A Dynamic Single Molecule Approach to Study Negative Supercoiling Induced G-quadruplex Formation in B-DNA

A Thesis

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by

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Certificate

This is to certify that this dissertation entitled **"A Dynamic Single Molecule Approach to Study Negative Supercoiling Induced G-quadruplex Formation in B-DNA"** towards the partial fulfilment of the BS-MS dual degree programme at the Indian Institute of Science Education and Research, Pune represents study/work carried out by Shankar Krishna V R at Indian Institute of Science, Bengaluru under the supervision of Dr. Mahipal Ganji Assistant Professor, Department of Biochemistry, during the academic year 2023-2024.

Dr. Mahipal Ganji

Committee:

Dr. Mahipal Ganji

Prof. Saikrishnan Kayarat

Declaration

I hereby declare that the matter embodied in the report entitled **"A Dynamic Single Molecule Approach to Study Negative Supercoiling Induced G-quadruplex Formation in B-DNA"** are the results of the work carried out by me at the Department of Biochemistry, Indian Institute of Science, Bangalore, under the supervision of Dr Mahipal Ganji and the same has not been submitted elsewhere for any other degree. Wherever others contribute, every effort is made to indicate this clearly, with due reference to the literature and acknowledgement of collaborative research and discussions.

Shankar Krishna V R

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Abstract

Strand separation by negative supercoiling of B-DNA is known to give rise to non-canonical DNA structures. It is hypothesized that negative supercoiling might give rise to the formation of G-quadruplexes. Current literature involving in vitro studies offer contrasting results both for and against the above hypothesis. Ensemble assays like chemical foot printing, S1 nuclease cleavage and 2D-gel electrophoresis show correlation between negative superhelical stress and G-quadruplex formation in only in certain cases. On the other hand, force-extension measurements using magneto-optical tweezers and single molecule studies using atomic force microscopy (AFM) support the hypothesis.

A real time dynamic assay will be useful to determine circumstances in which the hypothesis is true. Intercalation Induced Supercoiling of DNA (ISD) is a dynamic single-molecule technique to generate and visualize supercoiled DNA with the help of fluorescence microscopy. SG4 is a recently developed anti-G-quadruplex nanobody. We aim to use fluorescently labelled SG4 to probe G-quadruplex formation in negatively supercoiled DNA generated by ISD. Negatively supercoiled DNA was successfully generated through ISD assay. The purified SG4 nanobody however did not show G-quadruplex binding in gel mobility shift assay. Work needs to be done in optimizing conditions for the activity of the protein, only then can it be used as a probe.

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1. Introduction

1.1 G-quadruplexes

In thermodynamically favorable conditions, closely spaced guanine bases of DNA in the presence of monovalent cations such as potassium ions, come together to form structures known as G-quadruplexes. G-quadruplexes represent a class of non-canonical DNA structures, where multiple quartets of coplanar guanine bases stack on top of each other creating a stable complex (Sundquist and Klug, 1989). The quartets are stabilized by Hoogsteen hydrogen bonding and electrostatic interaction with the central cation. These structures emerge transiently and play an important role in cell regulation.

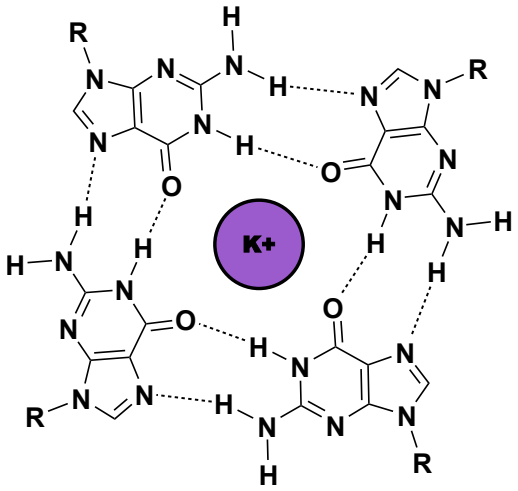


Figure 1.1: Chemical structure of a G-quartet stabilized by central potassium ion.

Inter-molecular G-quadruplexes arise from the assembly of guanines belonging to multiple ssDNA molecules, whereas Intramolecular G-Quadruplexes form from a single strand of DNA having a putative G-quadruplex forming sequence.

1.2 DNA Supercoiling

The B-form of DNA is a base-paired double helix with a diameter of 2 nm and rise of 0.34 nm per base. Twist refers to the number of crosses made by individual strands of the DNA double-helix. The individual strands of DNA in the relaxed B-form twist around the helical axis once every 10.4 base pairs. Writhe is the number of crosses the central axis of the DNA makes with itself.

Twist and writhe are related by the following relation,

$$\mathrm{Lk} = \mathrm{Tw} + \mathrm{Wr}$$

Linking number, Lk, is the sum of twist and writhe (Fuller, F. B, 1978). DNA supercoiling refers to the change in topology of B-DNA caused by change in the linking number. In a topologically constrained DNA, the twist and writhe are interchangeable whereas the linking number cannot be changed without introducing single or double stranded break.

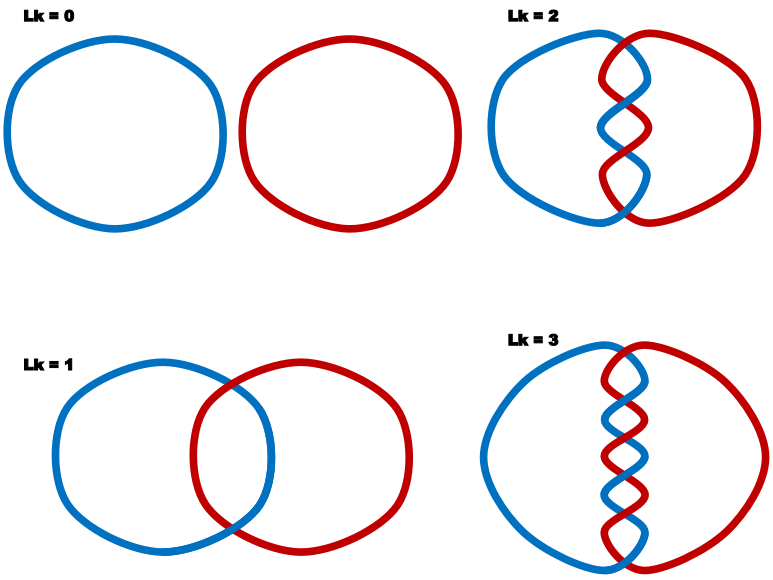


Figure 1.2: Linking number.

Strand separation due to negative superhelical stress is known to give rise to non-canonical structures in B-DNA, G-quadruplexes being one among them (Azorin et al., 1983), (Voloshin et al., 1987), (Panayotatos and Wells, 1981), (Sun and Hurley, 2009).

1.3 TIRF & HiLo Microscopy

In optics, total internal reflection (TIR) is a phenomenon in which light travelling from an optically denser media to an optically rarer media is reflected back to the denser media at the interface. TIR occurs when angle of the incident light is greater than a threshold angle called critical angle.

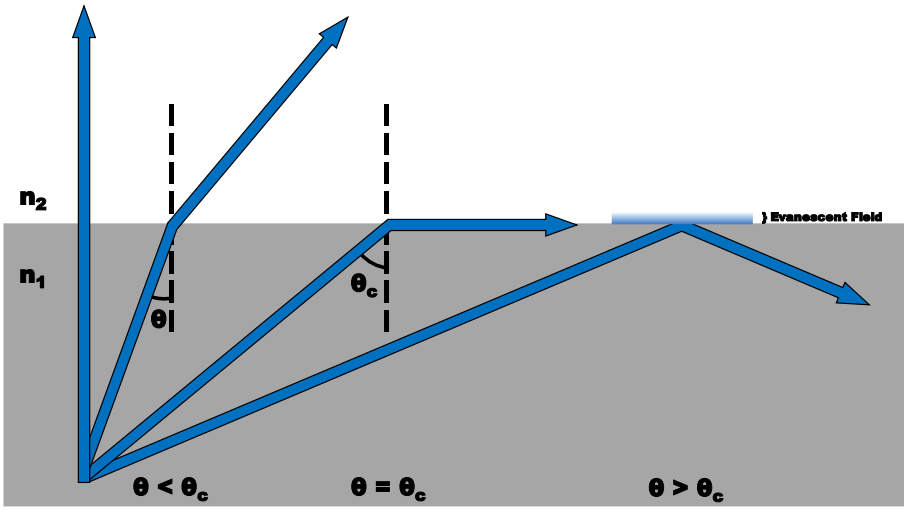


Figure 1.3: Total Internal reflection.

At the interface TIR creates a thin, standing electromagnetic field called an evanescent field, which is a direct consequence of electromagnetic waves being continuous at boundary conditions. The evanescent field is an exponentially decaying field off the surface.

The depth of the evanescent field is given by the following equation,

$$d = \frac{\lambda}{4\pi \sqrt{(n_1{}^2 \, sin^2(\theta) - n_2{}^2)}}$$

Where λ is wavelength of the light and n_1 & n_2 are the refractive indices of the media (Harrick N. J, 1967).

In conventional epifluorescence microscopy, the entire volume of the sample is illuminated by the light source which greatly impacts the signal to noise ratio (SNR). TIRF microscopy uses the evanescent field created by TIR is to illuminate only those fluorophores which are close to the surface (usually < 300 nm). This selective illumination significantly improves the SNR making TIRF-M a great tool for surface imaging.

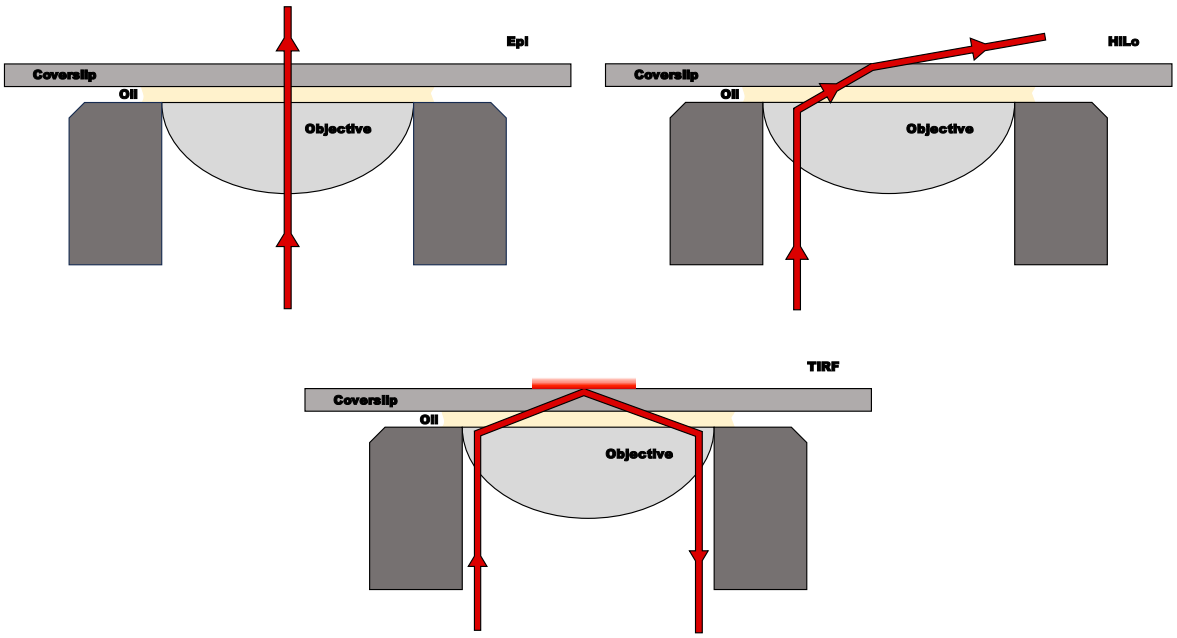


Figure 1.4: Objective-based TIRF-M. The curvature of the point where the incident beam hits the convex lens determines the angle of emergent beam. All three modes Epi, HiLo & TIRF can be achieved just by changing the x-y coordinates of the laser beam.

When the source laser is refracted through the specimen at an angle very close to the critical angle, a highly inclined sheet of light is produced which illuminates a greater depth of the sample still achieving better SNR than epifluorescence. This is the principle of HiLo (highly inclined and laminated optical sheet) microscopy. (Tokunaga et al., 2008)

1.4 Intercalation-induced Supercoiling of DNA (ISD)

ISD is a single-molecule technique with which supercoiled-DNA structures, aka, plectonemes can be generated, visualized, and studied with a fluorescence microscope (Ganji et al., 2016). In this technique large (>7 microns), linear, DNA molecules with biotinylated ends are immobilized on a streptavidin coated coverslip. This is followed by the introduction of an intercalating fluorescent dye. Individual dye molecules intercalate and induce twist between base pairs of the double helix, the local twists are now converted into a global writhe giving rise to plectonemes which can be seen as dynamic bright spots.

The above-mentioned method where the intercalating dye is added post immobilization generates positive supercoiling. To induce negative supercoiling, DNA is incubated with the intercalating dye and immobilized to the surface. After this the dye concentration is reduced which results in untwisting of the helix creating plectonemes. The technique solely depends on the intercalator concentration for the generation of plectonemes.

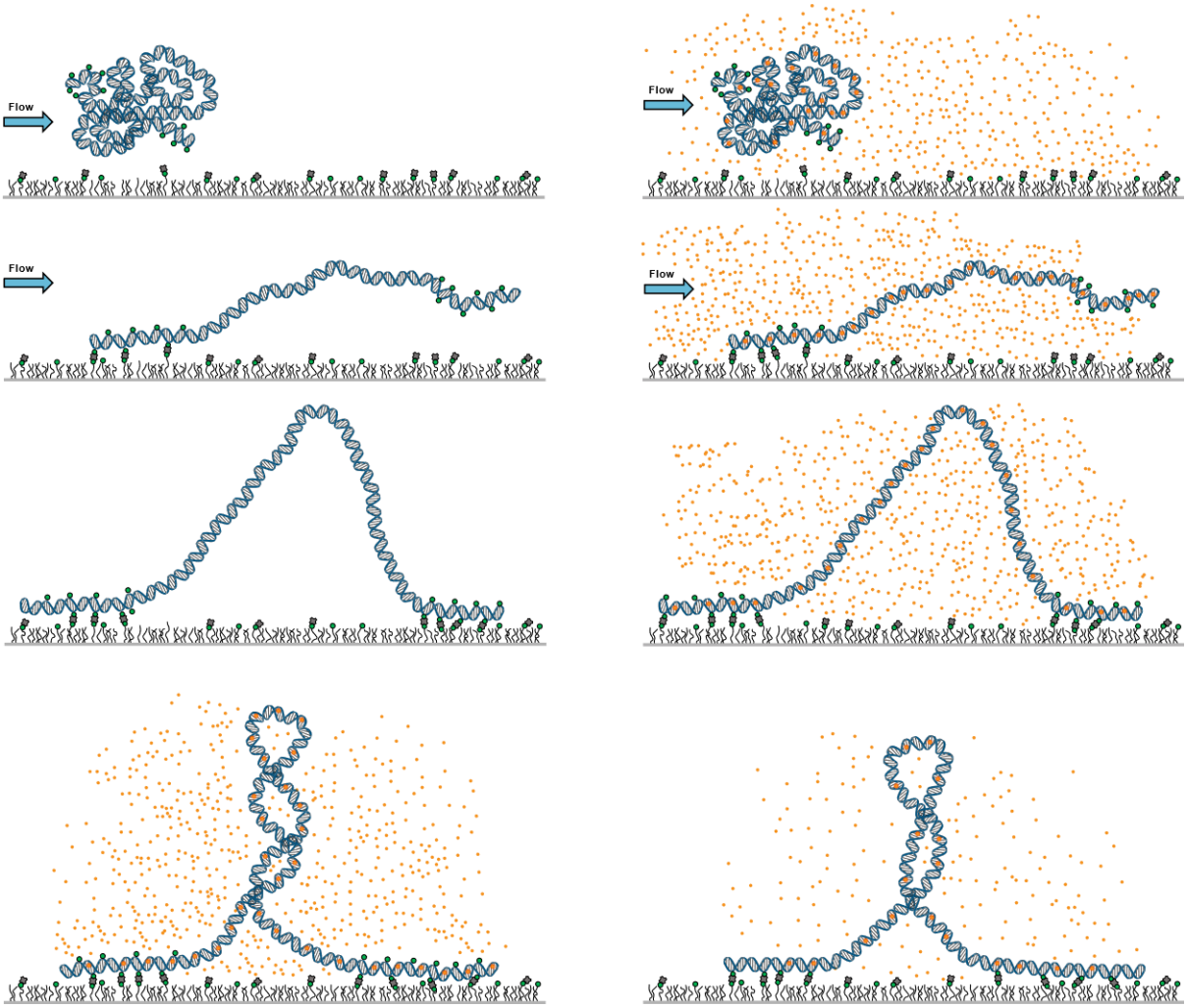


Figure 1.5: ISD Assay. (a) Positive Supercoiling of DNA. (b) Negative Supercoiling of DNA.

1.5 SG4 - A Nanobody Against G-quadruplexes

Unlike other jawed vertebrates, immunoglobulins of camelids and chondrichthyians only possess heavy chain and do not have a light chain (Hamers-Casterman et al., 1993), (Greenberg et al., 1995). The variable-domains of immunoglobulins from these animals are called single domain antibodies or nanobodies. Nanobodies are 12-15 kDa in size, almost one-tenth the size of conventional mammalian antibodies and can be recombinantly expressed in *E. coli*. This makes them a great tool for labelling targets for fluorescence microscopy. Galli et al developed a high affinity nanobody named SG4 (Addgene, #196071) against G-Quadruplexes using phage display technology (Galli et al., 2022). In this work fluorescently labelled SG4 nanobody is intended to probe G-quadruplexes. A structural study on nanobodies, revealed potential sites for introducing cysteines for site-specific labelling using thiol-maleimide click chemistry (Hansen and Andersen, 2022). Based on this serine 123 of SG4 was chosen to be mutated to cysteine.

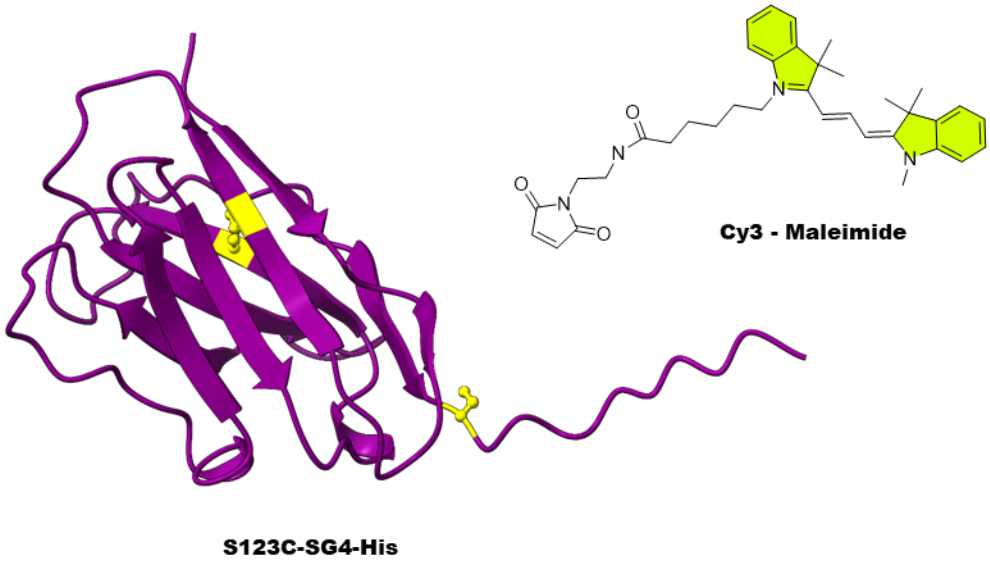


Figure 1.6: Alphafold2 prediction of S123C-SG4-His & chemical structure of cy3-maleimide. Cysteines are highlighted in yellow.

1.6 Objectives

The aim of this work is to see in real time whether negative supercoiling alone is sufficient to induce G-quadruplex formation using ISD technique. To achieve this, following are the specific objectives proposed:

a) Cloning, purification, and fluorescent labelling of S123C-SG4.
b) Cloning of a plasmid carrying putative G-quadruplex sequence.
c) Using fluorescent SG4 to probe G-quadruplex formation in negatively supercoiled DNA.

2. Materials and Methods

2.1 Site Directed Mutagenesis

Serine 123 of SG4 was mutated to cysteine using KLD site directed mutagenesis kit. The KLD reaction mixture contains kinase, ligase and DpnI. The DpnI enzyme chews off the methylated template plasmid, the kinase phosphorylates 5' ends of the amplicon and ligase joins the end.

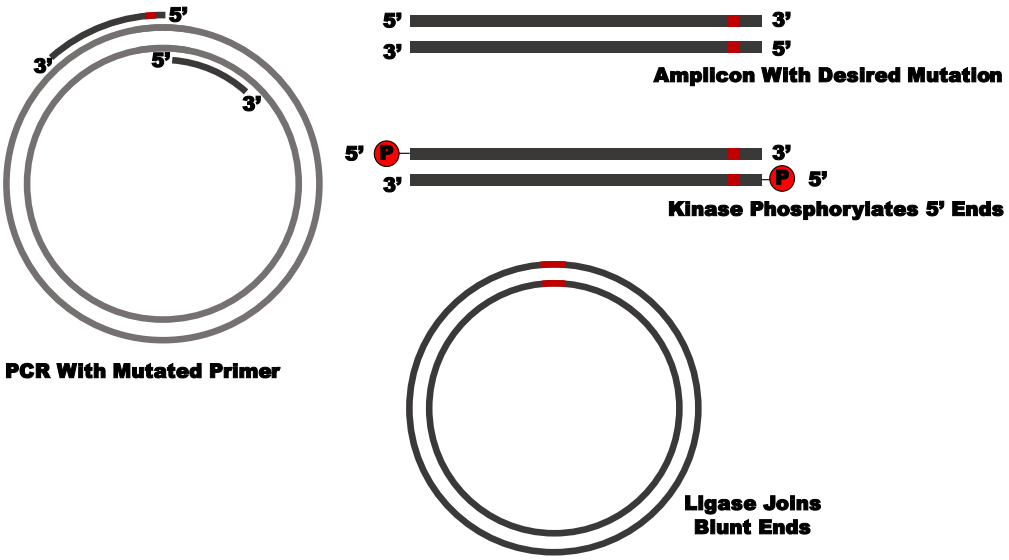


Figure 2.1: Site directed mutagenesis with KLD reaction.

pHEN2-SG4 plasmid was PCR amplified using primers with the desired mutation.

Primer	Sequence 5' - 3'
Forward-S123C-SG4	CACCATCACAAGCTGGACTACAAAGACCATGACGGT
Reverse-S123C-SG4	ATGATGATGTGCGGCCGCGCTACACACAGTTACCTGCGT

Table 2.1: Primers for KLD. The adenine in red indicates the mutation which changes AGT to TGT in the coding strand.

Components	Test	Control
5X Q5 Reaction Buffer	10 µl	10 µl
2.5mM dNTP mix	5 µl	5 µl
10µM Forward Primer	2.5 µl	2.5 µl
10µM Reverse Primer	2.5 µl	2.5 µl
pHEN2-SG4 Plasmid (264ng/µl)	1 µl	1 µl
MQ Water	28.5 µl	29 µl
Q5 DNA Polymerase	0.5 µl	-

Table 2.2: PCR components for cloning of pHEN2-S123C-SG4.

Step	Temperature	Time	
Initial Denaturation	98 ˚C	3 mins	
Denaturation	98 ˚C	15 s	30 cycles
Annealing & Extension	72 ˚C	3 mins	
Final Extension	72 ˚C	4 mins	

Table 2.3: PCR conditions for cloning of pHEN2-S123C-SG4.

Components	Test	Control
PCR Product	1 µl	1 µl
2X KLD Reaction Buffer	5 µl	5 µl
10X KLD Enzyme Mix	1 µl	1 µl
MQ Water	3 µl	3 µl

Table 2.4: Components for KLD site directed mutagenesis.

The PCR product was used for the KLD reaction and the control is to check DpnI digestion. The entire KLD reaction was used to transform chemically competent *E. coli* DH10β cells.

2.2 Restriction Cloning

S123C-SG4 was cloned into pET28b vector via restriction cloning. pET-28b-Cas9-His plasmid (Addgene ID #47327) was available in the lab. S123C-SG4 in pHEN2 plasmid had restriction sites NcoI & NotI, which were also present in pET-28b-Cas9-His. Both the plasmids were isolated and digested with the mentioned restriction enzymes.

Components	pET-28b-Cas9-His	pHEN2-S123C-SG4
Cut Smart Buffer (10X)	5 µl	10 µl
MQ Water	40 µl	68 µl
Plasmid	3 µl (1000 ng)	18 µl (2000 ng)
NotI	1 µl	2 µl
NcoI	1 µl	2 µl

Table 2.5: Components for restriction digestion of pET-28b-Cas9-His & pHEN2-S123C-SG4.

Post digestion, the reaction mixtures were run in an agarose gel and the desired bands were purified using gel extraction kit (QIAquick, Qiagen). The isolated insert and vector backbone were then ligated.

Components	Ligation reaction
T4 DNA Ligase	1 µl
T4 Ligase Reaction Buffer (10X)	3 µl
MQ	7 µl
Backbone	12 µl (20 nmoles)
Insert	6 µl (80 nmoles)

Table 2.6: Components for ligation of S123C-SG4 into pET-28b vector backbone.

2.3 Purification of S123C-SG4

SHuffle® T7 Express *E. coli* cells harbouring the pET28b-S123C-SG4-His plasmid were grown in 200 ml 2XYT media containing 50 µg/ml kanamycin at 28 ˚C overnight. The entire culture was then transferred to fresh 800 ml, 2XYT media containing 50

µg/ml kanamycin. After one hour of growth at 28 ˚C. The culture was induced with 500 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated at 25 ˚C. After 5 hours, the cells were harvested by centrifugation at 8000 g for 20 mins. The pellet was resuspended in lysis buffer (1X PBS + 10mM Imidazole + 1mM PMSF) and sonicated. Post sonication the lysate was centrifuged at 30000 g for 30 mins at 4˚C. The supernatant was subjected to Ni-NTA affinity chromatography.

2.3.1 Ni-NTA Affinity Chromatography

An empty gravity column with a porous polyethylene bed was manually packed with 0.5 ml Nickel-NTA Resin (G-biosciences). The supernatant was then passed through the column and the flow through was collected. The column was incubated for 1 hour. Post incubation, the column was washed with 50 ml lysis buffer, 10 ml Wash buffer (lysis buffer + 50 mM imidazole) and again with 10 ml lysis buffer. The protein was finally eluted in ten, 1 ml fractions of elution buffer (1X PBS + 250 mM imidazole).

2.3.2 Size Exclusion Chromatography

HiLoad 16/600 Superdex 200 pg column (Cytiva) was connected to an FPLC machine (Biorad NGC Chromatography system) with a UV-Vis Detector. The column was equilibrated with 1X PBS. 1 ml of the concentrated protein was injected in the column. After the first 35 ml (void volume) was flown, 180, 0.5ml fractions were collected.

2.4 Circular Dichroism Spectroscopy

CD spectra of the protein was recorded in the far UV region using a spectropolarimeter (Jasco J-810) to confirm secondary structure formation. After blanking with 1X PBS, 400 µl of 1 µM of protein in 1X PBS was transferred to a 10 mm quartz cuvette. The cuvette temperature was set to 25 ˚C using a Peltier thermostat which comes integrated with the cuvette holder. Absorbance was measured from 280 nm to 205 nm at a scan speed of 50 nm/min with a data pitch of 0.1 nm and digital integration time of 4 s.

2.5 Electrophoretic Mobility Shift Assay

EMSA was performed to check G-quadruplex binding of S123C-SG4. 6-FAM_14/23 was folded to G-quadruplex by heat ramping to 90 ˚C and slowly cooling down to 25 ˚C at a rate of 1 ˚C/ min. The binding buffer consists of 10% glycerol, 100 mM KCl and 50 mM KH_2PO_4. 250 nM of 6-FAM_14/23 and was incubated with increasing concentration (0-400 nM) of S123C-SG4 in binding buffer for 30 mins at 4 ˚C. The samples were loaded in a 1.5% agarose gel and run in 0.5X TBE buffer supplemented with 25 mM KCl at 4 ˚C.

Name	Sequence 5’ - 3’
6-FAM_14/23	6-FAM-TGAGGGTGGGTAGGGTGGGTAA

Table 2.7 Sequence of the 5’ fluorescein conjugated oligonucleotide used in EMSA.

2.6 Surface Passivation

Single molecule fluorescence microscopy demands platforms which are chemically inert to avoid unwanted surface artifacts. Surface passivation for single molecule imaging was performed following the protocol laid by Chandradoss et al (Chandradoss et al., 2016).

2.4.1 Cleaning

Glass slides and coverslips are placed in a custom-made Teflon slide holder and rested in a glass beaker. They are rinsed with MQ water and sonicated in an ultrasonic bath for 10 mins. MQ water in the beakers are then replaced with acetone and the slides are sonicated again for 20 mins. The acetone is discarded and the slides are rinsed with MQ three times to remove any acetone residue. The slides are then sonicated in 1M KOH for 20 mins. After discarding KOH, the slides are rinsed with MQ water thrice to remove any traces of KOH. The slides are then treated with piranha solution (3:1 ratio of sulphuric acid and hydrogen peroxide) in fume hood. The piranha solution is discarded once it cools down and the slides are washed with MQ water. Piranha etching incorporates hydroxyl groups in glass surfaces.

2.4.2 Amino-silanization

Post piranha etching the slides are thoroughly rinsed in methanol. Amine groups are now introduced in the glass surface by treating them with 3-APTES (3-aminopropyl trimethoxysilane). The amino-silanization reaction mixture is a 100:10:5 solution of methanol, 3-APTES, and acetic acid respectively. The methanol in the beakers is replaced with the reaction mixture and kept for 20 mins at RT. The reaction mixture is discarded and the slides are thoroughly washed with methanol.

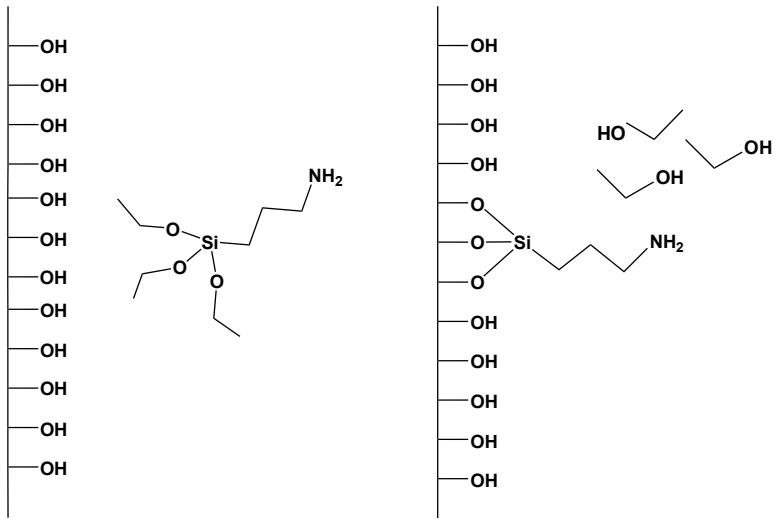


Figure 2.2: Amino-silanization. Ethoxy groups of 3-APTES reacts with the hydroxyl groups on the glass surface. Ethanol is released as a byproduct.

2.4.3 PEGylation

The slides and coverslips are now thoroughly washed with MQ water and dried with UHP nitrogen. 10 glass slides and coverslips are surface passivated in a batch. 2 mg of biotin-PEG-SVA (5000 MW) and 80 mg of mpeg-SVA are dissolved in 640µl of freshly prepared 0.1 M sodium bicarbonate. 70µl of this PEGylation mixture is added to each of the previously dried glass slides and then coverslips are gently placed on top of them. They are incubated at RT, overnight in a moist environment. The succinimidyl valerate (SVA) group reacts with primary amines, attaching valerate to the surface, releasing N-Hydroxysuccinimide. The ratio of Biotin-PEG:mPEG is kept

below 0.05, so that there are islands of Biotin-PEG in a sea of mPEG. This ensures that not all the valences of streptavidin are filled.

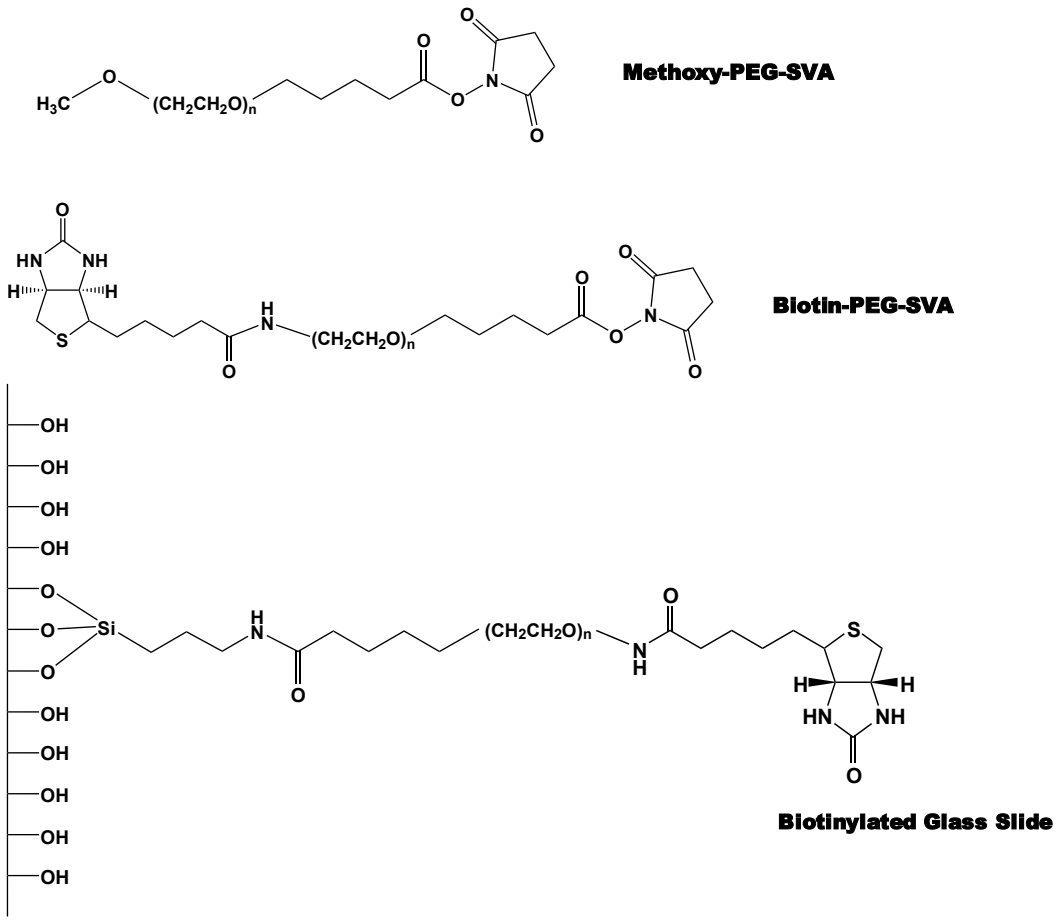


Figure 2.3: Chemical Structures of Biotin-PEG-SVA and mpeg-SVA.

After the incubation period the slides are again cleaned with MQ water, dried with UHP nitrogen, and stored in falcon tubes at -20 ˚C after evacuation and filling UHP nitrogen. These slides can be used up to 3 months.

2.7 Flow Cell Assembly

Double sided tapes (Scotch, 3M) are cut and stuck to the glass slide and the coverslip with the pegylated side is placed on top of it as shown in figure 2.4. The ends are then sealed with epoxy resin.

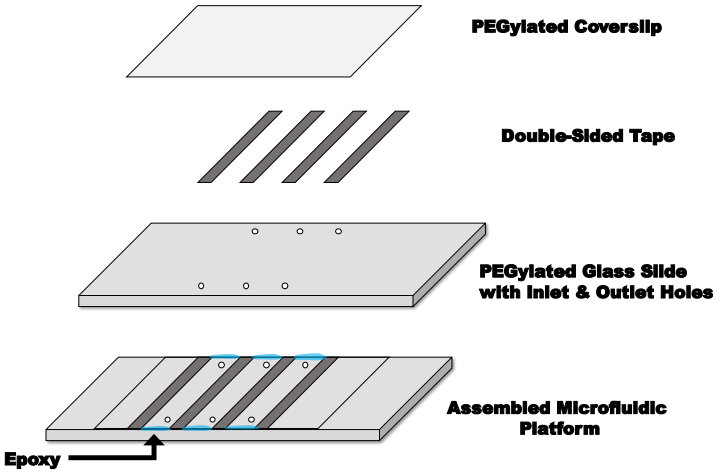


Figure 2.4: Assembly of Microfluidic Platform.

While performing experiments, a 200 µl tip is placed in the inlet hole and the outlet hole is connected to a syringe pump through a tubing. This helps in controlling the flow rate of liquids passed through the microfluidic cell.

2.8 TIRF-M Setup

For all single-molecule experiments, imaging was performed on a Nikon Ti-2 Eclipse Inverted Microscope. Five laser channels (405,488,532,561 & 640 nm) are available which are present together in a laser combiner (L6cc, Oxxius Inc). The laser enters the microscope via the modular illumination system (Nikon Ti2-LAPP), which controls the laser angle. An oil immersion TIRF objective (Apo SR TIRF 100X, NA 1.49, Nikon Instruments) magnifies the specimen and the images are captured in a sCMOS camera (PRIME BSI, Teledyne Photometrics).

2.9 DNA Preparation for ISD Assay

pSupercosλ-1, 2, a 21Kb plasmid carrying XhoI restriction site was used as substrate for ISD Assay. A 500 bp AT rich region from PBluescript phagemid was PCR amplified with Biotin-X-(5-aminoallyl)-dUTP (Jena Biosciences). The primer also contains XhoI restriction site at the end. Both the biotinylated amplicon and the plasmid are restriction digested and ligated. The amplicon serves as handles to tether the linear plasmid to the avidinated coverslip.

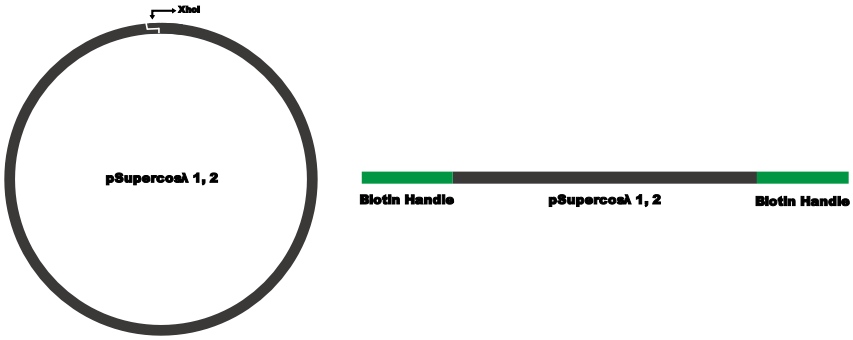


Figure 2.5: Linear pSupercosλ-1, 2 flanked by biotin handles.

Primer	Sequence 5' - 3'
B.H forward	GGTTTGCGTATTGGGCGCTCTTCCG
B.H reverse XhoI	CTCGAGAGACGATAGTTACCGGATAAGGCGC

Table 2.8: Primers used for amplification of biotin handles. Sequence highlighted in red indicates XhoI restriction site.

Components	Volume
10µM Forward Primer	2 µl
10µM Reverse Primer	2 µl
Template (pBlueScript)	2 µl
5mM dGTP	1 µl
5mM dCTP	1 µl
5mM dATP	1 µl
5mM dTTP	0.7 µl
1mM Biotin-dUTP	1.5 µl
10X Taq Reaction Buffer	5 µl
MQ Water	31.8 µl
Taq Polymerase	2 µl

Table 2.9: Components for PCR of Biotin-Handles.

Step	Temperature	Time	
Initial Denaturation	95 ˚C	3 mins	
Denaturation	95 ˚C	30 s	35 cycles
Annealing	61 ˚C	30 s	

Extension	72 ˚C	45 s	
Final Extension	72 ˚C	5 mins	

Table 2.10: PCR conditions for the synthesis of biotin-handles.

Components	pSupercosλ-1, 2	Biotin Handles
Cut Smart Buffer (10X)	5 µl	5 µl
DNA	50 µl (3000 ng)	50 µl (5000 ng)
XhoI	1 µl	1 µl

Table 2.11: Restriction digestion of pSupercosλ-1, 2 and biotin-handles.

Components	Ligation reaction
T4 DNA Ligase	1 µl
T4 Ligase Reaction Buffer (10X)	2 µl
pSupercosλ-1, 2	15 µl (30 fmoles)
Biotin Handle	0.217 µl (120 fmoles)
50% PEG	2 µl
100mM ATP	0.2 µl

Table 2.12 Ligation of pSupercosλ-1, 2 and biotin-handles.

2.10 ISD Assay

The volume of each microfluidic channel is approximately 10-15 µl. T50 buffer (pH-7.5, 50 mM Tris-HCl and 50 mM NaCl) is the default buffer to dilute any reagent in this assay. The channels are blocked with BSA & polysorbate-20 before avidination.

Step	Reagent	Volume	Incubation Time
Blocking	10% BSA	30 µl	5 mins
Blocking	1% Polysorbate-20	30 µl	5 mins
Avidination	0.5 mg/ml Neutravidin/ Streptavidin	30 µl	5 mins

Table 2.13 Blocking and Avidination of Coverslips.

All the steps mentioned in table are followed by washes with 100 µl T50 buffer. For generating positive supercoils, 100 µl of 50 pM DNA is flown through the channel at a

rate of 5-20 µl/min. The channel is then washed with T50 buffer to remove untethered-DNA. 100 µl of 100 nM SYTOX™ Orange (SxO) is then flown through the channel, which generates positively supercoiled DNA. The SxO buffer also contains 0.25 mg/ml Trolox, 0.4 mg/ml protocatechuic acid (PCA) and 300 nM protocatechuate 3,4-dioxygenase. PCA & PCD together make the oxygen scavenging system and Trolox is a triplet state quencher.

For generating negative supercoils, 100 µl solution containing 50 pM DNA and 250 nM SxO is flown through the channel. This is followed by flowing 100 µl of 50 nM SxO through the channel, this reduction in intercalator concentration results in negatively supercoiled DNA. 561 nm laser set at 1 mW source power was used to visualize the tethered DNA molecules.

3. Results & Discussion

3.1 ISD Standardization

When the DNA is flown through the channel, one end of the DNA attaches itself to the surface causing the molecule to linearly stretch, the other end subsequently binds once it finds avidin moieties. The tethering of DNA to the avidinated surface is a stochastic process and cannot be precisely controlled. The density of tethered DNA molecules on the surface mainly depends on the amount of DNA flown and the pressure at which the DNA containing buffer flows through the microfluidic channel. Adjusting these bulk parameters is a major challenge faced in optimizing the DNA density on the surface.

In ISD assay the tethered DNA molecules can be seen in three different topologies, single end tethered, supercoiled, and nicked. Because of the low persistence length, single end tethered DNA collapses and frays in the absence of flow. The plectonemes formed in the supercoiled DNA can be seen as dynamic bright spots moving back and forth. Prolonged exposure to laser causes nicking of DNA which is attributed to radicals produced by unknown photochemical processes.

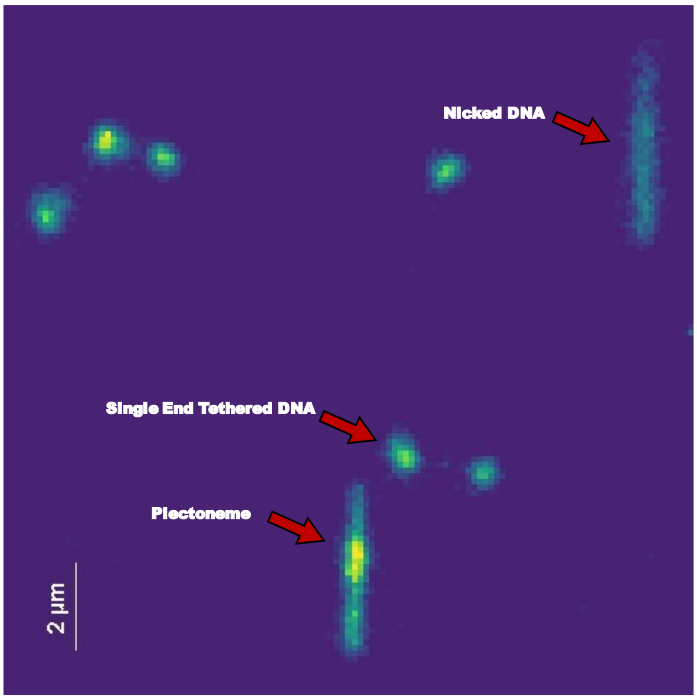
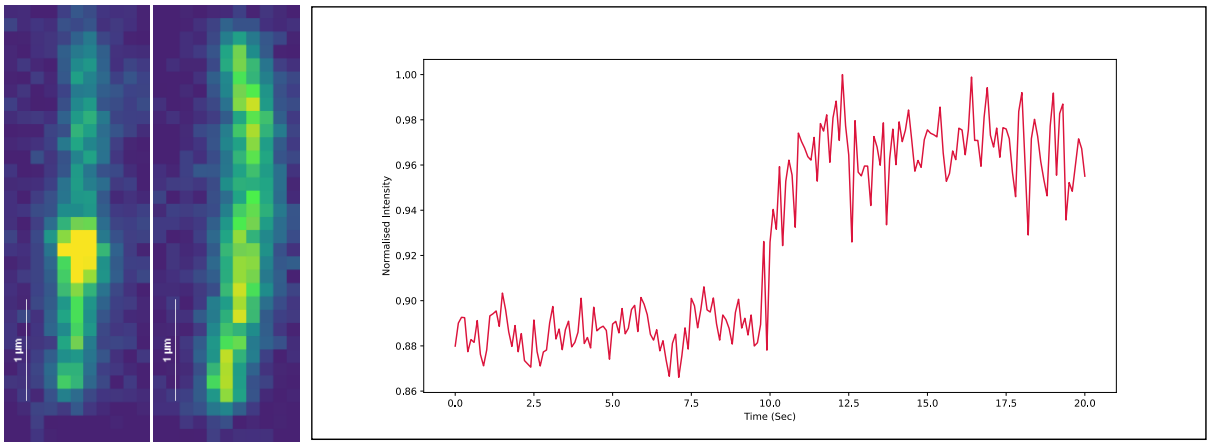


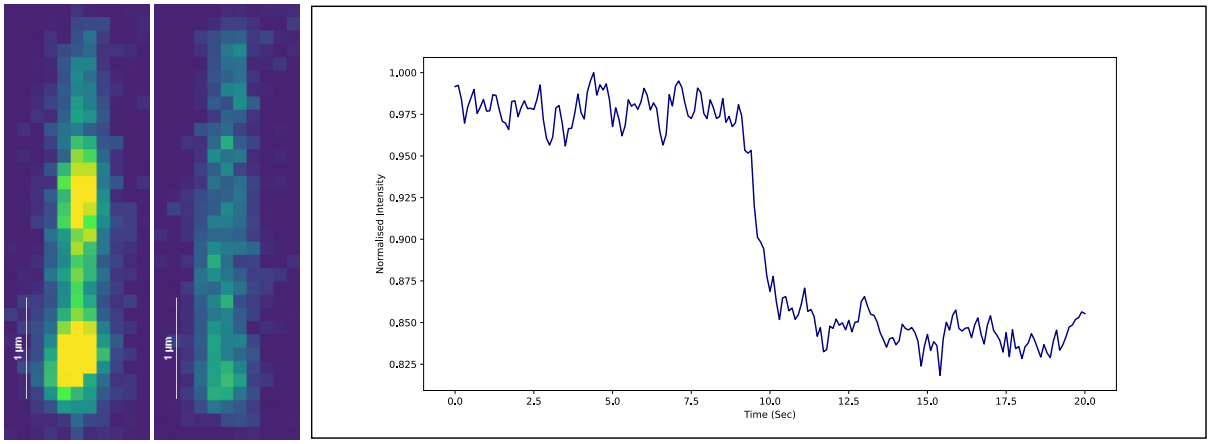
Figure 3.1: A typical field of view in ISD assay. Surface tethered, SxO stained, DNA molecules visualized with 561 nm laser in HiLo Mode.

To induce positive supercoiling, SxO is introduced after the tethering of DNA. Because of high twist rigidity of B-DNA, there is a limit to the twist that can be induced, thus the amount of SxO that can intercalate DNA is curtailed. Since photoinduced nicking relives torsional strain, more SxO molecules can now intercalate, this is observed as an increase in the fluorescence intensity as shown in fig.



(a) (b)

Figure 3.2: Nicking of positively supercoiled DNA. (a) Positively supercoiled DNA before (left) and after (right) nicking. (b) Normalized average intensity profile of the ROI shown in (a).



(a) (b)

Figure 3.3: Nicking of negatively supercoiled DNA. (a) Negatively supercoiled DNA before (left) and after (right) nicking. (b) Normalized average intensity profile of the ROI shown in (a).

To induce negative supercoiling, the linear DNA molecules are incubated with a high concentration of SxO. Since there is no torsional constraint, the linking number of the DNA decreases because of the induced twist. The DNA is then tethered and the SxO concentration is reduced. Because of torsional constraint, now there is a limit to the amount of dye molecules that can escape. Nicking of the DNA releases tension allowing SxO molecules to scarper until equilibrium is achieved, this is observed as a decrease in fluorescence intensity.

These results are in concordance with recent literature (Kolbeck et al., 2024).

3.2 Cloning & Expression Check of S123C-SG4

After KLD, four colonies were screened for mutation. One of them successfully incorporated mutation and was named pHEN2-S123C-SG4. It retains the C terminus His and FLAG tag. This plasmid was transformed in BL21-DE3 for expression check. Expression was induced at varying IPTG concentrations ranging from 0.25 mM to 1 mM IPTG, keeping the temperature conditions same as mentioned by Galli et al (Galli et al., 2022), but no over-expression was observed.

Thus, it was decided to clone the gene into another vector. Fortunately, the insert had same restriction sites (NcoI and NotI) as that of an available pET vector in the lab.

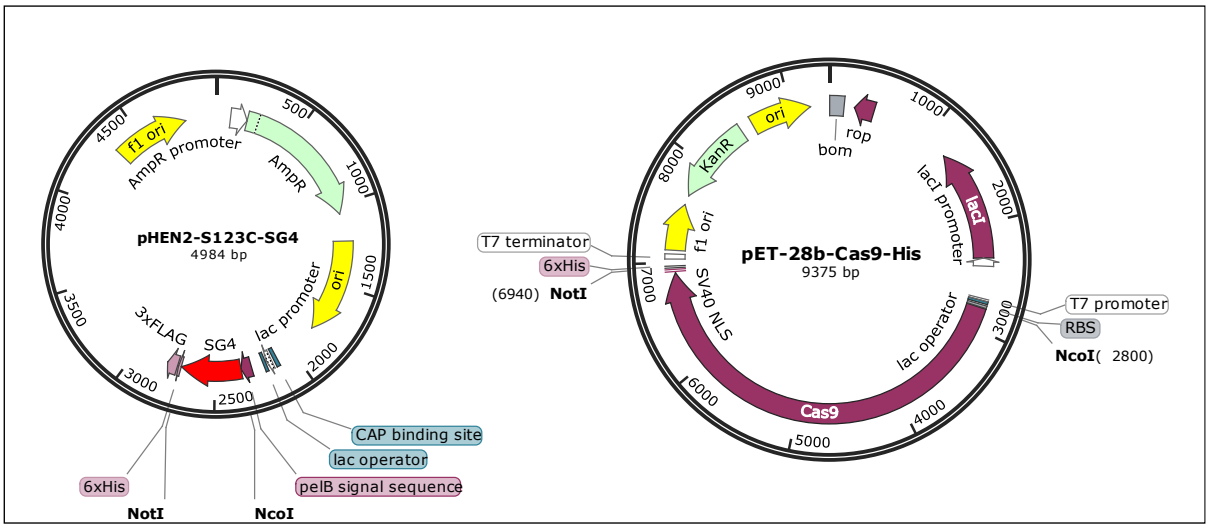


Figure 3.4: Vector maps of pHEN2-S123C-SG4 and pET-28b-Cas9-His.

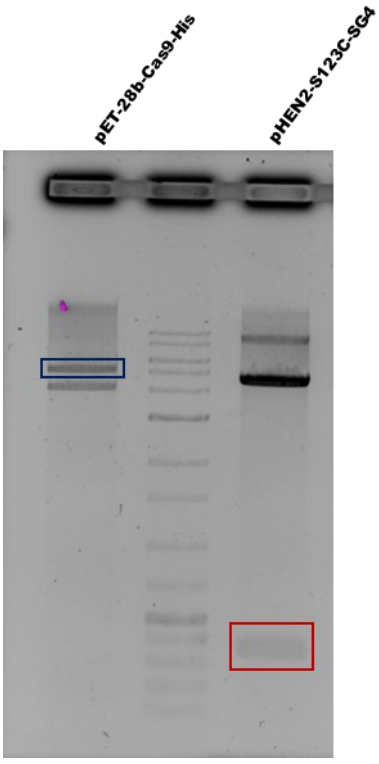


Figure 3.5: Plasmids pET-28b-Cas9-His & pHEN-S123C-SG4 digested with NcoI and NotI. Bands highlighted in red and blue indicates insert (372 bp) and backbone (5.2 kb) respectively.

After restriction cloning, four clones were sent for sequencing, of which two showed successful incorporation of the insert without any mutations. In pHEN2-S123C-SG4

the NcoI site cleaves Pel B which is an N-terminal leader sequence that exports unfolded proteins to the periplasm which provides an oxidative environment for folding. Since cloning removed the pel B sequence, the new plasmid was transformed into Shuffle® T7 Express *E. coli* cells. These cells constitutively express DsbC chaperone in the cytoplasm, which facilitates proper folding of proteins with disulphide bonds. These cells are commonly used for recombinant expression of nanobodies.

Over expression was confirmed but no significant increase in the expression profile of the protein was seen with increasing IPTG concentration.

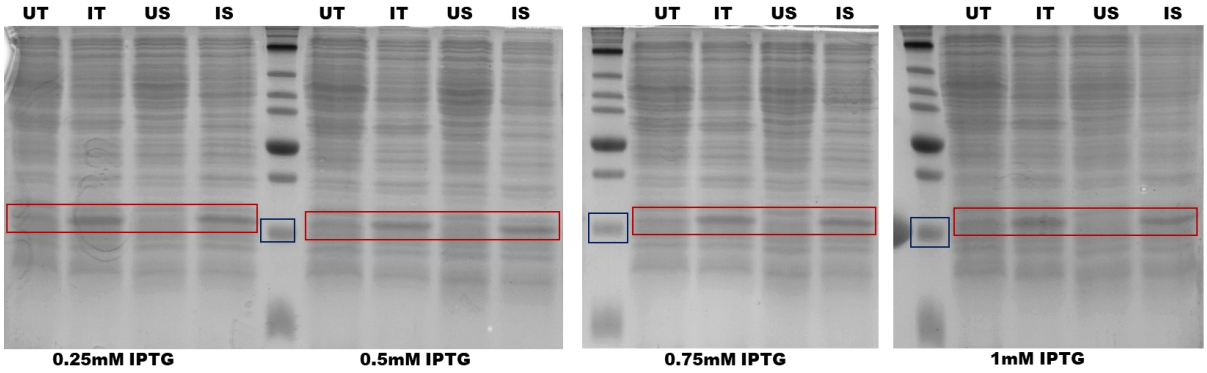


Figure 3.6: Expression Check of pET-28b-S123C-SG4. U - uninduced, I - induced, T - total, S - supernatant.

3.3 Purification of S123C-SG4

The expected protein size is 15 kDa. Even after Ni-NTA chromatography, the protein had contaminant bands above 20 kDa. To remove these SEC was performed. The first four elution fractions after Ni-NTA chromatography were concentrated to 1ml using a 10 kDa centrifugal filter (Viva-Spin, Sartorius). This concentrated protein was loaded onto size exclusion column. 65 fractions starting from E115 were pooled and concentrated.

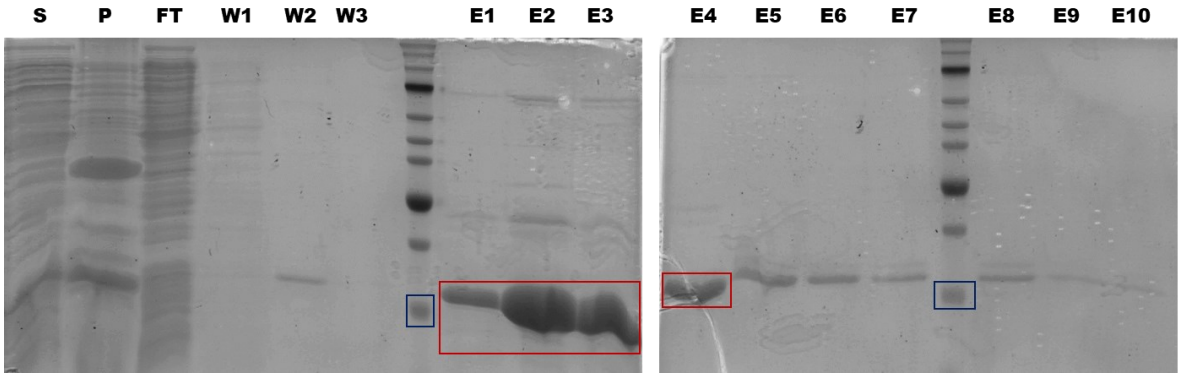


Figure 3.7: Ni-NTA purification of S123C SG4. Fractions highlighted in red were processed for SEC. Ladder band highlighted in blue is of 15 kDa.

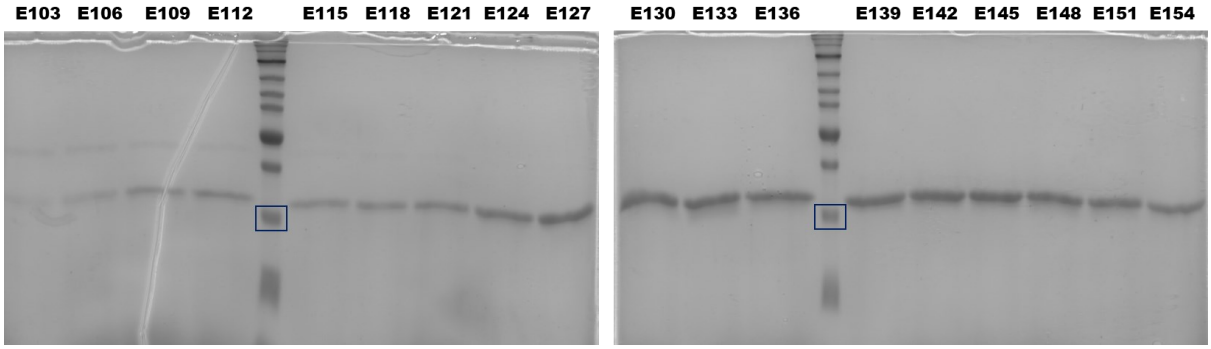


Figure 3.8: SEC purification of S123C SG4. Ladder band is highlighted in blue is of 15 kDa.

The molar extinction coefficient at 280 nm of the protein was the calculated by adding the individual extinction coefficients of tyrosine, tryptophan, and disulphide bonds at 280 nm. The concentration of protein was estimated based on the absorption measured at 280 nm in a nanophotometer (Implen). The final yield was 2 ml of 27 µM S123C-SG4. The protein was flash freezed in liquid nitrogen and stored in -80 ˚C.

3.4 Characterisation of S123C-SG4

3.4.1 CD Spectra

CD spectra of S123C-SG4 could only be measured till 205 nm from 280 nm. Even at 1µM protein concentration, the high-tension voltage exceeded limits restricting measurements at wavelengths lower than 205 nm. The negative peak at 217 nm followed by high rise near 200 nm confirms beta-sheet formation. The spectra is also very well comparable with published literature (Galli et al., 2022).

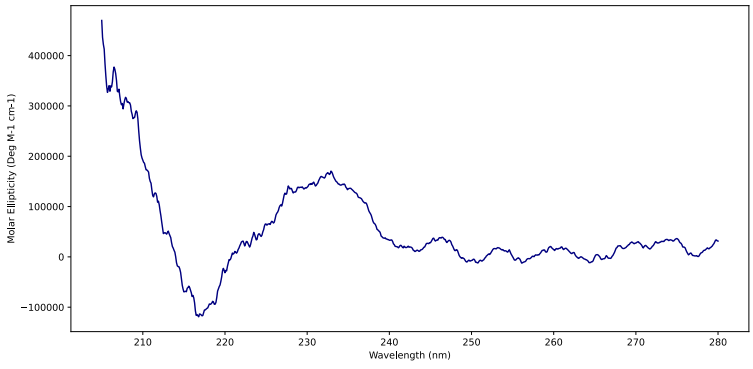


Figure 3.9: CD spectra of S123C-SG4.

3.4.2 G-quadruplex Binding Assay

To check the activity of purified protein, electrophoretic mobility shift assay (EMSA) was performed. 14/23 is a 22 bp DNA sequence known to form a parallel G-quadruplex (Hatzakis et al., 2010). The nanobody was developed against the same G-quadruplex sequence and has a dissociation constant of 2.6 nM (Galli et al., 2022). 6-FAM modified 14/23 was used as substrate for EMSA. No shift in mobility was observed with increasing concentration of protein. Multiple trials even with a freshly purified batch of S123C-SG4 was performed with no success.

Figure 3.10. EMSA with 6-FAM_14/23 G-quadruplex.

4. Conclusion & Future Prospects

ISD assay is standardized, optimizing conditions needed for creating negative supercoils. The purified S123C-SG4 is not binding to 6-FAM-14/23. Work needs to be done in standardizing conditions required for protein activity. Once protein activity is confirmed, it is planned to incorporate various G-quadruplex forming sequences in pSupercosλ-1, 2 and check whether fluorescently labelled SG4 can bind such DNA molecules upon negative supercoiling.

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