the preliminary steps for developing a drug to control specific G-quadruplex stability that may influence its gene expression.
Acknowledgements

“Hate it or Love it, You still have to do it.”

These were the exact words that I heard from my graduating seniors last July. Having spent close to 8 months completing my Final Year Project as a Physics undergraduate, I really loved the entire journey and it is the best academic highlight of my student life in NUS.

I can still recall vividly how this memorable journey started. I was in my grandpa’s flat in China, lying on the sofa deciding which project to choose for my FYP. I was lost and clueless and just 20 minutes to the closing dateline. I looked at the list again and decided to narrow down to the projects that were not taken. Then, I saw the project on G-quadruplex using magnetic tweezers. I thought it sounded really cool so I chose it.

I entered this FYP with absolutely zero knowledge of biophysics, zero experimental experience in a biological laboratory and zero confidence. However, my mindset totally changed when I first met Assoc. Prof. Yan Jie. When he first introduced me to the details of the project, the word that sparked my interest was CANCER. He mentioned that this research would have huge impact on cancer treatment and that really drew me in. The biggest take away from my first meeting with him was “In research, we do not manage people. You do things at your own pace at your own time but
to be a successful researcher you need to be a workaholic.” This quote is
the motivation that shaped my mindset throughout this FYP.

Then I met Dr. You Huijuan, who came from a biological background. Ini-
tially, I was afraid because I thought we would have very different opinions
when it comes to research and technicalities. I was totally wrong. She is
really one of the best in the field. Her knowledge in both disciplines were
extremely strong and I learnt so much from her that I am really grateful
that I have her as my mentor. Discussions with her pertaining experiments
were extremely fruitful and they helped in strengthening my understand-
ings of this field. She never fail to help me if I had any technical problems
from experiments to using MATLAB codes. I am truly in touched by her
dedication and commitment as my Co-Supervisor.

Another important aspect of me that went through this period together is
my family. I need to apologise to my mother for not coming home to have
meals with the family because I was too busy in school. I also feel sorry
for my brother for not been able to guide him in his school work and show
more care and concern for him especially during this phase as a rebellious
teenager. I just want to take this opportunity to say that I love them very
much.

The new friends that I met in Yan Jie’s group are also instrumental to the
success of my FYP. I wish to show my appreciation to Ricksen for teaching
me how to use the AFM, providing me with constructive feedbacks and
sharing with me the wonderful places to visit in Indonesia. I will definitely
visit there this year. Also to Mingxi, Shimin, Chenjin and Durgarao for
helping me troubleshoot, finding missing chemicals and freeing up slots for
me to use the magnetic tweezers. To everyone in the group thank you so
much for everything.

The last group of people that I want to show my deepest appreciation to
are those that have made Chuyuan who I am today. My closest secondary
school friends that have been through more than 12 years of friendship.
Boyuan, Haiyuan, Jin Xuan and Zhipeng for reminiscing those awesome old memories whenever we meet up. My three NPCC brothers Buo Wei, Wei Xiong and Jeffrey for all the late night suppers and overseas trip. My couchsurfing guru Yong Kuang. My adventurous brother Nigel and my home visit brother Sunbo. To my JC classmates that always meet once a semester to update how is life in all departments in the Faculty of Science, from Statistics to Applied Maths are doing. To my closest brothers in arms in 7th Company, Wang Xuan, Ervin, Jian Ting, Desmond, Hong Qing and Nicholas. Life will never be the same without you guys. To my university physicists, Marcus, Boon Kiat, Jufri, Kevin, Jack, Ji Xiong, Samuel, Zhong Hao, Qing Yuan, Zhi Xun, Yi Heng, Fu Kang, Peng Peng and many more. Thank you for making my time in NUS more lively and intellectual.

Lastly, I would like to dedicate this to a special someone, if you know who you are, I just want to say I hope when I look back at this many years later I can have a smile on my face and be with you.

This would most probably mark the end of my pursuit as a physicist but I will always be one at heart!

Zhao Chuyuan
April 2015
Abstract

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Chapter 1

Introduction

In this thesis, we are conducting a single molecular study on the structural stability of G-quadruplex, G4 in short, and how it can be influenced by the addition of G4 binding drugs. This chapter aims to provide a basic understanding of G4 structures, the motivations behind our research and the objectives of our study.

1.1 Background of G4

The double helix structure of DNA, first published by James Watson and Francis Crick in 1953 [1], is well-known to us since our early classes in biology. The standard B-form DNA folds into a right-handed helix [1] that is found in most of human DNA. However, other alternative structures also exist, such as the Z-form DNA in a left-handed helix and even DNA triplexes [2]. The alternative structure that is of strong interest to us is the G4 structure. G4 structures are formed particularly in guanine-rich sequences in our human DNA [3]. These regions include: telomeric, transcription and promoter regions [4–6].
The structure of G4 is thermodynamically stable because its physical properties [3]. In the G4 structure, four guanine nucleotides form a square planar G-quartet that can stack one on top of another [7]. The interactions between the guanine nucleotides are strengthened by four pairs of hydrogen bondings [3]. There is a presence of a central cation that further stabilises the G4 structure [8]. Factors that can influence the stability of the G4 structure includes: length and arrangement of the DNA sequence, size of the loops connecting the guanines and the characteristics of the stabilising cation [3]. G4 structures can be folded from a single molecule or combination of molecules [9]. Since we are conducting a single molecular study, we will focus on intramolecular G4 formed from a single DNA sequence.

1.2 Motivations of research

As G4 structures are usually found in telomeric and promoter regions [4,10] that are guanine rich, they are of high interests to scientists because these are regions that control programmed cell death [11]. They are extremely important because mutations that can result in cancer cells are often found in that regions [12]. With the presence of the G4 structures in these regions, through more scientific studies, we hope to understand the role G4
1.3 Objectives of research

The objectives of our research is to determine the stability of the B-cell CLL/lymphoma 2 (Bcl-2) gene G4 [14], first under standard buffer conditions similar to the human body and later with the addition of Phen DC3 drug ligand to investigate how it may alter the stability of the G4 structure. The measurement we are using to determine the stability of such structure is the force required for unfolding the G4 into a single stranded DNA. By repeating the stretching experiment for numerous trials, we are able to obtain a distribution of unfold forces which can be plotted into a distribution histogram. From this histogram, we are able to evaluate important constants such as the unfolding probability and the transitional distances of the thermodynamic reaction [15].

1.4 Thesis outline

In Chapter 1, we have already covered the main motivations and objectives for our research.

Chapter 2 will be discussing the literature review of the latest findings in this field that could provide us with a deeper understanding of the biological aspects of the G4 in our human genome. We would also relate how have these information sets direction our study.
Chapter 3 will focus on the physical frameworks that are used in this single molecular study that will be useful to us in understanding the experimental techniques used and how they can help us in analysing our results that we have obtained.

Chapter 4 describes the procedures that we have carried out during the three stages of our experiment. We will provide detailed information about the methodology as well as the principles in each important step that we have taken.

Chapter 5 illustrates the results that we have collected from our experiments in determining the unfolding force of the G4 structure. We will also be discussing the validity of our results and the implications of reaction constants that we have obtained.

Finally, we will consolidate our conclusions in Chapter 6 by summarising the knowledge that we have gained from our research and also highlighting further steps that can be taken to explore deeper into this research.
Literature Review

In this Chapter, we will be discussing the recent developments in the field of G4 structures and the human DNA sequences that have been studied. These discoveries and advances form as the foundation for our study in a relatively new area of research.

2.1 G4 in our human genome

As mentioned in the previous chapter, G4 structures are formed in guanine-rich regions in the DNA [3, 8]. Therefore, it is important to understand how prevalent these structures are in our human genome and whether do they exist in vivo. Research in bioinformatics have demonstrated that so far that there are 375,000 possible candidates for G4 formation in our human genome [10, 16]. However, the stability and the physiological conditions were not taken into account thus the actual ones that exist in vivo maybe much lesser [12]. Currently, G4 structures have been found in vivo in yeast and human DNA [13, 17].
2.2 Human telomere

The first human DNA G4 sequence that is widely studied is the human telomere G4. Its DNA sequence consists of repeating (TTAGGG) [7].

![Schematic topological drawing of the human telomere G4.](image)

Figure 2.1: Schematic topological drawing of the human telomere G4.

Scientists are especially interested in the human telomeric region because they control the programmed cell death due to the presence of the telomeric caps at the end of human chromosomes [18]. At the same time, telomeric region is associated with cancer-associated enzyme telomerase is that is able to lengthen the telomeric cap that enable the cell to divide uncontrollably forming a tumour [19–21]. The existence of G4 structures on the telomere sequence may help to stall telomerase elongation [20–22]. By understanding how to stabilise these G4 structures may give us a glimpse how it can act as a noble form of cancer treatment [3,12].

2.3 Human Bcl-2 gene

After understanding the widely-researched telomere G4, we move on to our research objective. The sequence that is the main focus of my research is the Bcl-2 gene that is a mitochondrial membrane protein involved in the control of programmed cell death [23–25]. The GC-rich region upstream of
the P1 promoter enables it to fold into G4 structures [14,26–28]. The main function of Bcl-2 is to inhibit apoptosis [14,29]. However, when it is overly expressed it can result the formation of tumour cells thus if we can inhibit its expression by using G4 targeting drugs, we maybe to able to influence its gene expression to our advantage [26].

The specific segment of the Bcl-2 that we using in our experiments are (GGGCGCGGGAGGAAGGGCCCGGG) with the underlined guanines forming a G4 structure with three G-quartets [14, 26, 28]. Among the four G-strands of this G4 structure, three of them are parallel namely the first, third and fourth strands and the second one is anti-parallel [26]. The unique feature is the looping strand connecting the second to the third strand has 7 nucleotides unlike the rest which have one or three nucleotides [26,28]. And in the case of telomere, all the looping strands have three nucleotides [22]. Thus, it could display interesting properties of this G4 sequence which can be determined from its unfolding force.

Comparing the structure of the Bcl-2 G4 to the telomere G4, we predict to see generally similar structural stabilities with only small derivations.
Chapter 3

Theoretical Background

This Chapter introduces the theoretical framework of physical systems in the understanding of G4 and how they are applied in the context in our research. The areas that will be covered are: paramagnetism, power spectrum and thermodynamics of reaction kinetics.

3.1 Worm-like chain model under force

To study the extension of DNA structures under external applied force, we make use of the worm-like chain model which models a polymer as a thin elastic rod that follows Hooke’s Law with negligible deformations [30–32]. Using the interpolation solution of the worm-like chain model, the Marko and Siggia formula [33] is given by:

\[
\frac{FA}{k_B T} = \frac{z}{L} + \frac{1}{4 \left( \frac{1-z}{L} \right)^2} - \frac{1}{4}
\]  

(3.1)

Where \( F \) is the external force, \( A \) is the bending stiffness, \( L \) is the contour length, \( k_B \) is the Boltzmann’s constant, \( T \) is the absolute temperature and \( z \) is the extension. This equation validates the increase in step size of the unfolded single strand DNA (ssDNA) under external force to confirm that we have unfolded the G4 structure.
3.2 Magnetic Tweezers

Magnetic tweezers have been used in single molecular studies for more than 10 years [34]. The working principle of magnetic tweezers is essentially using permanent magnets to pull a magnetic object to exert a force on it. The magnetic object used is a paramagnetic bead that is attached to a biopolymer that we are investigating. For example, a protein, a polymer or in our case a DNA tether. Depending on the structure of the DNA tether, when we apply an external force to it, we will perturb its structure and cause changes to its extension [35,36].

![Figure 3.1: Setup of magnetic tweezers](image)

However, this method also posts some limitations. For tethers that are less than 1 µm, when they are stretched at a high force, it will be difficult to determine the force exerted on the DNA tether with high accuracy [37]. The solution to this limitation will be explained in Section (3.6).
3.3 Force Generation

3.3.1 Production of a magnetic force

When the paramagnetic bead is in the presence of the non-uniform magnetic field produced by the pair of permanent magnets it will experience a magnetic force. The non-uniform magnetic gradient field will first induce a magnetic dipole moment of the bead and then once the moment is saturated as shown in Figure (3.2), the bead with experience a force that is directed perpendicularly upwards [34, 36].

The magnetic moment of the bead will be saturated in the presence of the magnets used in our experiment, which has a magnetic field strength of 0.2 T, thus the direction of the magnetic moment is constant.

![Schematic diagram of the paramagnetic bead in the presence of the magnetic field viewed from the x-z plane.](image)

Figure 3.2: Schematic diagram of the paramagnetic bead in the presence of the magnetic field viewed from the x-z plane.

Next we can calculate the magnetic potential energy and magnetic force
exerted on the bead:

\[
\begin{align*}
U &= -\mathbf{m} \cdot \mathbf{B} \quad (3.2) \\
F &= -\nabla U \\
&= -\mathbf{m} \frac{\partial B}{\partial z} \hat{z} \quad (3.4)
\end{align*}
\]

Where \( z \) is the distance in the vertical direction of the setup as shown in Figure (3.2).

3.4 Force Calibration at low force region

3.4.1 Calculation force using Brownian motion

In order to determine, the force acting of the paramagnetic bead, we are unable to measure the rate of change of magnetic field strength \( B \) with respect to \( z \) directly. Hence, we have to make use of the Brownian motion in the \( y \)-direction to calibrate the magnetic force [35, 37]. Note that the viewing angle is shifted to the \( y-z \) plane with the positive \( x \) axis directing outwards.

Figure 3.3: Fluctuations in the \( y \)-direction due to Brownian motion viewed from the \( y-z \) plane.
Due to the Brownian motion in the glass slide, it will produce a pendulum-like motion along both the x and y-axis [37,38]. However, only the pendulum motion in the y-direction would affect the magnetic force. The motion in the x-direction is pointing in and out of the plane which will not be affected by the magnetic field gradient.

By modelling the DNA tether as a spring, we can determine its elastic potential energy:

\[ U = \frac{1}{2} k \langle \delta_y^2 \rangle \] (3.5)

Where \( k \) is the spring constant and \( \langle \delta_y^2 \rangle \) is the variance of fluctuation in the y-direction. Using the Equipartition theorem for an one dimensional system with one degree of freedom [38], its internal energy is:

\[ U = \frac{1}{2} k_B T \] (3.6)

Combining Equations (3.5) and (3.6) we can express the spring constant \( k \) as a function of the bead fluctuations in the y-direction \( \delta_y \).

\[ k = \frac{k_B T}{\langle \delta_y^2 \rangle} \] (3.7)

The drift force exerted in the y-direction will be:

\[ F_y = k \delta_y \] (3.8)

From the angular displacement of the DNA tether due the drift force, we can able to relate the magnitudes of the magnetic force and the drift force:

\[ \tan \theta = \frac{F_y}{F_{mag}} = \frac{\delta_y}{l} \] (3.9)

Where \( l \) is the length of the DNA tether.
Hence, by putting Equations (3.8) and (3.9) together we can express the magnetic force in terms of the temperature $T$, length of the DNA tether $l$ and the bead fluctuations in the y-direction [37,38]:

$$F_{mag} = \frac{k_B T l}{\langle \delta^2_y \rangle}$$

(3.10)

One important precaution while doing this calculation is that if the length of the DNA tether is short (i.e. less than 1 $\mu$m), then the diameter of the paramagnetic bead (2.8 $\mu$m) [39] needs to be taken account of in the length of the tether. Then, the pendulum motion of the bead can be factorised into two components: the pendulum motion of the tether $\langle \delta_{tether}^2 \rangle$ and the rotational motion of the bead $\langle \delta_{bead}^2 \rangle$ about the y-axis [36,38]. Since these two motions are independent of one another, the effective motion of the bead is given by:

$$\langle \delta_{tether}^2 \rangle + \langle \delta_{bead}^2 \rangle = \langle \delta_y^2 \rangle$$

(3.11)

Therefore, the effective magnetic force would be the sum of forces due to the motion of the tether and the bead which are different in magnitude due to the difference in length.

$$F_{mag} = \frac{k_B T (l + r)}{\langle \delta_y^2 \rangle}$$

(3.12)

Moreover, for short DNA tethers, this method is only accurate for low force regions, less than 15 pN [37]. For calibration of a larger force, another method is used to calibrate the force extension relationship that will be discussed in the Section (3.6).

### 3.4.2 Measurement of the tether extension

To determine the length of the DNA tether including the diameter of the paramagnetic bead, we make use of imagine processing software together
3.4 Force Calibration at low force region

with a high resolution speed camera to track the imagine and z-position of the bead [34,35,37].

Firstly, a calibration is done to the paramagnetic bead with reference to another non-magnetic bead that is attached to the surface of the glass slide. This is achieved by increasing the z-position of the stage that shifts the sample out of focus with the help of a piezoelectric motor that is moving at a constant speed [35,40]. The library of images are recorded for both of the beads to set as reference.

![Comparison diagram](image.png)

**Figure 3.4: Changes in image due the constant motion of the stage caused by the piezo-electric motor.**

After calibration, the in-house software written with LabView will match the new z-position of the extended DNA tether to images collected during the calibration to match the extension in length. This comparison can be done to a high accuracy because when the light source is shined onto a bead that is out of focus, diffraction rings can be seen [34]. Similarly, the intensity of the centroid peaks of each image can be analysed to high accuracy. Thus by comparing the diffraction ring patterns the software allows us to determine with high accuracy the changes in length of the DNA tether.
3.5 Calibration at high force region

3.5.1 Paramagnetic Beads

The paramagnetic beads we used in our experiment are Dynabeads M-280 Streptavidin obtained from Life Technologies. They have a diameter of 2.8 µm and are coated with a monolayer of streptavidin [39]. These beads are made of identical-sized polymer beads that are deposited with maghemite (\(\gamma-Fe_2O_3\)) nano-particles that are 8 nm in diameter, which are encapsulated by an additional coating of uncharged polymer [39].

![Paramagnetic Beads Diagram]

Figure 3.5: Steps in producing the paramagnetic beads containing maghemite nano-particles.

The maghemite nano-particles are evenly distributed on the bead surface and the magnetic moment of each of them are randomly orientated [36,39]. Since the magnetic moment of the one maghemite nano-particle is given by \(\vec{m}_0\), then the total magnetic moment of a single paramagnetic bead would be zero:

\[
\vec{M} = \sum_{i=0}^{N} \vec{m}_{0i} = 0
\]  

(3.13)

But under the influence of an external magnetic field \(\vec{B}\), the magnetic moment directions of the individual nano-particles will be affected. The net individual magnetic moment produced have potential energy \(U\):
3.5 Calibration at high force region

\[ U = -\vec{m}_0 \cdot \vec{B} \quad (3.14) \]

\[ = -m_0 B \cos \theta \quad (3.15) \]

The partition function of this classical paramagnetic system is:

\[ Z = \int_{0}^{2\pi} d\phi \int_{0}^{\pi} \sin \theta \exp \left( \frac{m_0 B \cos \theta}{k_B T} \right) d\theta \quad (3.16) \]

To simplify, let \( y = \frac{m_0 B}{k_B T} \) and perform a change of variable \( x = \cos \theta \):

\[ Z = 2\pi \int_{-1}^{1} \exp (yx) dx \quad (3.17) \]

\[ = \frac{4\pi}{y} (\sinh y) \quad (3.18) \]

Next, we need to determine the statistical average of the magnetic moment in the direction of the external magnetic field \( \hat{x} \):

\[ \langle \vec{m}_0 \rangle = \frac{1}{Z} \int_{0}^{2\pi} d\phi \int_{0}^{\pi} (m_0 \cos \theta) \sin \theta \exp \left( \frac{m_0 B \cos \theta}{k_B T} \right) d\theta \quad (3.19) \]

\[ = m_0 \left[ \coth \left( \frac{m_0 B}{k_B T} \right) - \frac{k_B T}{m_0 B} \right] \quad (3.20) \]

Hence, to calculate the total magnetic moment \( \vec{M} \) in the x-direction of the external magnetic field for one paramagnetic bead would be:

\[ \vec{M} = \sum_{i=0}^{N} \langle \vec{m}_0 \rangle_i = Nm_0 \left[ \coth \left( \frac{m_0 B}{k_B T} \right) - \frac{k_B T}{m_0 B} \right] \quad (3.21) \]

When the magnetic field strength is strong, \( \frac{m_0 B}{k_B T} \gg 1 \), hence:

\[ \vec{M} = Nm_0 \left( 1 - \frac{k_B T}{m_0 B} \right) \quad (3.22) \]
In the case of our experiment, \( m_0 \) is approximately \( 1.5 \times 10^{-16} \text{ J} \cdot \text{T}^{-1} \) [15,39]. Hence, the critical magnetic field strength required for the magnetic moment of the paramagnetic bead to be saturated would be \( 3 \times 10^{-5} \text{ T} \). The pair of permanent magnets that we use in our experiments has a magnetic field strength of 0.2 T thus the paramagnetic beads have a saturated magnetic moment aligned in the direction of the magnetic field.

After understanding the magnetisation of the paramagnetic beads, we can show how a force can be exerted on them. A magnetic object will always experience a magnetic force when placed in a region of magnetic field and the magnitude and direction of the force is given by:

\[
\vec{F} = \vec{M} \frac{\partial B}{\partial z} \hat{z} = Nm_0 \frac{\partial B}{\partial z} \hat{z}
\]  

(3.23)

The magnetic force exerted on the paramagnetic beads depends on the rate of change of magnetic field in the z-direction at a position \( z \) and the total magnetic moment of the individual beads \( Nm_0 \).

Therefore, for any two given beads at same distance \( d \) away from the permanent magnet, they can be related using the relationship below:

\[
\frac{F_1(d)}{F_2(d)} = \frac{M_1}{M_2}
\]  

(3.24)

\[
lnF_1(d) = lnF_2(d) + ln\frac{M_1}{M_2}
\]  

(3.25)
As a result, if the force against distance graph of any bead is plotted in a logarithm scale, they will related to one another by a translation in the y axis [37, 41]. This fact serves as an important part when calibrating the force extension for short tethers which will be further elaborated in following Section (3.6).

![Graph of the calibration curve and its relation with actual experimental (the y axis is Force in the logarithmic scale).](image)

Hence, when plotting the actual force distance curves from our experimental results, we will be able to find the accurate force measurement by comparing to the standard calibrated curve by a shift in the y axis as shown in the graph above [37].

### 3.6 Stretching of short DNA tethers

#### 3.6.1 Power spectrum

For measurement of short tethers at high force regimes, the fluctuation of the paramagnetic bead will be too high [35,37] as a result, the camera capture rate is not high enough to record its motion precisely. Hence, we need to study the power spectrum of this signal to overcome this limitation by short tethers that we are using in our experiments.

In any signal, a power spectrum can be plotted to determine signal’s power at various frequencies [34,35]. We can calculate the energy spectral density
in the y component by using:

\[ |\tilde{y}(f)|^2 = \frac{C}{1 + (f/f_c)^2} \]  

(3.26)

Where \( C \) is a numerical constant and \( f_c \) is the Lorentz corner frequency given by [37]:

\[ f_c = \frac{F}{2\pi z\gamma} \]  

(3.27)

Where \( F \) is the external force, \( z \) is the length of the tether and \( \gamma = 6\pi \eta r \) where \( \gamma \) is the drag coefficient that is related to viscosity of the medium \( \eta \) and the radius of the paramagnetic bead \( r \). For our experiments using short tether, it will result in a high value of corner frequency which is posted as a problem as the sampling rate of our camera is limited at 100Hz [37].

![Figure 3.7: Characteristic graphs of the power spectrum of a signal.](image)

The average of y-fluctuation square is given by the entire area under the power spectral graph [35].

\[ \langle \delta_y^2 \rangle = \int_0^{f_c} |\tilde{y}(f)|^2 df \]  

(3.28)

However, in our experiment, the upper boundary of the frequency is limited
by the sampling frequency. If the sampling frequency is much lower than corner frequency, the area covered will be much lesser than the entire spectrum, hence the $\langle \delta r^2 \rangle$ square value will be lower than the true value [37]. As a result, the measured magnetic force will be greater than the true force thus our data will not be accurate [35, 37]. It is therefore critical for the sampling frequency to be equal or larger than the corner frequency but once that have reached its limit, another solution is need to overcome this problem.

### 3.6.2 Force extension calibration using λ DNA

In order to overcome the limitation of high Lorentz corner frequency, we use a calibration curve of force against magnet position for a long DNA tether then use it to extrapolate for a shorter one [31, 37]. This method allow us to directly measure force between 1 to 100pN for short DNA tethers [37].

![Graph of force against distance for 8 different individual beads shifted to overlap together.](image)

This is carried out by calibrating the force using 48,502bp λ DNA at high force regions. For each different bead the force against distance graph is plotted on the same scale. As each individual bead has its unique heterogeneity, this will result in different curves [37]. Thus to account for these differences, we translate the curve such that they all overlap one another.
3.6 Stretching of short DNA tethers

This forms our calibrated curve and we used a double exponential function to fit it [37].

![Calibration Curve Diagram](image)

Figure 3.9: Using the calibration curve in the low force region to extrapolate the force to the high force region.

With the help of this fitted equation for long $\lambda$ DNA, we are able to extrapolate the curve to short DNA tethers. Using the force measurement of short tethers in the low force range, it is accurate up to 15 pN at the maximum sampling rate of our camera at 100 Hz that is calculated using Equation (3.27) [35, 37]. Hence, we can determine the $y$ translation from the calibration curve. With that, we can correspond the respective distances to the high force regions once we found the translational distance.

3.6.3 Overstretching transition of dsDNA

Other than using the low force fluctuation to calibrate force, we can also use another unique property of the double stranded DNA (dsDNA) handles to calibrate. This is done using the overstretching transition that stretches the dsDNA to two ssDNA when a sufficient force is applied [30]. The requirement is that the dsDNA sequence has to be a 60% GC-rich sequence [37, 42–44].
Figure 3.10: Graph of Extension against Force at the overstretching transition.

The overstretching transition occurs at force of 65 pN that have been widely reproduced and the extension in length is about 1.7 times [42–44]. Hence, if we are able to stretch the DNA tether to observe this drastic increase in extension, we can use the z-position of the magnet at this known force to calibrate our force position graph accurately. By using this approach, we have overcome the limitations of the Lorentz corner frequency and are able to calibrate forces for short tethers to high accuracy [37].
3.7 Bell’s model

The free energy landscape of the G4 transition from a folded state to unfolded ssDNA state can be shown below.

![Free energy landscape](image)

At low force, the transition is rarely seen due to high activation energy but at high force the transition is more likely to occur due to lower activation energy and higher stability of the unfolded state [15].

Bell’s model allows us to predict the probability of overcoming an energy barrier when a force is applied to the system using the Bell’s equation given by [40,45]:

\[
k(f) = k_0 \exp\left(\frac{x_0 f}{k_B T}\right)
\]

(3.29)

Where \(k_0\) is the unfolding rate at zero external force and \(x_0\) is the distance from the folded state to the transition state. Using this model, we need to fit it into the parameters of our experiment. When we stretch the DNA tether with a constant loading rate, we can firstly determine the unfolding
3.7 Bell’s model

probability and then the distribution of the unfolding force [15].

Starting with the basic relations of loading rate $r$ and force $f$:

\[ f = rt \quad (3.30) \]

We can re-express the Bell’s equation into:

\[ k(t) = k_0 \exp \left( \frac{x_0 rt}{k_B T} \right) \quad (3.31) \]

Next, using the relationship of the probability of unfolding with respect to time will give us:

\[ \frac{dp(t)}{dt} = k(t)[1 - p(t)] \quad (3.32) \]

By substituting Equation (3.31) into the above relation we are able to do the following simplification:

\[
\int_0^p \frac{d(1 - p(t))}{1 - p(t)} = - \int_0^t k(t)dt \quad (3.33)
\]

\[ p(f) = 1 - \exp \left( k_0 \frac{k_B T}{x_0 r} \left( 1 - \exp \frac{x_0 f}{k_B T} \right) \right) \quad (3.34) \]

\[ P(f) = \frac{\partial p(f)}{\partial f} = \frac{k_0}{r} \exp \left( \frac{x_0 f}{k_B T} + k_0 \frac{k_B T}{x_0 r} \left( 1 - \exp \frac{x_0 f}{k_B T} \right) \right) \quad (3.35) \]

Where $P(f)$ is the probability of unfolding at a given force. By plotting a graph of $P(f)$ against force, the distribution curve will have one peak at:

\[ f = \frac{k_B T}{x_0} \ln \left( \frac{x_0 r}{k_B T k_0} \right) \quad (3.36) \]

Through data fitting into Equation (3.35), we are able to determine the rate of unfolding at zero external force $k_0$ and the distance to the energy barrier $x_0$ which are important constants to understand the stability of the G4 structure that will form useful applications for our research [15,45].
3.7 Bell’s model

3.7.1 Loading rate

With reference to Equation (3.35) and (3.36), a change in the loading rate \( r \) can shift the peak position as well as the spread of the distribution graph. When a constant loading rate is applied to the tether, it will change the free energy landscape of the reaction \([40,46]\). If the loading rate is smaller, 1 to 5 pN/s, it will produce a more precise and sharp peak when plotting the force distribution histogram shown below.

![Force distribution histograms for different loading rates of the same reaction.](image)

On the other hand, when loading rates are too large, greater than 100 pN/s, the large step increases cause it to be difficult to pin point the actual force needed to break the bond as the incremental rate is too high for the particular molecule to respond \([40,45,46]\). That is reflected by the broad histogram with an inaccurate peak value for the unfolding force at higher loading rates. Hence, in our experiments in order to improve accuracy of our data we would use loading rates at 1-5 pN/s since the typical unfolding rates of G4 is within the range of 10-30 pN \([15]\).
Experimental Methodology

In this Chapter, we will be discussing the different stages of our experimental procedure: the synthesis of the DNA tether, preparation of glass channel, attaching the tether to the channel and the operation of the magnetic tweezers. The steps can be summarised in the flow chart below.

![Flow chart of experimental procedures]

**Stage 1**  
Synthesis of DNA tether

**Stage 2**  
Sample preparation

**Stage 3**  
Magnetic tweezers experiment

Figure 4.1: Sequence of experimental procedures used in my research.
4.1 Synthesis of G4 with dsDNA handles

The DNA tether used in our experiments consists of 3 different segments. The top and bottom tethers are dsDNA synthesis from λ DNA which act as handles connected to the G4 sequence that we are trying to investigate. The centre segment is the ssDNA sequence that can be folded into the Bcl-2 G4 structure. The 3 segments are connected together by ligation to form a single DNA tether.

![Schematics of DNA tether synthesised.](image)

**Figure 4.2:** Schematics of DNA tether synthesised.

4.1.1 PCR of dsDNA handles

Polymerase Chain Reaction (PCR) is widely used in many biological experiments to reproduce a specific DNA sequence in large quantities to carry out large scale experiments. It involves using a template DNA sequence, in our case lambda DNA, DNA polymerase, dNTP, two complementary primers and a suitable buffer. The whole process involves 3 stages: denaturing, annealing and elongating stage (details are found in Appendix).

The length of DNA handles that we are using are 473bp and 581bp respectively. The composite of the two handles are designed such that they contain more than 60% GC-rich sequences so that they can undergo overstretching transition [42–44].
After obtaining the PCR product, DNA purification is done using the Pure-link PCR Purification Kit (details in Appendix) to filter out the DNA fragments from PCR as well as to obtain the desired DNA sequence at a higher concentration.

![Figure 4.3: Pictures of Nanodrop 2000 Spectrophotometer used to analysis DNA concentration.](image)

With the purified DNA product, we need to determine the concentration of our product and confirm that the product we have collected is indeed DNA. This is done using the Nanodrop 2000 Spectrophotometer machine that does a wide spectral analysis of a sample using ultraviolet visible spectroscopy. In the case of DNA, the absorbent spectral is at 260 nm and the software can calculate the concentration of DNA for a sample droplet of 1 µL.

### 4.1.2 Cutting of the DNA sequences

After the purified DNA of the two handles are obtained, we need to cut the handles at specific ends to allow it to ligate with the Bcl-2 G4. This cutting is done by adding 0.5 µL of restriction enzyme BstXI and 5 µL of NEBuffer 3.1 to the 2 tubes of tethers (45 µL) and incubated at 37 °C for 2 hours. The resulting solution will contain the DNA tethers with ends.
that will match the centre segment together with some fragment DNA. Following that we would run gel electrophoresis to yield the final product and do purification one more time.

4.1.3 Ligation of G4 to handles

The central segment is the most important segment that contains the Bcl-2 G4 which is the key focus of my research. It is firstly annealed with DNA linkers that are complementary to the cut dsDNA handles. With the central segment ready, it is then mixed with the other two dsDNA handles that were prepared from the previous section. The ligation is done with the molar ratio of each segment been equal. The reaction would require: the three DNA segments in equal molar ratio, T4 ligase and T4 ligation buffer that are incubated at 16 °C overnight. The final step is to run gel electrophoresis and do purification again. The resulting DNA tether should have 1,100 base pair with an estimated length of 350 nm. The final gel electrophoresis result is shown.

![Figure 4.4: Gel electrophoresis results of the final ligation product.](image)
4.2 Preparation of channel

In our experiment, the DNA is tethered to a glass surface inside a channel where we are free to change its buffer conditions and move its position on the microscope stage.

4.2.1 Cleaning and treatment of glass surface with APTES

Since the lower end of our DNA tether is attached with a thiol functional (-SH) group, its purpose is to be chemically attached to a fixed point on the glass slide that can withstand high forces when it is stretched. In order for this thiol group to be attached to the glass surface, the surface needs to be treated chemically.

Firstly of all, we need to clean the glass slides to a sterile condition before we can treat it chemically. This is done by ultra-sonication which is a process of using ultra-high frequency sound energy to excite particles on surfaces so that it can remove impurities. The cleaning steps are as follow:

1. Place the glass slides into a jar then add 5ml of detergent (Decon 90) and fill it up with DI water.

2. Submerge the jar into the ultrasonic bath of the ultra-sonication machine for at least 20 minutes.

3. Wash the treated glass slides with DI water for 6 times and then with 100% ethanol.

4. Put the wet glass slides in an oven for 20 minutes or until they are completely dried.
4.2 Preparation of channel

Figure 4.5: Ultra-sonication machine for ultra cleaning and FEMTO Science machine for surface modification.

Going through these procedure will ensure the glass slides are of sterile condition and ready to be further treated with chemicals. As glass slides are made up of silicon dioxide (SiO$_2$), on the surface layer, usually a hydroxyl (-OH) group is been exposed. By plasma cleaning the glass surface, we are able to remove any organic chemicals left on its surface and oxidise the hydroxyl group into O$^-$ group which is negatively charged. This is carried out by putting the jar of glass slides into the FEMTO Science machine and allow it to plasma clean with oxygen gas for 20 minutes.

Once that is done, we would add 1 % (3-Aminopropyl)triethoxysilane (APTES) in methanol solution to the glass slides to react APTES with the O$^-$ group present on the glass surface followed by washing with methanol once and with DI water 6 times then by ethanol for one last time. As APTES consists of one silicon atom bonded to an amine (-NH$_2$) group and three other (O-CH$_3$) groups, it will react with the glass surface to form covalent bonds that will attach it to the glass surface. The exposed amine group is important because it will further react with Sulfo-SMCC to form a cross-linker at the later steps to bond with the thiol group found on the lower end of our DNA tether.
4.2 Preparation of channel

4.2.2 Constructing the channel

A single glass channel consists of one glass slide coated with APTES and one cover slip cleaned by ultra-sonication. A sample diagram shows how the designed channel will look like. The cover slip is placed on top the glass slide with two thin strips of parafilm at the top and bottom to seal the channel. This is achieved by heating the glass slide with the cover slip and parafilm on a heated surface, when the parafilm starts to melt, we gentle press the cover slip down to seal and attach the two glass slides closely. The estimated thickness of the channel is 10 $\mu$m and the channel has a total volume of 20 $\mu$L. Next, silicon gel is added around the exposed sides of the glass slide not covered by the cover slip to form an enclosed perimeter overlapping with the cover slip to facilitate the addition of liquids to the channel without leakage.

4.2.3 Preparing channel with DNA tether for experiment

In this next stage, we are trying to attach the DNA tether to the modified glass surface. A overview of the chemical interactions occurring the glass slide from the beginning to the final step can be shown in the following flow chart.
4.2 Preparation of channel

Figure 4.7: Flow chart of surface modifications to the glass channel throughout our sample preparation.

The detailed procedure of preparing the channel to perform experiments can be carried in the following steps:

1. White polybead beads in 1X PBS buffer solution used as reference markers are added into channel and left to incubate for 15 minutes.

2. After incubation, the channel is washed with 100 µL of 1X PBS buffer solution to remove excess beads.

3. Prepare a solution of Sulfo-SMCC, by first dissolving 0.1 mg of Sulfo-SMCC in 6 µL of DMSO, as it is not completely soluble in water. Then dilute the solution with 94 µL of 1X PBS.

4. Add the final solution to the channel and incubate for another 30 minutes. Following that, wash with 1 mL of 1X PBS buffer solution to remove excess chemicals.

5. Dilute the DNA tether (5 µL) with 45 µL of 1X PBS buffer solution and add it to the channel. Incubate for another 30 minutes.
6. Using blocking buffer BSA and Beta-Me, wash the channel for the last time and leave in the fridge overnight.

7. On the actual experiment day, add paramagnetic beads M280 coated with steptavadin in to the channel. Followed by 200 µL of working buffer (Tris + KCl 100 mM) required for G4 formation.

4.3 Magnetic tweezers operation

4.3.1 Experimental procedure

Once we have the sample channel prepared, we are ready to conduct the experiment using the magnetic tweezers setup. The photographs below show the actual setup.

![Figure 4.8: Photographs of experimental setup from the top and side view.](image)

Next, we would need to calibrate the tweezers, find a desired target bead and finally make a data collection for many stretch cycles. The detailed steps can be summarised in the flow cycle shown to provide a clearer picture.
4.3 Magnetic tweezers operation

The detailed steps are described as the following:

1. Firstly, we need to calibrate the position of the magnet that is fixed onto a lever connecting to the motion motor. This is to ensure that the magnet is symmetric about the x and y direction and is at the centre when viewed from the microscope lens. A 10X magnification lens is used to calibrate this.

2. Following that, we need to calibrate the z-direction of the tweezers. We use a normal glass slide without a cover slip and place it onto the stage of the microscope. Then we lower the magnet to a point just before touching the glass slide and set this point as the zero point.

3. Before mounting the prepared channel into the stage of the microscope, we need to colour the transparent channel black using a black
nail polish. By doing so will sure make the background of the channel is black thus allowing us to see the beads with sharper contrast.

Figure 4.10: Photographs of the channel mounted to the stage and ready to conduct experiment.

4. Using the 40X oil dispersion microscope lens, we are able to see the beads in the channel. The reference beads are white and the paramagnetic beads are yellow in colour.

5. Next, we set the magnet position to 4.5 mm, and search for magnetic beads that we see fluctuating. That means they maybe attached to DNA tethers fixed to the glass slide.

6. After finding a good bead, we use the LabView program (detailed operation details of this software are included in the Appendix) to the calibrate relative z-positions by using the piezo-electric motor.

7. Finally, we need to ensure that the DNA tethered really contains the dsDNA handles, since the synthesis process may produce many fragmented and complex derivatives. The is done using the overstretching transition that occurs at a force of 65 pN with an extension of 1.7 times its original length [42–44].

Next, depending on the nature of the experiment we are conducting, we can stretch the DNA tether at a constant loading rate to determine the force needed to unfold the G4 structure on the DNA tether. The program
4.3 Magnetic tweezers operation

...does this automatically for us when we key in the loading rate, start and end positions of the magnet. The output will be a single stretch cycle with constantly increasing loading force and the interval time between each cycle, known as the relaxation time is another important parameter in our experiment. In most cases, we usually investigate 32 cycles on each individual bead to obtain a reliable and reproducible data.

4.3.2 Addition of drug

At the second stage of our experiment, after determining the unfolding force for Bcl-2 G4, we will investigate the effects of adding drugs to it. After stretching a few beads and confirming that G4 structures can be found on most of the DNA tethers, we will add the Phen DC3 drug at different concentrations to change the environment of the buffer.

We would firstly prepare the drug at the target concentration with a volume of 200 $\mu$L. Then move the magnet z-position to 1.5 mm to exert a high force on the paramagnetic bead so that its tether will not be broken by the drift force when the drug is added to the channel. The drug is added very slowly and carefully to ensure it does not affect the calibration of the tweezers.

4.3.3 Data collection

For a single bead we will test it for at least 32 cycles. The output data will have the numerical values for all the parameters: x, y, z, magnet and piezo positions throughout the duration of the entire experiment. The two main variables that are important to our data analysis is the z and magnet positions. These data are then further processed using Origin and Matlab to determine the force corresponding to each G4 unfolding signal seen from the z-extension graph that will be covered in the next chapter.
Results and Discussion

In this Chapter, we will be discussing the main components of our results and how the data is treated to obtain the information that is useful to us. We will be focusing on the force required to unfold the G4 structure and how we can determine the rate of unfolding at the equilibrium and the probability of refolding. In the second part we will be discussing the effect of ligands on the stability of the G4 structure and how it will affect the unfolding force.

5.1 Data collection

5.1.1 Accurate force determination

As previously mentioned in Chapter 3, the way we can calibrate the force exerted by the magnets is by using the property of the overstretching transition of the dsDNA handles. This is the first confirmation we need to achieve in order to know that our tether is synthesised correctly. At the same time, once this overstretching transition is identified, we will correspond the z-position of the magnet to produce a force of 65 pN [42–44]. Then we will find the constant in the double exponential equation to get the true force displacement graph as shown in Figure (3.9).
5.1 Data collection

Figure 5.1: Graph of Extension and z position of magnets against Time to illustrate overstretching transition.

We will move the z-position of the magnet until the overstretching transition can be observed as shown in Figure (5.1). Once the exact z-position at which the transition occurs is pin-pointed, we can calibrate our magnetic tweezers such that it can produce the force that we desire.

5.1.2 Stretch short tether at a constant loading rate

After force calibration is done, we would want to stretch the DNA tether at a constant rate from low force to higher force to unfold the G4 structure. This is done by the software program, which will control the movement of the magnet to produce a constant loading force on the targeted tether. Once the stretch cycle begins, we will be able to observe any signal of unfolding of G4 from the sudden stepwise extension of the length of the tether as shown.
Figure 5.2: Extension curves showing the signal of the G4 unfolding step.

As the experiment repeats for 32 cycles for a single tether, we can find the individual forces needed for the unfolding in every cycle when the signal is been observed. The output data file is analysed with a MATLAB program to determine the position of the magnet at the instant the folding signal is been recorded. Hence, from that, we can calculate the force using the calibration curve we have obtained from the previous step.
5.2 Data analysis

5.2.1 Determining the force of unfolding

How the MATLAB software works is by the following steps:

1. Firstly, we run the first MATLAB code “CutDataLoadingRateContinues” to cut the continuous 32 stretching cycles into individual cycles for ease of analysis.

2. Next, using each individual cycle we will run the second code “verticalanalysis_FLNa20_21”, that will give us the step jumps in the stretch cycle that may correspond to the G4 unfolding signal.

3. We need to manually make sure the step size increase is in the order of 10-20 nm for it to qualify as a possible candidate.

4. We will choose the best step jump and find its respective magnet position at the instant (Highlighted row in Figure 5.3).

5. Finally, using the calibration equation we have gotten from the previous section, we can find the unfolding force for that particular cycle. This is repeated for the rest of the data.
5.3 Results

5.3.1 Force distribution of Bcl-2 G4

For the Bcl-2 G4 sequence, we conducted the more than 10 successful experiments and the results collected are from 20 different beads to ensure reproducibility in our data. All the 147 individual unfolding force are tabulated in the histogram shown below.

![Unfolding force histogram of Bcl-2 with its fitting curve.](image)

Figure 5.4: Unfolding force histogram of Bcl-2 with its fitting curve.

The mean unfolding force from this distribution is \((23.5 \pm 0.6)\) pN, this value is in the order of magnitude that we have expected because the unfolding force for human telomere G4 is 12 pN [47,48].

\[
P(f) = \frac{k_0}{r}e^{x_0f/k_BT} + k_0 \frac{k_BT}{x_0} \left( 1 - e^{x_0f/k_BT} \right) \quad (5.1)
\]

Next, using Equation (3.39) that we have derived in Chapter 3, we can perform a curve fitting to determine the parameters \(k_0\) and \(x_0\), which are important experimental constants that tell us the unfolding rate at equilibrium and the transition distance [15]. The fitted curve is the one shown in
black and the parameters that we have found are: \( k_0 = (0.011 \pm 0.003) \text{ s}^{-1} \) and \( x_0 = (0.58 \pm 0.06) \text{ nm} \).

Hence, from these results, we are able to determine a preliminary value of the unfolding rate at equilibrium for the Bcl-2 G4 sequence and at the same time determined the transition distance between the equilibrium state and the transitional state.

### 5.3.2 Refolding probability

The refolding probability can depend on two factors, which are the relaxation time and the force exerted on the tether during relaxation [19,46,48].

In our experiment, we should to use relaxation time as the variable because we want to determine the average time the tether needs to be held so that the unfolded G4 structure will refold such that in the following cycle we are able to unfold the G4 again. Hence, we keep the relaxation force to be constant at 1 pN for all our experiments. Then, we varied by 1, 5, 10, 30, 45, 60, 90, 120 seconds and repeated the experiment for 3 different beads of 32 cycles.

Next, we calculated the fraction of the unfolded cycles out each 32 cycle runs and plotted the probability of refolding for each relaxation time. The data points are then fitting into a negative exponential decay equation for a first order rate reaction to the determine the refolding constant. The fitting equation is given by:

\[
y = 1 - e^{-kt}
\]

\( k \) is the refolding constant with units per second, \( t \) is the relaxation time and \( y \) is the refolding probability.
The value for $k$ determine from our curve fitting is $(0.017 \pm 0.002) \text{ s}^{-1}$. Therefore by taking the reciprocal of this constant we obtained the average relaxation time of 59 seconds and that is the standard relaxation time we used in our later experiments.
5.3 Results

5.3.3 Force distribution of Bcl-2 G4 with drugs added

We aim to find out how the magnitude of the unfolding force can be affected by addition of drugs. The drug we are using is Phen DC, which is a G4 ligand drug. It has an electronic charge of +2 and an overall planar structure [49]. Due to its planar structure, Phen DC3 molecules can stack on top of G4 that will further stabilizes the G4 structure due to stacking interactions [50].

In our experiments, Phen DC3 is added to the channels containing the DNA tethers at different concentrations. Firstly, 50 nM of Phen DC3 was used to test its effect on the stability of the Bcl-2 G4. The results of the force distribution histogram is shown below.

![Unfolding force histogram of Bcl-2 in 50 nM Phen DC3 with its fitting curve.](image)

The peak of the graph have shifted to the right to an average force of $(49 \pm 2)$ pN. This is in line with our prediction because the drug stabilises the G4 structure causing the force to unfold it almost two times of that without drug [22,50]. The concentration of Phen DC3 is almost at saturation because majority of the unfolding force is at the higher region that
corresponds to the bind state of G4 [22]. Hence, we would lower the concentration to find the point where the possibility of unfolding is half at the unbind state and another half at the bind state [22, 50].

A series of other concentrations were tested i.e. 0.5, 5, 10 and 20 nM. Unfortunately, only at 20 nM it gave us the clearest unfolding signals and enough counts to plot a distribution histogram. The results from 2 separate experiments were tabulated, with a total of 3 different beads used. The extension time graph of a cycle without unfolding signal, unfolding at high force due the binding of the Phen DC3 molecule and unfolding at a lower force without binding are shown:

![Graph showing extension curves](image)

Figure 5.7: Extension curves showing a stretch cycle with no G4 unfolding signal, unfolding of the bind state at high force and unfolding of unbind state at low force.

The graphs clearly illustrate that there are two force ranges that the G4 structure unfolds due to the addition of the drug molecules at a non-saturated concentration.
Next, the unfolding force histogram, for 20 nM of Phen DC3 is tabulated from 103 cycles that registered unfolding signals, are plotted as shown:

![Unfolding force histogram of Bcl-2 in 20 nM Phen DC3 with its fitting curve.](image)

Figure 5.8: Unfolding force histogram of Bcl-2 in 20 nM Phen DC3 with its fitting curve.

The force distribution diagram when 20 nM of Phen DC3 is added to the DNA tether shows clearly that, instead of a single peak distribution we obtained in Figure (5.4) when no drugs were added, there are two distinct peaks. The lower force peak is at \((19.4 \pm 0.3) \text{ pN}\) and the higher peak is at \((44.0 \pm 0.4) \text{ pN}\).

This is close to the results we obtained for first histogram in Figure (5.4) where the unfolding force is at 23.5 pN. Thus the stability of Bcl-2 G4 that is bind with the Phen DC3 molecule requires a mean force of 44.0 pN to unfold. This is an important confirmation as the addition stability could hinder gene expression of Bcl-2 [12], therefore very useful to pharmaceutical research areas in drug targeting [3,12].
5.4 Discussion

From our results, we have determined the unfolding force of the Bcl-2 G4 sequence that has not been published. Therefore, the validity and accuracy of this result cannot be compared since there are no published data from previous research. However, we can evaluate the accuracy and precision of our data by the methods used and comparing to other known data of G4 unfolding force such as the human telomere. We would also propose some sources of errors and areas of limitations for our experiments.

The unfolding force of the human telomere G4 sequence is in the value of 12 pN \cite{47, 48} while the result that we have found for the Bcl-2 G4 is 23.5 pN. These two values are well in the same force range which reinforces the relative stability of the G4 structures. We can also cross reference our results we obtained for the non-drug experiments with those with the addition of drugs, we can see that the unfolding force for the unbind state is comparable at a force of 19.4 pN. Nevertheless, the results still differ by a margin, this is because most of our experiments recorded only 100 counts. In order to get a better force distribution histogram, more data points are needed to increase the precision of our determined values.

The precision of our instrument, the magnetic tweezers, is extremely high and stable \cite{34, 35, 37}. It can produce a force with a precision of up to 0.1 pN \cite{37}. Moreover, with the calibration technique developed to overcome the limitation of using short tethers, the accuracy of the instrument is extremely reliable \cite{35, 37}. However, problems may result from specific experimental parameters. One of such parameter is the loading rate. The loading rate in all of our experiments are fixed at 2 pN/s which is in the acceptable range for our experiment because the typical unfolding force is the region of 10-20 pN. But the loading rate directly affects the precision
of our force measurement because if it is too fast the DNA tether may not respond to the change in force that quickly [40, 45, 46]. Thus ideally we should use the slowest loading rate possible i.e. 0.01 pN/s but that will be too time consuming for us to collect data. With the limitation of time for conducting our experiments, using 2 pN/s as the loading rate is a reasonable compromise.

We found for the bind state Bcl-2 G4 the average unfolding forces are 49 pN and 44.0 pN. Again they are similar in magnitudes. However, one difficulty faced is determining the step jumps at the high force region in the extension time graphs due to the presence of other step jumps that look similar to the G4 unfolding steps as shown.

![Figure 5.9: Extension curve in the high force region that is difficult to determine the G4 unfolding signal.](image)

The is due to the peeling transitions near the overstretching transition of the dsDNA handles in the tether at a high force region [42–44]. This is because the overstretching transition occurs at 65 pN and our unfolding force is about 40-50 pN. Therefore, unless the signal is extremely clear that it is a G4 candidate, or else we will not to use that cycle for analysis.
Conclusion

6.1 Summary of research work

Through the method of single molecular experiments using the magnetic tweezers, we were able to investigate the structure stability of G4 sequences, in our case Bcl-2 the human promoter gene. In our experiments, we determined the force needed to unfold the Bcl-2 G4 is $(23.5 \pm 0.6)$ pN which is larger than that of the human telomere G4 at 12 pN. The refolding constant at equilibrium is also determined to be $(0.017 \pm 0.002)$ s$^{-1}$. Finally, we added the presence of Phen DC3 drug ligands to the interact with the G4, the bind state of Bcl-2 with the ligand is found to have a higher unfolding force at $(49 \pm 2)$ pN for 50 nM concentration of Phen DC3 and $(44.0 \pm 0.4)$ pN for 20 nM Phen DC3.

6.2 Future work

The immediate step that we can take is testing the unfolding force distribution of concentrations between 50 nM and 20 nM of Phen DC3 to find the probability of 50% bind state and 50% unbind state of the G4. By finding that, we can determine the dissociation constant of this reaction. However, due to the lack of time, this was not able to be accomplished within the
time frame of this project and due to numerous failed experiments.

Moreover, for the Bcl-2 gene, there are many other segments in the sequence that can also form G4 structures. We can also mutate the sequence of the long connecting strand (AGGAAGG) in our sequence to determine its effects on stability too. These would give us a more thorough understanding of the Bcl-2 gene as a whole and the comparison between the wild type and its mutants.

Finally, different G4 ligand drugs could also be used to test their different binding affinities with Bcl-2 G4. With a whole spectrum information, it may provide us with insights to which one will be most effective in producing the desired stability hence acting as the most specific targeting drug.
Appendix

7.1 Procedure of PCR

The chemicals used are: the PCR product, Binding Buffer (B2), Binding Buffer HC (B3) Wash Buffer (W1) and Elution buffer (E1). The procedure are as follows:

1. Add 4 times the volume of B2 and B3 to the PCR product in a tube and mix well.

2. Transfer the mixture into the spin column from the purification kit and centrifuge at 14000 rpm for 1 minute.

3. After centrifuging, discard the flow through and re-insert the column.

4. Add 650 µL of W1 to the spin column and centrifuge for 1 minute.

5. Discard the flow through and centrifuge for another 3 minutes at max speed.

6. Place the spin column into a new elution tube and add 50µL of E1 and incubate at room temperature for 1 minute.

7. Centrifuge the column at maximum speed for 2 minutes and the purified product is in the elution tube.
7.2 Procedure of gel electrophoresis and purification

1. Prepare an Agarose gel slab using the Agarose Powder.

2. Pipet the marker DNA into the left most well and the rest of the sample into adjacent ones.

3. Place the gel slab into the electrophoresis buffer and connect the terminals. Run it for 35 minutes.

4. After the run, place the gel onto the viewing platform of the UV machine.

5. Conduct an initial scan to identify the band of DNA for the tethers and print out the results.

6. Place the UV shield on the platform and cut the segment band and transfer into a tube.

After getting the slice of DNA gel, we need to further purify it using the same procedure done before in previous section. The only additional step we need to carry out is to dissolve the gel so that we can obtain a solution with the DNA tether we need. This is done by:

1. Weigh the slice of gel using the digital weighing machine.

2. Add Gel Solubilisation Buffer (L3) at a 3:1 ratio with respect to the mass of the gel.

3. Incubate the tube at 50°C for 10 minutes and invert the tube every 3 minutes to ensure it is thoroughly dissolved.

4. Continue with Step 2 of the PCR purification procedure till the end.

The final product will be the cut dsDNA handles required to form our DNA tether.
7.3 Software interface for magnetic tweezers

The software we are using to gather our data is created by one of our research group members and designed with LabView. The program allows us to obtain data such as: the image from the microscope, the position of the target bead in the x, y, z coordinates, the z position of the magnet and the position of the piezo-electric motor displayed in the five graphs on the right of the screen.

The program allows us to measure the extension of the paramagnetic with reference to the white beads that are attached to the glass surface. We can adjust the z-position of the magnet by changing the difference parameters and even set a program for the magnet movement to produce a constant load of the DNA tether using the command window on the left of the screen.


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