Optical tweezers are used to investigate small particles from a few micrometers to subnanometer in size at the molecular level. The optical tweezers manipulate molecules tethered between micrometer-sized particles and measures the force of these interactions, which is on the scale of piconewtons. With these advantages, optical tweezers are widely used to study structures and functions of DNA, RNA, and proteins as well as viruses, bacteria, and cells. In this dissertation, use of optical tweezers to study lipid droplet fusion as a single particle research will be presented. The lipid droplet fusion mechanism is relevant to understand growth of lipids inside the cell and its relation with diseases like obesity and fatty liver. Besides lipid droplet fusion, investigation of DNA G-Quadruplexes and its interaction with ligands at a single molecule and sub-molecule level will be presented. This research on the G-Quadruplex gives insight into the structural-stability relation relevant to target it as a means of new cancer therapy.
FUSION OF LIPID DROPLETS AND SUBMOLECULAR
DISSECTION OF DNA G-QUADRUPLEX USING OPTICAL
TWEEZERS

DISSERTATION

Submitted to Kent State University in Partial Fulfillment of the Requirements for
Doctor of Philosophy Degree in Chemistry and Biochemistry

BY

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Chapter I: Introduction and Background

1.1 Introduction of Lipid Droplets

Lipid droplets (LDs) are found in most of the cells, for example skeletal and adrenal cortex, in varying amounts.\textsuperscript{1-2} LDs contain a neutral lipid core such as triacylglycerol (TG), steryl esters, and retynyl esters coated with phospholipids and proteins in varying composition depending on tissue and cell type.\textsuperscript{3-5} LDs are prevalent in adipose tissue, liver, and intestine which are specialized in energy storage and sequestration.\textsuperscript{6} These lipids are utilized by cells in time of necessity to generate energy, create membrane components and signaling lipids.\textsuperscript{7} Recently, LDS are considered as a functional organelle inside cells with their main function to be an intracellular protein storage organelle.\textsuperscript{8-14} Protein exchange between lipid droplets and the nucleus has been shown to affect the transcription factor.\textsuperscript{15} Lipid droplets play important role in membrane functions and signaling in the nervous system.\textsuperscript{16} LDs also help cells to control toxic species and change them into an inert form.\textsuperscript{17} Due to their role in fat storage (adipose tissue), LDs are important in the pathogenesis of obesity and related diseases such as type-2 diabetes and fatty liver disease (steatohepatitis).\textsuperscript{8-10, 18-21} Some viruses utilize LDs as a host to gain a supply of lipids.\textsuperscript{22}

These important biological implications demand the elucidation of the formation and maturation of intracellular lipid droplets, in which fusion of LDs is proposed as one of the routes.\textsuperscript{23-24} Compared to biomembrane fusion, however, little is known about the fundamental
interactions important for LD fusion in aqueous solutions. Lipid droplets are being studied mostly in the cell, and various conditions play key roles for their growth and dynamics.\(^5,25-26\)

Such conditions are ions, proteins and other cellular species interacting with lipids. To understand the combined effect of all the components is important, but it is also very important to know the role of individual species so that we can explain the combined effect of all the species together. This helps us to understand the mechanism of lipid droplets dynamics that can be used to understand intracellular lipid droplets growth and could be utilized in pathogenesis.

Still little is known about the lipid droplets growth by fusion. The insufficient information is due to the difficulty to track individual droplets of similar size before and after the fusion process. Collision between neighboring droplets is random and synchronization among collisions, which is necessary for accurate kinetics measurement, is very difficult for ensemble methods. A special difficulty for an ensemble technique lies in the fact that it measures average properties of different droplets. Each cellular lipid droplet is likely to be different in size or composition, either of which may affect the fusion process. Because of these technical difficulties, a detailed profile of lipid droplet fusion is largely unknown.\(^27-28\)

1.2 Lab-In-A-Trap (LIAT) System to Study Lipid Droplets

In this research, we used an optical trap method, lab-in-a-trap or LIAT, to evaluate fundamental interactions important for the fusion between lipid droplets. Previously, optical traps and microfluidic devices have been used to facilitate the fusion of droplets.\(^29-31\) However, the collision, the docking, and the fusion processes cannot be well differentiated in the few kinetics studies reported.\(^31\) Also, measurement of fusion kinetics has been rather limited by the frame rate of the camera used in these studies.\(^31\) As a light pathway is changed upon fusion of
two droplets, we used a position sensitive photodetector (PSD) to monitor the displacement of a trapping laser to follow the entire fusion processes in situ. The time resolution of our LIAT method is only limited by the response time of a photodetector, which can reach picoseconds. The capability of this method is well demonstrated by unprecedented observation of docking, and fusion stages between two phospholipids-coated triolein droplets.

1.3 Introduction of DNA G-Quadruplex

Oligonucleotides containing adjacent runs of three or four guanines make four-stranded, non-canonical DNA structures called G-quadruplexes (GQ). The completion of the human genome sequencing and further algorithms and experiments determined that GQs occur throughout the human genome as well as in the genes of other organisms, which further raised the curiosity of their functionality. Guanine-rich sequences are enriched in the telomere and promoter region of the chromosome.

In eukaryotes, the ends of chromosomes, called telomeres, are capped with a tandem repeat of DNA with a general sequence of (T/A (1-4) G (1-8)) in association with a complex of proteins called shelterin that protects the telomere. In somatic cells of mammals, except stem cells due to lack of telomere lengthening mechanisms, with each cell division cycle telomeres progressively shorten. Here, a DNA polymerase is unable to replicate the 3' part of linear DNA due to the replication end process and the nucleolytic process of telomeres. Because of continuous shortening of the telomere, at a certain critical length, the cell cycle becomes irreversible, and the cell dies. This process is called cellular senescence. Another polymerase called telomerase is a reverse transcriptase protein which adds telomeric repeat units onto the chromosome ends to prevent replication related loss of telomere length.
Cancer cells are immortal, as the telomere lengthening mechanism is reactivated and senescence stops. Formation of GQ at the 3’ single-stranded region makes this part unable to hybridize with the telomerase RNA template. Thus, the first step in the telomere lengthening process is stopped. GQ stabilization using small molecules is well demonstrated. Many of these GQ specific small molecules have shown anticancer activity in tumor xenograft models.

Not only the telomere regions but also the promoter regions have the potential to form GQs. The promoter region in the genome is a sequence where RNA polymerase binds to start transcription downstream. Siddiqui-Jain et al. have demonstrated that GQs can form within the promoter region of c-MYC, which can be stabilized by using small molecule ligands to repress transcription of c-MYC. Other proto-oncogenes such as HIF-α, VEGF, KRAS, BCL-2, c-Kit, HIV-I, and c-MYB also have GQ motifs in their promoter regions. The GC-rich regions in these promoters are very sensitive to nucleases, and may form many secondary structures with single-stranded character. It has been shown that the GQ formed within the promoter region plays an important role in transcriptional regulation. Thus, these sequences with GQ motifs are promising targets for anti-cancer drugs.

A well-defined structure and dynamics for GQs will help in understanding their role in vivo and their modulation for treatment of various diseases such as cancer, and HIV. GQs are also considered for their roles in catalysis and as a nanomaterial in nanotechnology. Differing from Watson and Crick base pairing in duplex DNA, a GQ is formed by Hoogsteen H-bonding, which is a H-bond between the amino proton of one base and the nitrogen N7 of the adjacent base and an imino proton of one base to the oxygen O6 atom of the other base (Figure 1.1). The guanine residues are in either syn or anti position.
with respect to the sugar residue.\textsuperscript{64} This creates a planar G-tetrad of four guanines. These tetrads stack over each other through $\pi-\pi$ interaction between them. Each of these tetrads is stabilized by a coordinating cation such as K$^+$ or Na$^+$ and connected to each other by loops of nucleotides.\textsuperscript{65-66} The presence of certain ions in the solution and the type and number of nucleotides in the loops determine the conformation and stability of the GQ with stunning polymorphism. \textsuperscript{64, 67-68}

Figure 1.1 Some examples of DNA G-quadruplex conformations.

1.4 Conventional Methods of G-quadruplex Studies

Structural understandings about GQs are mainly obtained from ensemble based methods such as NMR.\textsuperscript{33, 69} However, ensemble-based methods provide only average information of the molecules in the bulk and not about the structural and kinetic information
for single molecules. Lack of synchronization in bulk methods makes it more difficult to get insights at the molecular level at physiological concentrations.

1.5 Single Molecule Methods of G-quadruplex Studies

Single-molecule methods such as single-molecule fluorescence or single molecule force spectroscopy can probe subpopulations in a reaction, and investigate the conformational dynamics of the individual species. Using force-based single molecule methods, one can also probe the mechanical stability, determine structural characteristics, and find transition kinetics. Atomic Force Microscopy, laser tweezers, and magnetic tweezers are the highly used single molecule techniques based on force.

This research utilizes a home built laser tweezers instrument as a single-molecule force spectroscopy method to obtain the sub-molecular structural information of G-quadruplexes in the human telomeric region. Same method has also been used to determine the interaction between G-quadruplex structures and small-molecule ligands. Laser tweezers are particularly suitable to measure force with picoNewton (pN) and distance with nanometer (nm) resolutions, making this method highly appropriate to study G-quadruplex structures by mechanical unfolding-refolding approach. As G-quadruplex structures have nanometer scale dimension, typically, the DNA or RNA sequence of interest is sandwiched between two long dsDNA handles, and the construct is then tethered between two polystyrene beads in the optical traps via affinity or covalent linkages. The structure is then unfolded by moving one of the optical traps, thereby recording the force and the change in extension of the unfolding events. Such experimental information on force and the extension change can be used to interpret the mechanical stability and the size of the structure respectively. Similar experiments in the presence of ligands provide the information on ligand induced stabilization or destabilization effects on G-quadruplex structures.
Chapter II: Materials and Methods

2.1 Materials

1,2,3-Trioleoylglycerol (Triolein, >99%), 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC, >99%), and 1-palmitoyl-2-(dipyrrometheneboron difluoride) undecanoyl-sn-glycero-3-phosphocholine (TopFluor PC, >99%) were purchased from Avanti Polar Lipids (Alabaster, AL), stored at −20 °C, and used without further purification. Various sodium salts of 99% purity were purchased from VWR (Randor, PA). These salts were heated to within 50 °C of their respective melting points for 7 h before use to remove any organic contaminants according to literature. Enzymes and plasmids were purchased from New England Bioabs (NEB). Nucleotides were purchased from Integrated DNA Technology (IDT). Polystyrene beads coated with streptavidin or anti-digoxigenin antibody were purchased from Spherotech (Lake Forest, IL). All other chemicals (>99% purity, unless specified) were purchased from VWR. Synthesis and characterization of telomestatin derivatives, and pyridostatin (PDS), were according to previous reports.

2.2 Laser Tweezers Instrument

The instrument was described previously. Briefly, a diode pump solid state (DPSS) laser (1064 nm, 4 W, CW mode, BL-106C, Spectra-Physics) was used as a trapping laser. The p- and s-polarized laser light from the same laser source constituted two traps. The s-polarized light was controlled by a steerable mirror (Nano-MTA, Mad City Laboratories) at a conjugate plane of the back focal plane of a focusing objective (Nikon CFI-Plan-Apochromat 60×, NA 1.2, water immersion, working distance ~320 μm). The exiting p- and s-polarized beams were collected by
an identical objective and detected by two position-sensitive photodetectors (PSDs, DL100, Pacific Silicon Sensor) separately.

2.3 Preparation of Triolein Droplets

2 μL of triolein was mixed with 4 mL of water under nitrogen. The sample was vortexed for 30 s and homogenized for 45 s at 14,000 rpm using a Polytron PT 3000 homogenizer (Brinkmann Instruments, Inc., Westbury, NY). 1 mL of the obtained solution was injected into a microfluidic chamber in the laser tweezers instrument.

2.4 Preparation of Lipid Droplets Coated with Phospholipids

A mixture of triolein and POPC with 10:1 molar ratio was mixed with 1 mL of chloroform under nitrogen. The mixture was vortexed for 30 s. The chloroform was then evaporated with a continuous flow of nitrogen gas for half an hour at room temperature. The sample was kept under vacuum overnight to remove any trace of chloroform. The LDs were prepared as described for the triolein droplets (see above). Fluorophore-labeled lipid droplets were prepared by mixing TopFluor PC into the triolein−POPC mixture with a ratio of 100:10:0.01 (triolein:POPC:TopFluor PC). The TopFluor PC-labeled lipid droplets were visualized by a Nikon Eclipse TE 2000-U (Kanagawa, Japan).

2.5 Observation of the Fusion Events in the Lab-in-a-Trap (LIAT) System

A two-channel microfluidic chamber was prepared according to the literature. The sample was injected in a side channel which is connected to the main channel via a glass pipet with an internal diameter of 20 μm. A continuous flow of a desired buffer was maintained in the main channel. We used 50 mM phosphate buffer at pH 7.4 for size effect and convergence
experiments. For buffers containing Hofmeister anions, we added 90 mM of respective monovalent anions to 10 mM phosphate buffers at pH 7.4. Two triolein or lipid droplets of identical sizes (0.5–2.5 μm in diameter) were trapped by laser tweezers in the main channel and taken to the upstream of the buffer flow to avoid subsequent trapping of other droplets. To start the fusion, the bottom laser trap was fixed while the top trap was brought closer (Figure 3.2). Once the two droplets were within ~500 nm in distance, the top droplet was released by turning off the top laser trap. The released droplet joined the bottom droplet in the fixed laser focus in ~11 ms (Figure 3.2).

After a specific time, two droplets at the bottom laser focus fused together, triggering a signal change in the position sensitive photodetector due to a change in the light path. The complete fusion was confirmed with the size and contrast changes when the two droplets merged into one (Figure 3.2).

For each fusion under a specific condition, the fusion time was sorted in increasing order for >100 pairs of fusion droplets. In a fixed time-range (see x-axis in Figure 3.3a, for example), fusion events were counted and converted to the percentage probability of fusion (y-axis in Figure 3.3a) with respect to the total pairs of lipid droplets. A single-exponential fit of the data (eq 6) gives an overall docking or fusion rate constant k. Fusion experiments were repeated three times to give standard deviations (error bars) for each measurement.

2.6 Synthesis of 5′-O-Dimethoxytrityl-N2-tert-butylphenoxyacetyl- 2′-deoxyriboguanosine 3′-O-Ethynylphosphinoamidite

Bis(N,N-diisopropylamino)ethynylphosphine (compound II in Figure 4.1c) was prepared by Grignard reaction using bis(N,Ndiisopropylamino) chlorophosphate and ethynylmagnesium
bromide as reported previously. The solution of 5′-DMT-N2-tert-butylphenoxyacetyl-2′-deoxyguanosine (0.82 mmol, 1.0 equiv, compound I in Figure 4.1c) and 1H-tetrazole was prepared in anhydrous dichloromethane (10 mL) under argon. Bis(N,N-diisopropylamino)ethynylphosphine (1.64 mmol, 2.0 equiv) in anhydrous dichloromethane (20 mL) was placed in a 100 mL two-neck round-bottom flask with a 50 mL addition funnel. To a solution of bis(N,N-diisopropylamino) ethynylphosphine was dropwise added 2′-deoxyriboguanosine and 1H-tetrazole in dichloromethane over 15 min and the mixture stirred for 30 min at room temperature under argon. After completion of the reaction, triethylamine was added to neutralize the reaction mixture and the solvent was removed in a vacuum. The residue was purified by column chromatography using a 50–100% gradient of ethyl acetate in hexane containing 1% triethylamine to afford an amorphous, ivory solid (341 mg, 45% yield). 31P NMR (CDCl3): δ 97.2, 96.0. 1H NMR (CDCl3): δ 7.83 (d, 4JHH = 1.4 Hz, 1H), 7.41–7.37 (m, 4H), 7.30–7.24 (m, 7H), 7.19 (td, 3JHH = 7.1 Hz and 4JHH = 1.4 Hz, 1H), 6.92 (d, 3JHH = 8.9 Hz, 2H), 6.79 (dd, 3JHH = 5.8 Hz and 4JHH = 2.7 Hz, 4H), 6.32 (ddd, 2JHH = 13.6 Hz, 3JHH = 6.1 Hz and 4JHH = 1.3 Hz, 1H), 4.70–4.66 (m, 1H), 4.64–4.63 (m, 2H), 4.30–4.26 (m, 1H), 3.77 (s, 6H), 3.37–3.28 (m, 2H), 3.07 (dd, 3JHH = 11.9 Hz and 4JHH = 1.7 Hz, 1H), 2.66–2.53 (m, 2H), 1.32 (s, 9H), 1.19–1.17 (m, 9H), 1.12 (t, 3JHH = 6.8 Hz, 3H). 13C NMR (CDCl3): δ 169.9, 158.8, 155.6, 154.4, 147.9, 146.3, 146.2, 144.6, 137.2, 135.8, 130.2, 128.3, 128.1, 127.1, 127.0, 122.3, 114.6, 113.4, 92.3, 86.8, 86.3, 86.1, 85.6, 83.9, 76.4, 67.4, 63.7, 55.4, 34.5, 31.6, 24.5. HRMS (ESI): calcd for C51H59N6NaO8P [M + Na]+ 937.4030, found 937.4024.

2.7 Oligodeoxynucleotide (ODN) Synthesis

ODNs (compound III in Figure 4.1c) were synthesized on solid supports using alkynemodified phosphinoamidite and commercially available O5′-dimethoxytrityl-2′-
deoxyribonucleoside-O3’-phosphoramidites. Solid-phase oligonucleotide synthesis was performed on an ABI DNA synthesizer (Applied Biosystem, Foster City, CA). The alkyne-modified phosphinoamidite was chemically synthesized as described above and incorporated without purification into oligonucleotide through coupling reactions for 10 min. Coupling yields with alkyne-modified phosphinoamidites were equal to the ones obtained with standard phosphoramidite building blocks. Cleavage from the solid support was performed with 50% of anhydrous ethylenediamine in toluene at room temperature for 2 h. The cleavage mixture was discarded and the solid support was dried using a SpeedVac. The crude oligonucleotides were obtained by washing the support with 5% acetonitrile in water (1 mL) and purified by RP-HPLC using a linear gradient of 0 to 100% acetonitrile over 40 min at a flow rate 1.5 mL/min (50 mM TEAA solution was used). The trityl-on fractions were collected and dried using a SpeedVac. The deprotection of trityl group was carried out with an 80% acetic acid solution for 2 h. After a second purification by RP-HPLC, alkyne-modified oligonucleotide was collected by freeze-drying. DNA concentrations were determined by using the Nano drop ND-1000 (Nanodrop Technologies, Wilmington, DE).

2.8 Synthesis of DNA Constructs for Mechanical Unfolding Experiments

To mechanically unfold and refold structures formed in telomeric DNA, the ethynyl-modified ssDNA fragments [the G4-G22 (top), G5-G23 (middle), and G6-G24 (bottom) constructs] prepared above were sandwiched between the two dsDNA strands of 2028 bp length. These two 2028 bp dsDNA handles were prepared separately by PCR from a pBR322 plasmid using two sets of primers. One set of primers contains azide and biotin, while the other contains azide and digoxigenin at the 5’-ends. Two 2028 bp dsDNA handles were mixed with alkyne-
modified G4-G22, G5-G23, or G6-G24 in equimolar ratio (0.5 μM) in 10 μL aqueous solution, followed by adding freshly prepared 3 μL of a solution that contains DMS/t-ButOH 3:1 v/v with 33 mM CuBr and 67 mM TBTA (tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine, Sigma).95-96 The reaction mixture was incubated overnight in the absence of light. The CuBr was removed by addition of equimolar amounts of EDTA, followed by ethanol precipitation. As a result of cyclic addition via click chemistry,97 the ssDNA sequence was sandwiched between the two dsDNA handles.

To prepare the [L1-3′] construct96 used for the determination of G-quadruplex conformation, we introduced an EagI overhang at the 5′ dsDNA terminal in a dsDNA-ssDNA hybrid similar to that described above. The ssDNA in this hybrid contained the same human telomeric G-quadruplex sequence as described above, except that T8 was replaced by an azide-modified uridine (IDT). The dsDNA-ssDNA hybrid was ligated to the 2690-bp dsDNA handle prepared above via a T4 DNA ligase (NEB) through the EagI overhang in the dsDNA section. The azide-modified uridine was attached to the 2028-bp handle terminated with the alkyne via the copper-catalyzed azide-alkyne cycloaddition (CuAAC, detailed reaction conditions see above).98 The 2028-bp handle was prepared by PCR using the pBR322 template (site 629-2961th) and two primers labeled with alkyne (hexynyl) and biotin at the 5′ ends, respectively.

The [5′-3′] DNA construct was used to determine the structure of human telomeric G-quadruplex with or without bound ligands. The detailed method to prepare this construct has been reported previously.81 Briefly, we first hybridized a ssDNA fragment (83-nt) that contained the human telomeric G-quadruplex sequence (5′-TTA(GGG TTA)4-3′) with two ssDNA pieces that were complementary to the two terminal regions of the 83-nt fragment.
The resultant dsDNA-ssDNA hybrid contained EagI and XbaI overhangs in the termini of the two dsDNA regions. The dsDNA-ssDNA hybrid was then ligated with the 2690-bp dsDNA handle (terminated with an EagI overhang, which was prepared from the pEGFP plasmid (NEB) through sequential digestions with EagI and SacI endonucleases) and the 2028-bp dsDNA handle (terminated with an XbaI overhang, prepared from the site 629\textsuperscript{th}-2691\textsuperscript{th} of the pBR322 plasmid) sequentially using T4 DNA ligase (NEB). The final construct contained a single-stranded G-quadruplex hosting sequence sandwiched between two dsDNA handles that were end-labeled with digoxigenin (the 2690-bp handle, digoxigenin was introduced by incorporation of digoxigenin-modified dUTP using terminal transferase) and biotin (the 2028-bp handle, see above for preparation), respectively.

To prepare the [L1-3'] construct\textsuperscript{96} used for the determination of G-quadruplex conformation, we introduced an EagI overhang at the 5' dsDNA terminal in a dsDNA-ssDNA hybrid similar to that described above. The ssDNA in this hybrid contained the same human telomeric G-quadruplex sequence as described above, except that T8 was replaced by an azide-modified uridine (IDT). The dsDNA-ssDNA hybrid was ligated to the 2690-bp dsDNA handle prepared above via a T4 DNA ligase (NEB) through the EagI overhang in the dsDNA section. The azide-modified uridine was attached to the 2028-bp dsDNA handle terminated with the alkyne via the copper-catalyzed azide-alkyne cycloaddition (CuAAC, detailed reaction conditions see above).\textsuperscript{98} The 2028-bp handle was prepared by PCR using the pBR322 template (site 629-2961\textsuperscript{th}) and two primers labeled with alkyne (hexynyl) and biotin at the 5' ends, respectively.

These DNA constructs were stored in a degassed buffer (100 mM KCl, 25mM EDTA, and 10 mM Tris, pH 7.4) at -80 °C for future use.
2.9 Synthesis of Telomestatin Derivatives

Synthesis and structural characterizations of the derivatives were performed according to the precedent report. All derivatives were prepared as 10 mM stock solutions in dimethyl sulfoxide (DMSO). Further dilutions to working concentrations were performed using 10 mM Tris buffer (pH 7.4) with 100 mM KCl.

2.10 Single Molecule Force Ramp Assay

Single molecule force ramping was performed with home-built dual-trap optical tweezers at 23 °C in 10 mM Tris buffer containing 100 mM KCl at pH 7.4, with and without telomestatin derivatives. Two kinds of polystyrene beads coated either with streptavidin or anti-digoxigenin antibody were separately trapped by laser tweezers in a microfluidic chamber. The antibody coated bead was incubated with the DNA construct prior to the laser trapping. One of the traps was fixed while the other was controlled by a steerable mirror. The DNA was tethered between the two polystyrene beads through affinity interactions between anti-digoxigenin antibody and biotin–streptavidin complexes. When the two beads were moved apart, the tension in the DNA tether increased, which was recorded in the force–extension (F–X) curves using the Labview program (National Instruments Corp., Austin, TX). We recorded the force range from 0 to 60 pN at 1000 Hz with a loading rate of 5.5 pN/s. The single molecular nature of the DNA was confirmed by the observation of the 65 pN plateau in the F–X curve.

The F–X curves were filtered using the Savitzky–Golay function with a time constant of 10 ms in the Matlab program (The Math Works, Natick, MA). The change in extension (Δx) at a given force was calculated from the difference between stretching and relaxing curves at that force.
The change in contour length ($\Delta L$) was calculated from $\Delta x$ using the wormlike chain model (WLC).100

$$\frac{\Delta x}{\Delta L} = 1 - \frac{1}{2} \sqrt{\frac{k_B T}{F P}} + \frac{F}{S}$$ \hspace{1cm} (1)

where $L$ is the contour length, $k_B$ is the Boltzmann constant, $T$ is the absolute temperature, $P$ is the persistence length (50.8 nm),101 and $S$ is the stretching modulus (1243 pN).101

Wherever appropriate, three sets of experiments were performed to obtain standard deviations, which are reported in the main text or as error bars in figures.

Calculation of Change in the Free Energy of Unfolding. The change in free energy of unfolding a G-quadruplex ($\Delta G_{\text{unfold}}$) was calculated using Jarzynski’s equality equation under nonequilibrated conditions.102

$$\Delta G_{\text{unfold}} = -k_B T \ln \sum_{i=1}^{N} \frac{1}{N} \exp\left(-\frac{w_i}{k_B T}\right)$$ \hspace{1cm} (2)

where $N$ is the number of repetitions and $w_i$ is the nonequilibrium work done to unfold the G-quadruplex. See the literature86 for a detailed calculation of the work.

The bias for $\Delta G_{\text{unfold}}$ was estimated from the histogram of work distribution according to the literature.103

2.11 Surface Plasmon Resonance (SPR) Binding Assay

The 5′-biotin-AGGG(TTAGGG)3-3′ telomere DNA sequence used in the SPR experiments was purchased from Sigma Genosis (HPLC grade). The SPR binding experiments were performed with Biacore T-200 (GE Healthcare, Chicago, IL). The binding experiments were carried out according to the report by Hurley and co-workers as follows.104-106 The biotin labeled
DNA was bound to a streptavidin-coated sensor chip (Series S Sensor Chip SA). One flow cell was used to immobilize the DNA [400 resonance units (RU)], while a second cell was left blank as a control. The binding experiments were performed in the sterile, filtered, and degassed HEPES buffer [0.01 M HEPES (pH 7.4), 3 mM EDTA, and 0.05% surfactant P20 with 100 mM KCl]. For binding experiments, an experimental solution of L2H2-6OTD monomer and its dimer was prepared in the HEPES buffer by serial dilutions from stock solution (10 mM). The experimental solutions at concentrations from 7.8 to 62.5 nM were injected through the DNA and blank flow cells at a rate of 100 μL/min at 25 °C until a constant steady-state response was obtained (300 s, Figure 4.6). Compound solution flow was then replaced by buffer flow, resulting in dissociation of the complex (350 s). To remove any remaining bound compound after the dissociation phase of the sensorgram, a low-pH glycine regeneration buffer was used (10 mM glycine at pH 2). The reference response from the blank cell was subtracted from the response of the sample flow cell to give an instrument response (RU) that is directly proportional to the amount of L2H2-6OTD monomer and its dimer bound to the immobilized DNA. Reference-subtracted sensorgrams for each concentration were analyzed using the kinetics 1:1 binding program using BIAevaluation software. The dissociation constant (KD) between G4 ligands and telomeric G4 is defined according to KD = kd/ka, where ka and kd represent the kinetic constants for association and dissociation, respectively (see Table 4.4 for values).

2.12 Circular Dichroism (CD)

CD spectra were collected in Jasco-810 spectropolarimeter (Easton, MD) using a quartz cuvette with 1 mm optical path length at room temperature. Five micromolar of DNA samples were dissolved in a 10 mM Tris buffer (pH 7.4) with 100 mM KCl. These DNA samples were heated at 95 °C for 10 min before rapid cooling in an ice-bath. They
were then mixed with five micromolar ligands for CD spectra collection. Each spectrum was recorded as an average of three scans over the wavelength range of 220-320 nm with a scan rate of 100 nm/min. The background signals from the buffer solution were subtracted from the spectra of DNA samples with or without ligands and smoothed using Savitzky-Golay function.

2.14 Pairwise Comparison Using Root Mean Square Deviation (RMSD)

To determine the binding mode of unknown ligands to telomeric G-quadruplex, we calculated root mean square deviation of the unfolding forces ($RMSD_F$) between ligands with unknown binding modes (the classification group, $F_{\text{classification}}$) and those with known binding modes (the training group, $F_{\text{training}}$) along the same unfolding geometry (eqn (2)),

\[
RMSD_F = \sqrt{\frac{\sum_{i=1}^{n} (F_{\text{classification},i} - F_{\text{training},i})^2}{n}}
\]

here $n=3$, which represents the types of rupture forces of G-quadruplexes mechanically unfolded from the top, middle, and bottom G-quartets, respectively. From the least $RMSD_F$ to the known binding mode, the binding mode for the ligands of interest was assigned.

Since the comparison was based on the shape of a profile that contains three rupture forces corresponding to the mechanical unfolding from the three different G-quartets, respectively, we normalized each profile so that average rupture force remained the same during each comparison.

RMSD was also used to determine the conformation of the G-quadruplex with or without various ligands. To this end, distances between the two click-chemistry-modified
phosphorus atoms ($x_{\text{tweezers}}$) were first retrieved from the change-in-contour-length ($\Delta L$) during the mechanical unfolding of a specific DNA construct using the equation,\textsuperscript{107}

$$x_{\text{tweezers}} = n \times L_{nt} - \Delta L,$$

where $n$ is the number of nucleotides in the folded G-quadruplex and $L_{nt}$ is the contour length per nucleotide (0.45 nm).\textsuperscript{108-109}

These distances were then compared with those measured from the known PDB structures ($x_{\text{PDB}}$) using the following equation,

$$\text{RMSD}_x = \sqrt{\frac{\sum_{i=1}^{n} (x_{\text{tweezers},i} - x_{\text{PDB},i})^2}{n}} \quad \text{.......................... (4)}$$

here $n$ represents the number of different unfolding geometries. We chose 5 geometries which include the unfolding through the 5'-3' (G4-G24), L1-3' (T8-G24), top G-quartet (G4-G22), middle G-quartet (G5-G23), and bottom G-quartet (G6-G24). The least deviation between the distance from the PDB structure and that from optical-tweezers measurement (RMSD$_x$) determines the most likely structure. Here the relative errors for the RMSD$_x$ values were calculated from error propagation.\textsuperscript{110}
Chapter III: Controlled Particle Collision Leads to Direct Observation of Docking and Fusion of Lipid Droplets in an Optical Trap


3.1 INTRODUCTION

Recently, intracellular lipid droplets (LDs) coated with phospholipids have emerged as a functional organelle inside cells.\(^8\)\(^-\)\(^9\) Intracellular lipid droplets are heterogeneous organelles that consist of a neutral lipid core of triacylglycerol (TG) and cholesterol esters (CE) with varying fatty acid composition and CE/TG ratios depending on tissue and cell type.\(^{111}\) Apart from serving as energy storage, LDs appear to function as an intracellular protein storage platform.\(^{27}\), \(^{112}\) Because of their role in fat storage (adipose tissue), LDs are important in the pathogenesis of obesity and related diseases such as type 2 diabetes and fatty liver disease (hepatic steatosis).\(^{113}\)

These important biological implications necessitate the elucidation of the formation and maturation of intracellular lipid droplets, in which fusion of LDs is proposed as one of the routes.\(^{23}\), \(^{114}\) Compared to biomembrane fusion, however, little is known about the fundamental interactions important for LD fusion in aqueous solutions. The paucity of knowledge can be in part ascribed to the difficulty to track individual droplets of similar size before and after the fusion process. Collision between neighboring droplets is random, and synchronization among collisions, which is necessary for accurate kinetics measurement, is nearly impossible for ensemble methods. A special difficulty for an ensemble technique lies in the fact that it measures average properties of
different droplets. Each cellular lipid droplet is likely to be different in size or composition, either of which may affect the fusion process. Because of these technical difficulties, a detailed profile of lipid droplet fusion is largely unknown.\textsuperscript{19, 27}

Herein, we used an optical trap method, lab-in-a-trap or LIAT, to evaluate fundamental interactions important for the fusion between lipid droplets. Previously, optical traps\textsuperscript{29-30} and microfluidic devices\textsuperscript{31, 115} have been used to facilitate the fusion of droplets. However, the collision, docking, and fusion processes cannot be well differentiated in the few kinetics studies reported. In addition, measurement of fusion kinetics has been rather limited by the frame rate of the camera used in these studies.\textsuperscript{31} As a light pathway is changed upon fusion of two droplets, we used a position-sensitive photodetector (PSD) to monitor the displacement of a trapping laser to follow the entire fusion process in situ (Figure 3.1 and 3.2). The time resolution of our LIAT method is only limited by the response time of a photodetector, which can reach picoseconds. The capability of this method is well demonstrated by unprecedented observation of docking and fusion stages between two POPC-coated triolein droplets (Figure 3.2). The rate-limiting docking process prior to the fusion is influenced by anions according to a general Hofmeister series, which indicates that shedding off water in the hydration layer of a droplet is the key step during the docking process. Compared to bare triolein droplets, POPC-coated droplets have slower fusion rates, suggesting that breaking of phospholipid coating is involved during the fusion. Direct observation of a hemifusion stage in the POPC-coated droplets, but not in the bare triolein droplets, provided further evidence for the importance of the phospholipid coating during the fusion.
3.2 RESULTS AND DISCUSSION

Lab-in-a-Trap (LIAT) Platform To Investigate Droplet Fusion. As the refractive index of triolein is higher than that of aqueous solutions, we could trap two triolein droplets in a microfluidic chamber by two 1064 nm laser beams in our instrument (Figure 3.1 and 3.2a, see materials and methods for the preparation of triolein droplets). We then brought two triolein droplets together without physical contact by moving one laser trap with a steering mirror in the instrument (Figure 3.2a).

![Figure 3.1 Estimation of the time required for a droplet released from the top laser trap to join the bottom laser trap.](image)

To start fusion, we released the top droplet by turning off the corresponding laser trap. Because of the trapping power of the bottom laser focus at proximity, the top droplet quickly joins with the bottom in ~11 ms (see Figure 3.1 for the time constant estimation). The joining of the two particles induced a signal variation in the PSD that records the position of the remaining trapping laser (top panel, Figure 3.2). This signal change allowed us to postsynchronize the docking process by precisely determining the start time of docking. Merging (fusion) between the
two collided droplets occurred at the end of the docking. It generated a sudden change in the light path of the bottom trap laser (Figure 3.2b,c). The resultant signal variation in the PSD signified the end of the docking and the start of the fusion. When fusion is complete, the merged droplet in Figure 3.2 The lab-in-a-trap (LIAT) platform. (a) Two spherical droplets are separately trapped at two laser foci that are placed side by side. (b) The top trap is turned off, releasing the top droplet to contact with the bottom one captured in the bottom laser trap. Notice the different contrast in the droplet image in the bottom panel is a result of the two overlapping droplets. (c) Two droplets are fused in the bottom laser trap after the docking stage in (b). Top panel: entire fusion processes (collision, docking, and fusion) are recorded by a position sensitive photodetector (PSD) in the laser tweezers instrument. Notice the change of the laser position in the PSD during different fusion stages. Images of the droplets (bottom panels) were taken by a CCD camera in the laser tweezers instrument. Scale bar in (c) represents 2 µm.
Figure 3.3 Docking rate constants retrieved from different number of triolein droplet pairs in a 50 mM phosphate buffer at pH 7.4. (a) Fusion probability vs docking time for different number of droplet pairs. For clarity, only fusion experiments with 15, 100, and 145 pairs of droplets are shown. Solid curves represent single exponential fit. (b) Docking rate constant \( k \) obtained from single exponential fitting in (a) is plotted against the number of droplet pairs. Black curve depicts a single exponential fitting to guide eyes. Errors in (a) and (b) represent the standard deviations from \( \geq 3 \) independent sets of experiments.
Figure 3.4 Size effect on the docking of triolein droplets in a 50 mM phosphate buffer at pH 7.4. (a) Fusion probability vs docking time for the droplets of 0.5-1.5 µm (grey solid circles) and 1.5-2.5 µm (red solid triangle) in diameter. Curves represent single exponential fittings. (b) Docking rate constants (k) for the droplets with different sizes. Values of k are obtained from the single exponential fittings in (a). Errors in (a) and (b) represent the standard deviations from ≥3 independent sets of experiments.
Figure 3.5 Effect of droplet size on the actual fusion of triolein droplets in a 50 mM phosphate buffer at pH 7.4. (a) Fusion probability vs fusion time. Solid curves represent single exponential fittings. (b) Comparison of fusion rate constants for droplets with different size. Errors in (a) and (b) represent the standard deviations from ≥3 independent sets of experiments.

in the bottom trap changed the light path of the trapping laser close to the original position before the top droplet was joined (Figure 3.2, top panel). From these variations in PSD signals, docking and fusion processes were clearly differentiated, and their lifetimes were precisely measured with a resolution of 10 μs.
To evaluate the kinetics of the docking or fusion process, fusion probability from a collection of triolein droplets was plotted with docking or fusion time (Figure 3.3, 3.4 and 3.5). The apparent docking or fusion rate constant $k$ was obtained by fitting with the single-exponential equation

$$p(t) = p(\text{teq}) - A \exp(-kt) \quad (1) \quad \ldots \quad (6)$$

where $p(t)$ and $p(\text{teq})$ are fusion probabilities at time $t$ and equilibrium time $\text{teq}$, respectively, and $A$ is a constant. The rate constant determined in this fashion denotes the time it takes for 63% of droplets to complete the docking or fusion process.

The number of triolein droplet pairs that can produce accurate and precise kinetic information was evaluated from the fitting of the fusion probability measured in a 50 mM phosphate buffer at pH 7.4. As shown in Figure 3.3, docking rate constants converge at around 100 droplet pairs. Below 100 droplet pairs, the uncertainty in the rate constant measurement becomes significant. Therefore, in the following experiments, a minimum of 100 pairs of lipid droplets were used to measure kinetics.

The effect of droplet size on the kinetics was investigated next. Triolein droplets ranging from 0.5 to 2.5 μm were split into two groups (0.5–1.5 and 1.5–2.5 μm). Docking and fusion rate constants from these two groups in the same phosphate buffer showed similar values, suggesting that the size effect in the range of 0.5–2.5 μm is insignificant for either docking (Figure 3.4) or fusion (Figure 3.5). Based on these results, we chose to follow the docking and the fusion events between droplets of 1–2 μm in diameter, which are consistent with the size of LDs in numerous cell types.

The lab-in-a-trap (LIAT) method described here allows well controlled collision between two droplets, which solves the synchronization problem encountered in ensemble kinetic
measurements. In addition, heterogeneous properties, such as size and shape of particles, can be well controlled as trapped particles with desired property can be selected prior to the kinetic evaluation. Fusion of individual droplets has been monitored previously by light scattering\textsuperscript{30} or high-speed camera\textsuperscript{31} in hollow optical fibers or optical traps, respectively. However, the light scattering approach cannot differentiate the collision, the docking, or the physical fusion stage. Depending on the viewing angle, a high-speed camera may separate these processes in principle. However, since it is angle dependent, a special setup must be accommodated to ensure effective measurement. In addition, the temporal resolution is limited by the frame rate (∼1 ms) of a camera.\textsuperscript{31} Given that the docking time and the actual merging time of two particles can be precisely determined by monitoring the position of a trapping laser in situ and in real time, our LIAT platform enables an easy differentiation of the collision, docking, and fusion stages. The temporal resolution is only limited by the response time of a PSD, which can easily reach tens of picoseconds when visible laser traps are used.

3.2.1 Docking and Physical Fusion between Triolein Droplets

With the LIAT method firmly established, we set out to interrogate the mechanism of the docking and fusion processes. Previously, it has been found that many chemical and biological processes, such as folding of macromolecules and solvation of hydrophobic compounds, are affected by ions according to a Hofmeister series.\textsuperscript{118} In the past, it was considered that Hofmeister ions influence an aqueous process by changing water structures, which is a rather indirect effect.
However, evidence now suggests that direct interaction between ions and molecules in aqueous processes can be effective.\textsuperscript{118} By evaluating the effect of Hofmeister ions on various

Figure 3.6 Effect of anions on the docking of triolein droplets. (a) Fusion probability vs docking time for different anions. Solid curves show single exponential fittings. Inset shows a blow-up region for the fusion that occurs below 50 seconds. (b) Docking rate constant $k$ for different anions. Inset shows the blowup region. Values of $k$ are obtained from the single exponential fitting in (a). Error bars in (a) and (b) are the standard deviations from $\geq 3$ independent experiments.

However, evidence now suggests that direct interaction between ions and molecules in aqueous processes can be effective.\textsuperscript{118} By evaluating the effect of Hofmeister ions on various
fusion processes, we wish to reveal the role of water molecules on each step of lipid droplets fusion.

First, we investigated the effect of anions on the triolein droplets in phosphate buffer at pH 7.4. To maintain a constant effect of phosphate while evaluating different anions on the docking and physical fusion processes, we added 90 mM anions into a 10 mM phosphate buffer during each experiment (see Experimental Section). Using the LIAT approach described above, we found that in general docking rates of different anions follow the Hofmeister series (Figure 3.6). In contrast, when we analyzed the physical fusion rates, no clear trend was observed for the Hofmeister anions although their rates were faster than the corresponding rates for the docking (Figure 3.7). These results suggested that docking is the rate-limiting step that may involve water molecules.

3.2.2 Docking and Physical Fusion between Lipid Droplets

Next, we investigated the effect of Hofmeister anions on the docking and physical fusion between two lipid droplets in the same set of phosphate buffers (see Materials and Methods). To mimic intracellular lipid droplets, we mixed triolein with POPC (10:1) to prepare LDs coated with a phospholipid coating. Triolein and POPC are major components found inside and at the surface of intracellular lipid droplets, respectively. To confirm that POPC stays on the droplet surface, we introduced a fluorophore-labeled phospholipid, TopFluor PC, into the triolein–POPC mixture with a final ratio of 100:10:0.01 (triolein:POPC:TopFluor PC). As shown in the inset of Figure 3.9, line scan of fluorescence intensity confirms that phospholipid stays mainly at the periphery of a droplet.
Using the LIAT strategy, we found that, like the triolein droplets, anions influence the docking kinetics according to the Hofmeister series (Figure 3.9) while the same effect is much reduced in the physical fusion kinetics (Figure 3.10). Given the same concentration, polyvalent anions $\text{SO}_4^{2-}$ and $\text{PO}_4^{3-}$ have a more pronounced effect than the monovalent anions on the docking of either triolein or lipid droplets (Figures 3.6 and 3.9). This suggests that counterions may influence the docking process since the concentration of cation for $\text{SO}_4^{2-}$ or $\text{PO}_4^{3-}$ is 2 to 3 times

Figure 3.7 Effect of anions on the physical fusion of triolein droplets. (a) Fusion probability vs fusion time for different anions. Solid curves show single-exponential fittings. (b) Fusion rate constant $k$ for different anions. Values of $k$ are obtained from the single-exponential fitting in (a). Error bars in (a) and (b) are the standard deviations from $\geq3$ independent experiments.
higher than that for monovalent anions. Further experiments directly showed that docking rate increases with total ion concentration (Figure 3.8). Approximated by a linear concentration effect on the docking rate, however, the Hofmeister ranking still holds for anions with different charges (the total ion concentrations for buffers that contain trivalent, bivalent, and monovalent anions are 387, 297, and 207 mM, respectively; see Table 3.1 for respective k values). By computer simulation, it was found that kosmotropic anions in the Hofmeister series, such as SO\textsubscript{4}\textsuperscript{2−}, are excluded from the water layer in immediate proximity to the air–water interface whereas polarizable chaotropic anions are enriched. This leads to increased interfacial tension in the presence of kosmotropic ions with respect to chaotropic species. Because of the osmotic effect,

![Graphs](image.png)

*Figure 3.8 Effect of total ion concentration on the docking of triolein droplets in phosphate buffers at pH 7.4. (a) Docking probability vs time at phosphate concentration ranging from 10-100 mM. Solid curves represent single exponential fits and errors are the standard deviations from ≥ 3 experiments. (b) Docking rate constant k vs phosphate concentrations. Inset shows the blowup region of 10-25 mM phosphate. (c) A summary table of k at different phosphate concentrations.*
Figure 3.9 Effect of anions on the docking of the triolein droplets coated with POPC.  
(a) Fluorescence intensity profile of a triolein droplet coated with TopFluor PC labeled lipid (inset). Scale bar represents 1 µm.  
(b) Fusion probability vs docking time for different anions. Solid curves show single exponential fittings. Inset shows a blow-up region for the fusion that occurs below 50 seconds.  
(c) Docking rate constant k for different anions. Error bars in (b) and (c) represent standard deviations from ≥ 3 experiments.  
(d) Schematic drawing depicting water shedding during entire fusion processes. Water shedding is more obvious during the docking stage (left) with respect to later stages.
it becomes more difficult for water to enter the hydration shell from the bulk (demixing) and solvate hydrophobic surfaces in a solution that contains kosmotropic ions. A similar enrichment of chaotropic anions and depletion of kosmotropic anions was found at the interface between water and polar molecules such as amides and esters. Merging of two lipid droplets forms a bigger droplet with reduced surface area compared to those of two separate, but smaller droplets. The reduced solvent access area suggests that water molecules must be removed from the droplet

Figure 3.10 Effect of anions on the actual fusion of lipid droplets. (a) Fusion probability with fusion time for different ions. (b) Fusion rate constants for different anions.
surface before physical fusion can occur. The general trend of Hofmeister series on the docking kinetics observed here provides evidence that water shedding is involved in the docking process (Figure 3.9d). Further evidence of water shedding comes from the effect of the ion concentration on the docking rate (Figure 3.8). As phosphate concentration increases, the difference in the osmotic pressure between

![Diagram](image)

*Figure 3.11 Direct observation of a hemifusion stage by monitoring PSD signals. The bottom inset shows a PSD trace that contains collision, docking, and fusion events. The top inset shows a possible hemifusion stage in which phospholipid coatings from the two lipid droplets form a transient stalk structure.*

the droplet surface and bulk solution becomes larger, which facilitates the docking through easier mixing of interfacial water molecules to the bulk. Compared to bare triolein droplets (Figure 3.6),
we observed that it takes a longer time for phospholipid-coated LDs to complete the docking process in the presence of the same ions (Table 3.1). It is interesting to note that as the LD surface is covered by charged (zwitterionic) phospholipids, enrichment of anions at the interface may be increased due to the attraction of anions by positive choline groups that stay closer to the interface. This effect reduces the surface tension of phospholipid-coated LDs with respect to bare triolein droplets, slowing down the docking process as water shedding becomes sluggish. On the other hand, it is known that PC head groups are solvated by a clathrate shell of water molecules,\textsuperscript{121} which is energetically more difficult to remove compared to regular interfacial water molecules and therefore, reducing the rate of water shedding.

Compared to docking, the physical fusion is a much faster process that finishes within tens of milliseconds (Figure 3.10 and Table 3.1). This result demonstrates that docking is rate-limiting during the lipid droplet fusion. The critical role of water shedding during the rate-limiting docking stage has profound biological significance. It has been proposed that LD fusion represents an important route to equilibrate LDs inside cells.\textsuperscript{114} Proteins from outside LDs have been shown to play indispensible roles in this process.\textsuperscript{23} Given the importance of the water shedding during the docking of lipid droplets, the fusogenic proteins, such as SNARE,\textsuperscript{122} may perhaps be understood from their effects on the water shedding while bringing two droplets together.

We noticed that the kinetics of the fusion is independent of Hofmeister anions (Figure 3.10b), suggesting that during the physical fusion process involvement of water molecules is rather limited. Instead, comparable to lipid vesicle fusion, breaking of the phospholipid layer could be important for this process.\textsuperscript{123}
Table 3.1 Comparison of docking and fusion rate constants for POPC coated triolein droplets (lipid droplets) and pure triolein droplets.

<table>
<thead>
<tr>
<th>Ions</th>
<th>Lipid Droplets</th>
<th>Triolein Droplets</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Docking $k_{\pm sd} , (s^{-1})$</td>
<td>Fusion $k_{\pm sd} , (s^{-1})$</td>
</tr>
<tr>
<td>SO$_4^{2-}$</td>
<td>0.08 (0.03)</td>
<td>70 (10)</td>
</tr>
<tr>
<td>PO$_4^{3-}$</td>
<td>0.083 (0.03)</td>
<td>70 (10)</td>
</tr>
<tr>
<td>Cl$^-$</td>
<td>0.0175 (0.005)</td>
<td>80 (10)</td>
</tr>
<tr>
<td>NO$_3^-$</td>
<td>0.0170 (0.004)</td>
<td>90 (10)</td>
</tr>
<tr>
<td>I$^-$</td>
<td>0.015 (0.003)</td>
<td>70 (20)</td>
</tr>
<tr>
<td>ClO$_4^-$</td>
<td>0.0084 (0.0003)</td>
<td>65 (8)</td>
</tr>
</tbody>
</table>

The observation that fusion kinetics of lipid droplets is slower than those of bare triolein droplets is consistent with this scenario (Figure 3.6 and Table 3.1). The breaking of the lipid layer is also supported by the slowest fusion rate constant observed in the ClO$_4^-$ buffer (Figure 3.10b). Large chaotropic anions such as ClO$_4^-$ have been found to enter the hydrophilic part of a lipid layer$^{124}$ or even the hydrophobic region of the lipid$^{125}$ As a result, the increased surface pressure provides entropic stabilization of the lipid layer$^{124}$ decreasing the fusion rate constants as it becomes more difficult to break the layer.

Further support for the importance of the phospholipid coating came from direct observation of a possible hemifusion stage during the fusion. We observed that during the slowest fusion in perchlorate solutions $\sim 3\%$ of the fusion events contain a hemifusion state with a lifetime of $46 \pm 36$ ms (Figure 3.11). No such state has been observed in the faster fusion processes between
two pure triolein droplets. This indicates that the short-lived intermediate state is associated with
the phospholipid coating. The hemifusion state was also supported by the observation that after
turning off the laser trap, two lipid droplets stayed together occasionally. One possible hemifusion
structure could be a transient connection between phospholipid coatings of the two lipid droplets
(Figure 3.11, top inset). Similar stalk-like connection has been observed in the hemifusion of lipid
vesicles.\textsuperscript{126}

3.3 CONCLUSIONS

In summary, the controlled particle collision and the position measurement of a trapping
laser in the LIAT platform allow to synchronize various processes in the lipid droplet fusion. The
unprecedented temporal resolution in this method results in direct observation of the docking,
potential hemifusion, and full fusion processes of lipid droplets for the first time. While the rate-
limiting docking process involves interfacial water shedding, evidence has suggested that physical
fusion event is associated with the breaking of phospholipid coatings. While these results provide
hitherto unavailable insights into the important cellular process of lipid droplet fusion, we
anticipate the method described here is readily expandable to investigate the fusion of other
organelles, such as lipid vesicles, inside cells.
Chapter IV: Direct Quantification of Loop Interaction and $\pi-\pi$ Stacking for G-Quadruplex Stability at the Submolecular Level


4.1 Introduction

With firm demonstration of in vivo formation of DNA G-quadruplexes, research focus in the field has now shifted to their biological functions. It has been shown that G-quadruplex formed in the single-stranded telomeric region can inhibit telomerase, an enzyme overexpressed in 80% of cancer cells to elongate telomere overhang, which leads to cell immortality. The inhibition of telomerase can be tuned by G-quadruplex binding ligands, such as telomestatin. Due to potential pharmaceutical applications, this finding has attracted an intensive research interest. As a motor protein, telomerase can be stopped by a roadblock whose mechanical stability is higher than the stall force of the enzyme. Elucidation of the mechanical property of the G-quadruplex/ligand complex is therefore instrumental to design more effective molecules that can strengthen G-quadruplex as a mechanical blocker not only to telomerase but also to other motor proteins, such as RNA and DNA polymerases.

G-quadruplex has a rather unique topology. It is composed of a stack of G-quartet planes, each of which is joined by four guanine residues through Hoogsteen base pairs (Figure 4.1a, inset). It has been generally accepted that the stacking between G-quartets provides stability to a G-quadruplex, whereas the size and the orientation of the loops determine the type and the flexibility
Figure 4.1 Dissection of DNA structures at the submolecular level. a) Experimental setup for the quartet-by-quartet dissection of G-quadruplex. A G-quartet structure and a click chemistry linkage (triazole) are shown in the left and right insets, respectively. b) Human telomeric sequences with alkyne modified guanine residues (underlined) in the top, middle, or bottom G-quartet of an expected hybrid-I G-quadruplex. c) Synthesis of alkyne modified telomeric sequences. An alkyne modified guanine residue for solid phase synthesis (II) was prepared from compound (I). After incorporation of (II) into oligodeoxynucleotides (III) via solid phase synthesis, dsDNA handle terminated with an azide group (IV) was attached to (III) through click chemistry.
of the structure. The importance of the G-quartet stacking to the structural stability has been supported by the binding geometry of many ligands that stack onto G-quartet planes. Recent findings, however, have suggested that the loop interaction is also important for the structural stability. Due to the lack of appropriate techniques, however, the relative contribution of G-quartet stacking and loop interaction to the G-quadruplex stability has not been resolved unambiguously. We reasoned that by submolecular dissection of a G-quadruplex in a quartet-by-quartet fashion, we may directly distinguish the contribution of the G-quartet stacking and loop interaction to the stability of G-quadruplex.

Previously, we have probed the mechanical property of DNA G-quadruplexes with and without ligands using optical tweezers. Assisted by click chemistry, we have been able to attach pulling handles to one or two loops, from which the unfolding and refolding of telomeric G-quadruplex can be investigated along specific geometries. To dissect a G-quadruplex quartet-by-quartet, pulling handles are designed to attach to the 3′-phosphate of the particular pair of guanine residues in the top, middle, or bottom G-quartet separately (Figure 4.1c). The mechanical unfolding experiments via these pulling handles revealed that unfolding forces of the overall G-quadruplex are smaller than those through loops, suggesting that loops contribute more to the structural stability than the G-quartets. Among the three G-quartets in a telomeric G-quadruplex, the middle quartet presents the weakest mechanical stability. This is consistent with the least interaction between the loops and the middle G-quartet, confirming that the quartet stacking is weaker than the loop interaction. Binding of telomestatin derivatives corroborates this finding. When a telomestatin dimer binds to G-quadruplex by stacking on both the top and bottom G-quartets, it leads to similar mechanical stabilities for all three G-quartets, probably due to the prevention of loop–quartet interactions. Whereas a telomestatin monomer and dimer demonstrate
different mechanical stabilities for the bottom G-quartet, they share the same stability for the top G-quartet. This result is consistent with the NMR structure in which the monomer ligand prefers to stack on the top G-quartet. The submolecular dissection method, therefore, can be used conveniently to probe the ligand binding sites of biological molecules.

4.2 RESULTS AND DISCUSSION

4.2.1 Quartet-by-Quartet Dissection Suggests That Loop Interaction is a Predominant Factor for G-Quadruplex Stability

The submolecular dissection of DNA G-quadruplex structure was carried out by laser tweezers, with which the mechanical unfolding of a biomacromolecule was carried out along specific trajectories defined by two residues that serve as pulling handles (Figure 4.1a). To clearly differentiate different G-quartet layers, handle residues must be selected from the known G-quadruplex conformations. The human telomeric G-quadruplex structure can form a variety of different conformations dependent on solvent conditions or flanking sequences.

Figure 4.2 a) Schematic to show force is exerted through the upper G-quartet while unfolding the G-quadruplex from G4 to G22. b) Schematic of the chemical bonding in the blow-up circles shown in a).
In our previous investigations, we have determined that hybrid-1 G-quadruplex is the most likely conformation formed in the same sequence used here under single-molecular conditions.\textsuperscript{101} Assuming this conformation, we chose G4 and G22 to attach the pulling handles for the top G-quartet, G5 and G23 for the middle G-quartet, and G6 and G24 for the bottom G-quartet (Figure 4.1b). We modified these G-residues with terminal alkynes through phosphate groups,\textsuperscript{95} which allow the attachment of double-stranded DNA as pulling handles via click chemistry (see Materials and Methods and Figure 4.1c). Since the distance between the modified phosphate and the 5′-end guanine base in a specific G-quartet is shorter than that between the same phosphate and the 3′-end guanine base in another G-quartet (see Figure 4.2 and Table 4.1), the tension felt by the G-quadruplex likely propagates through the 5′-end guanine pairs. By choosing the specific guanine pairs discussed above, we can unfold desired G-quartets in the hybrid-1 G-quadruplex.

*Table 4.1 Distance measurements between the G-quartets and the modified phosphorous atoms.*

<table>
<thead>
<tr>
<th>Position</th>
<th>phosphorus to 5′ G-quartet (nm)</th>
<th>phosphorus to 3′ G-quartet (nm)</th>
<th>Difference (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G4</td>
<td>0.52</td>
<td>0.57</td>
<td>0.05</td>
</tr>
<tr>
<td>G5</td>
<td>0.54</td>
<td>0.55</td>
<td>0.02</td>
</tr>
<tr>
<td>G6</td>
<td>0.61</td>
<td>N/A, in the loop</td>
<td>N/A</td>
</tr>
<tr>
<td>G22</td>
<td>0.49</td>
<td>0.60</td>
<td>0.05</td>
</tr>
<tr>
<td>G23</td>
<td>0.53</td>
<td>0.62</td>
<td>0.02</td>
</tr>
<tr>
<td>G24</td>
<td>0.45</td>
<td>N/A, in the loop</td>
<td>N/A</td>
</tr>
</tbody>
</table>
To probe the G-quadruplex structures after the modification, we performed CD experiments (Figure 4.3a). A valley at 240 nm and two peaks at 260 and 290 nm suggested that the hybrid-1 G-quadruplex conformation is maintained for all three mutants. Further confirmation of the hybrid-1 structure came from the single-molecule structural identification method based on the end-to-end distance measurements (see below).96, 101

Figure 4.3 Mechanical unfolding of a hybrid-1 G-quadruplex. a) Circular dichroism spectra of 5 µM wild type (WT) and the Top, Middle, and Bottom telomeric sequences (see Figure 4.1b) in a 10 mM Tris (pH 7.4) buffer with 100 mM KCl. (b-d) Single-molecule force ramp experiments for the Top, Middle, and Bottom telomeric sequences in the same buffer. b) Typical force-extension (F-X) curves. Scale bars in the blowup insert represent 10 nm. c) Change-in-contour-length (ΔL) of unfolded features. d) Rupture force of unfolded features. e) Root Mean Square Difference (RMSD) of the appropriate inter-residue distances between known PDB structures and experimental measurements.
With this structural clarification, each set of the alkynemodified telomeric sequences was reacted with two dsDNA handles labeled with azide at one of their ends through click chemistry (see Materials and Methods). The attachment of the DNA constructs to two optically trapped polystyrene particles was achieved through the affinity linkage between the digoxigenin-anti-digoxigenin antibody and biotin–streptavidin complexes (Figure 4.1a).

Using the single molecular force-ramp assay (see Materials and Methods), we observed rupture events that represent the unfolding of G-quadruplexes (Figure 4.3b). From each unfolding event, we measured the change in contour length (ΔL, Figure 4.3c) and the rupture force (Frupture, Figure 4.3d), which depict the size and the mechanical stability of a G-quadruplex, respectively. The ΔL agrees with expected value of ~6.6 nm from all three unfolding geometries (PDB code: 2HY925). Based on these ΔL values, we calculated the end-to-end distance (x) between the two handle residues by the function x = L − ΔL. Here L is the contour length of the structure. After combining these distances with those between different pairs of residues measured previously, we compared the corresponding distances obtained from the known human telomeric G-quadruplex structures. Evaluation using the RMSD (Figure 4.3e) between these two sets of data allowed us to conclude that the best matching conformation is the hybrid-1 G-quadruplex, therefore, confirming the CD results. The Frupture histograms for the three G-quartets revealed (Figure 4.3d) that the rupture forces for the top and bottom G-quartets are similar, while that for the middle quartet is the smallest. This result suggested that the loop interaction is a predominant factor for the stability of G-quadruplex. Since the top and the bottom quartets interact more extensively with the loops than the middle quartet, the rupture forces are higher for the top and the bottom G-quartets.
Figure 4.4 Loop interaction is stronger than the G-quartet stacking. a. Rupture force for different unfolding geometries of human telomeric G-quadruplex. “Top”, “Middle”, or “Bottom” indicates unfolding through a particular G-quartet (see Figure 4.1a). “L1”, “L2”, and “L3” depict T8 in the first loop, T14 in the second loop and T20 in the third loop respectively, counted from the 5′-end of the G-quadruplex. The sequence of the G-quadruplex forming sequence is 5′-T1T2A3G5G6G7T8T9A10 G11G12G13T14T15A16G17G18G19T20T21A22G23G24G25T26T27 A28-3′. b. Schematic drawing depicts that loop-loop interaction is stronger than quartet stacking in a hybrid-1 telomeric G-quadruplex.

When we summarized the rupture force for each pair of handle residues, we found that indeed, unfolding through loop residues has a higher rupture force than that through G-quartet residues (Figure 4.4a; see Table 4.2 for sequences). In fact, the highest rupture force is from the unfolding via two loop residues, T9 in loop1 and T21 in loop3. These results strongly supported that loop interactions have stronger mechanical stability than the stacking between G-quartets (Figure 4.4b).
These loop interactions could have conceivably originated from hydrogen bonding between loop bases and the stacking interaction of the bases in the loop and the G-quartet.\textsuperscript{132-133}

\textit{Table 4.2 Sequences of G-quadruplexes with different unfolding geometries used in Figure 4.4a.}

<table>
<thead>
<tr>
<th>Geometry</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’-3’</td>
<td>5’-TTA GGG TTA GGG TTA GGG TTA GGG TTA-3’</td>
</tr>
<tr>
<td>L1-3’</td>
<td>5’-TTA GGG T/AzideN//A GGG TTA GGG TTA GGG TTA-3’</td>
</tr>
<tr>
<td>5’-L3</td>
<td>5’-TTA GGG TTA GGG TTA GGG T/AzideN//A GGG TTA-3’</td>
</tr>
<tr>
<td>5’-L2</td>
<td>5’-TTA GGG TTA GGG T/AzideN//A GGG TTA GGG TTA-3’</td>
</tr>
<tr>
<td>L2-3’</td>
<td>5’-TTA GGG TTA GGG T/AzideN//A GGG TTA GGG TTA-3’</td>
</tr>
<tr>
<td>L1-L3</td>
<td>5’-TTA GGG T/5-Oct-dU//A GGG TTA GGG T/5-Oct-dU /A GGG TTA-3’</td>
</tr>
</tbody>
</table>

Note: /AzideN/ represents deoxyuridine with azide modification and /5Oct-dU/ depicts 5-Octadiynyl-deoxyuridine. These modifications are incorporated to facilitate the attachment of the pulling handles to the sequences via click chemistry.

4.2.2 Dissection of Telomestatin-Bound G-Quadruplex Confirmed That the Loop Interaction is Stronger Than the G-Quartet Stacking

To provide further evidence that the loop interaction is a predominant stabilization factor for human telomeric G-quadruplex, we proceeded to investigate the effect of ligand binding on the G-quadruplex stability. We chose telomestatin derivatives as model ligands, which are known to bind tightly with G-quadruplexes by stacking with terminal quartets.\textsuperscript{128} NMR structures have revealed that a telomestatin monomer, L2H2-6OTD (Figure 4.7, blue), prefers to stack on the top
G-quartet in a hybrid-1 telomeric G-quadruplex. On the other hand, we designed an L2H2-6OTD dimer (Figure 4.7, green) with a flexible linker that matches with the height of the human telomeric G-quadruplex. This ligand is expected to stack on both the top and bottom G-quartets. Due to this stacking, we reasoned that top or bottom G-quartet(s) may be shielded from loop interactions, which may result in similar rupture force as that for the middle G-quartet. This scenario was exactly observed. To ensure the complete stacking of the dimer, we used a saturation concentration of 100 nM (KD for the dimer is 8.3 nM; see Materials and Methods and Figure 4.6 and Table 4.4). CD spectrum confirmed that the hybrid-1 G-quadruplex conformation is maintained after ligand binding (Figure 4.5).

Comparison of the rupture forces revealed that they increase to the same value for all three G-quartets (38 ± 3, 37 ± 2, and 36.8 ± 0.4 pN for the top, middle, and bottom G-quartets, respectively, Figure 4.7), confirming that due to the stacking of the telomestatin dimer, the loops are shielded from interacting with both top and bottom G-quartets. The increased rupture force is a result of ligand binding, which stabilizes the G-quadruplex. The stabilization has been corroborated by the measurement of the change in free energy of unfolding (ΔGunfold) (Table 4.3; see Materials and Methods for calculations), which showed increased stability of G-quadruplex after binding with the telomestatin dimer (≈14 vs ≈9 kcal/mol for free G-quadruplex). When we used 100 nM L2H2-6OTD monomer (KD = 10.8 nM, Figure 4.6a and Table 4.4), we found that G-quadruplex assumes the expected hybrid-1 conformation from CD signatures (Figure 4.5a). Comparison of the rupture force revealed that the top G-quartet (37 ± 1 pN) showed similar mechanical stability as that for the dimer binding (38 ± 3 pN, Figure 4 top). In contrast, the bottom G-quartet had a lower rupture force (34 ± 2 pN) than that bound with the dimer (36.8 ± 0.4 pN, Figure 4.7, bottom). This result is consistent with the fact that the L2H2-6OTD monomer prefers
Figure 4.5 Circular dichroism (CD) spectra of 5 μM telomeric G-quadruplexes with ligands in a 10 mM Tris (pH 7.4) buffer with 100 mM KCl. “WT” depicts wild type sequence. “Top”, “Middle”, and “Bottom” represent G-quadruplexes modified with the alkyne group in the top, middle, and bottom G-quartets, respectively. “Monomer” and “Dimer” depict CD spectra in the presence of 5 μM (a) L2H2-6OTD monomer and (b) L2H2-6OTD dimer, respectively.

to bind to the top, instead of the bottom, G-quartet,\textsuperscript{129} which reinforces the mechanical stability of the top G-quartet only. Similar to the dimer binding, the rupture forces for the monomer-bound structures are higher than for free G-quadruplexes along the same unfolding geometry, indicating
Figure 4.6 Binding assays of telomeric G-quadruplex sequence with L2H2-6OTD monomer and L2H2-6OTD dimer by SPR. (a) Sensorgrams of L2H2-6OTD monomer at the concentrations of 7.8, 15.6, 31.3, 62.5 nM, respectively; (b) Sensorgrams of L2H2-6OTD dimer at the concentrations of 7.8, 15.6, 31.3, 62.5 nM, respectively. Concentrations are represented by different colors, and fitting curves are shown in black. The fitting curves were obtained using drifting baseline fitting in the BIAevaluation software.

again that ligand binding increases the overall stability of G-quadruplex. The increased $\Delta$Gunfold upon monomer binding (1–2 kcal/mol; see Table 4.3) confirmed this stability variation.
Table 4.3 Change in free energy of unfolding telomeric G-quadruplex ($\Delta G_{\text{unfold}} \pm \text{sd (bias)}, \text{kcal/mol}$) with and without telomestatin derivatives.

<table>
<thead>
<tr>
<th></th>
<th>No Ligand</th>
<th>Monomer</th>
<th>Dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top</td>
<td>9.7±0.4 (-0.4)</td>
<td>11±4 (-0.3)</td>
<td>15±2 (0.1)</td>
</tr>
<tr>
<td>Middle</td>
<td>9.0±0.6 (-0.5)</td>
<td>13±2 (-0.3)</td>
<td>14±2 (3.5)</td>
</tr>
<tr>
<td>Bottom</td>
<td>9.9±0.9 (1.4)</td>
<td>11±2 (-0.1)</td>
<td>13±2 (-0.3)</td>
</tr>
</tbody>
</table>

Table 4.4 Summary of rate constants ($k_a$ and $k_d$) and dissociation constants ($K_D$) for L2H2-6OTDs with telomeric DNA determined by SPR.

<table>
<thead>
<tr>
<th></th>
<th>L2H2-6OTD Monomer</th>
<th>L2H2-6OTD Dimer</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_a$ (1/Ms)</td>
<td>$6.39 \times 10^5$</td>
<td>$5.74 \times 10^5$</td>
</tr>
<tr>
<td>$k_d$ (1/s)</td>
<td>$6.89 \times 10^{-3}$</td>
<td>$4.79 \times 10^{-3}$</td>
</tr>
<tr>
<td>$K_D$ (M)</td>
<td>$1.08 \times 10^{-8}$</td>
<td>$8.34 \times 10^{-9}$</td>
</tr>
</tbody>
</table>

Note: We noticed that although $K_D$ for the L2H2-6OTD dimer is smaller than the L2H2-6OTD monomer, the difference is not obvious. This result can be ascribed to the surface effects experienced by the immobilized G-quadruplex, which cause inactive binding sites, especially for the G-quartets facing the surface.\(^{135}\)

The overall $\Delta G_{\text{unfold}}$ trend (Table 4.3) revealed that G-quadruplex bound with telomestatin dimer is strongest in stability, while the free G-quadruplex is weakest. Such a trend agrees qualitatively with the binding constant measurement in which L2H2-6OTD dimer ($K_D = 8.3 \text{ nM}$) shows
stronger binding than the monomer (KD = 10.8 nM). Interestingly, ΔG\textsubscript{unfold} is similar for different G-quartets incubated with the same ligand, which reflects the fact that ΔG\textsubscript{unfold} is the state function independent of unfolding pathways.\textsuperscript{96}

Figure 4.7 Effect of telomestatin ligands on the stability of G-quadruplex. Left panel shows rupture force histograms from different G-quartets in the presence of either telomestatin monomer (blue) or dimer (green). Rupture force histograms of free G-quadruplexes are shown in red. Right panel depicts structures of telomestatin derivatives.

4.2.3 Quartet-by-Quartet Unfolding of G-Quadruplex Involves Two Energy Barriers

It is interesting that the difference in the rupture force between the G-quadruplex/telomestatin monomer and the G-quadruplex/telomestatin dimer complexes is more obvious from the unfolding via the middle G-quartet than those through terminal G-quartets.
(Figure 4.7). Such a difference can be rationalized by an unfolding model that involves two energy barriers (Figure 4.8). The first (inner) barrier involves the unfolding of the G-quartet that is directly subject to mechanical stress. During the unfolding from the top or bottom G-quartet, due to the weaker contribution of the quartet stacking with respect to the loop interaction, the middle G-quartet is not as strong as the terminal quartets that interact more extensively with loops. Thus, the second (outer) barrier in these cases is dominated by the unfolding of the distally located terminal G-quartet (Figure 4.8, top and bottom panels, solid curves). Given that telomestatin dimer stacks to the two terminal quartets with similar strength, both the first and the second energy barriers...
barriers increase their magnitudes to a similar level (Figure 4.8, top and bottom panels, green solid curves). On the other hand, due to the preferential binding of the telomestatin monomer to the top G-quartet, only the top quartet increases the energy barrier. Therefore, during the unfolding from the top G-quartet, the first energy barrier (top quartet) is higher than the second (bottom quartet; see the blue solid curve in Figure 4.8, top panel), whereas this trend is reversed during the unfolding from the bottom G-quartet (see the blue solid curve in Figure 4.8, bottom panel) when monomer is bound.

During the unfolding through the middle G-quartet, the first energy barrier corresponds to the unfolding of the middle quartet, while the top and the bottom G-quartets represent the second barrier (Figure 4.8, middle). This geometry leads to a reduced distance between the two energy barriers. Because the terminal G-quartets are stronger than the middle quartet due to the loop interaction (see above), the second (outer) energy barrier is higher than the first (inner). Such an energy landscape renders the longest distance from the folded state to the highest energy barrier for the middle G-quadruplex among three G-quadruplexes (Figure 4.8). Since both top and bottom G-quartets bound with the dimer must be overcome before the whole G-quadruplex can be unfolded in this geometry, the second energy barrier in the presence of the telomestatin dimer is significantly higher than for the monomer, which binds to the top quartet only.

When force (F) is applied through a particular G-quartet, it reduces the free energy landscape with a magnitude of FX (solid lines in Figure 4.8; the slope of each line represents force F), where X is the reaction coordinate of the unfolding. During the unfolding through the top or bottom G-quartet, due to the relatively large distance between the two energy barriers (ΔX), the outer energy barrier is more reduced than the inner barrier by FΔX. This leads to a predominant inner energy barrier after application of force (Figure 4.8, top and bottom panels, dotted curves).
Thus, while unfolding through the top G-quartet, similar rupture forces exist between the telomestatin dimer and monomer, as both species stack to strengthen the top quartet, which is the inner barrier for this geometry. The difference in rupture forces becomes larger during the unfolding through the bottom G-quartet, since this quartet is the predominant inner barrier, which is strengthened by the telomestatin dimer but not the monomer.

In contrast, during the unfolding from the middle G-quartet, due to the much reduced distance between the two energy barriers, the outer energy barrier remains predominant, even after application of the force (Figure 4.8, middle panel; compare solid and dotted curves). Since the outer energy barrier represents both the top and bottom quartets in this geometry (see above), it leads to well-separated unfolding forces of the free G-quadruplex (weakest), the monomer-bound G-quadruplex, and the dimer-bound G-quadruplex (strongest). Finally, being the G-quadruplex with the longest distance (X) from the folded state to the highest energy barrier (see above and Figure 4.8), the unfolding force of the middle G-quadruplex is lowest among three G-quadruplexes due to the largest reduction of the energy barrier by a value of FX (see above). Such a prediction is well-validated by experimental observation in Figure 4.3b–d.

4.3 CONCLUSIONS

Using submolecular dissection of individual G-quartet planes in human telomeric DNA G-quadruplexes, we found that loop interaction contributes more to the G-quadruplex stability than the G-quartet stacking. These results suggest that in the design of ligands, molecules that interact with loops perhaps affect the structural stability more than those that intercalate with top or bottom G-quartet. As stacking modes of telomestatin derivatives can be well-differentiated at the submolecular level, we anticipate that this dissection method provides an alternative approach to
probe ligand binding sites of biomacromolecules that are recalcitrant to conventional structural characterization methods.
Chapter V: Identification of Binding Modes of Ligands in G-Quadruplex by Sub-molecular Mechanical Dissection

5.1 Introduction

Guanine (G)-rich DNA sequence can self-assemble into a planar G-quartet in presence of monovalent cations, such as potassium (Figure 5.1, top). Stacking of at least two G-quartets forms a G-quadruplex, which has shown conformational polymorphism attributed mainly to different sizes and orientations of the loops by which G-rich tracts are connected. G-quadruplexes exist in vivo, particularly in the promoter and telomeric regions. It has been found that G-quadruplex in promoters of human genes can regulate gene expression, among other biological functions. In telomeric regions that serve as protecting caps of chromosomes, formation of G-quadruplex has shown to inhibit telomerase, a ribonucleoprotein that maintains the telomere length by reverse transcription. Since the telomere extension by telomerase is commonly found in the majority of cancer cells, reducing telomerase activity by stabilizing the G-quadruplex becomes a viable strategy to fight cancer.

Small molecules that interact with the G-quadruplex have shown high therapeutic potentials as they can regulate biological activities associated with G-quadruplexes. To improve the potency and reduce the side effect, it is ideal that small ligands selectively bind to G-quadruplex over duplex DNA, which is the predominant DNA conformation inside cells. Stabilization of the G-quadruplex by ligands is reported mainly via $\pi-\pi$ interaction between the ligands and the G-
quartets or by electrostatic interactions. Ensemble methods such as NMR and X-ray are highly useful to profile the binding sites at the atomic detail. However, these methods often require sample quartets or by electrostatic interactions. Figure 5.1 Top, experimental setup for mechanical unfolding of a human telomeric G-quadruplex through the top, middle, or bottom G-quartet (see right inset for the structure) in a 10 mM Tris buffer with 100 mM KCl at pH 7.4. The G-quadruplex was attached to the two optically trapped polystyrene beads via two dsDNA handles by using copper assisted cycloaddition (CuAAC) (see left inset). Bottom, to identify different G-quadruplex binding modes, the unfolding force patterns of the G-quadruplex bound with telomestatin L2H2-6OTD monomer or L2H2-6OTD dimer (left) were compared with those bound with Phen-DC3 or PDS (right).
concentrations significantly higher than physiologically relevant levels while the signals are not always resolvable.

Recently, we have pioneered submolecular mechanical dissection (SMD) methods to reveal the conformation and transition dynamics of G-quadruplexes or proteins. By combining this strategy with a supervised multivariate classification, here we have presented a novel method to differentiate G-quadruplex binding modes of small-molecule ligands (Figure 5.1 and Table 5.1). To this end, a biomacromolecule is first attached to two DNA handles through which mechanical unfolding of

Table 5.1 List of the number of features (N) and the number of molecules (n) in mechanical unfolding experiments. Top, Middle, and Bottom represent unfolding from the top, middle, and bottom G-quartets, respectively.

<table>
<thead>
<tr>
<th></th>
<th>No Ligand</th>
<th>Monomer</th>
<th>Dimer</th>
<th>Phen-DC3</th>
<th>PDS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>n</td>
<td>N</td>
<td>n</td>
<td>N</td>
</tr>
<tr>
<td>Top</td>
<td>366</td>
<td>53</td>
<td>157</td>
<td>27</td>
<td>125</td>
</tr>
<tr>
<td>Middle</td>
<td>245</td>
<td>51</td>
<td>157</td>
<td>27</td>
<td>139</td>
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<tr>
<td>Bottom</td>
<td>261</td>
<td>52</td>
<td>154</td>
<td>34</td>
<td>275</td>
</tr>
</tbody>
</table>

the biomacromolecule can then be carried out in laser tweezers. By comparing different folding/unfolding geometries, not only conformations of G-quadruplexes can be quickly identified, information on the transition kinetics can also be retrieved along a particular trajectory. However, these pioneering efforts have targeted G-quadruplex structures without implications
from bound ligands, which present extra degree of complexity to the system. To address this problem, here, we have innovated a simplified version of supervised multivariate classification to differentiate G-quadruplex binding modes of ligands.

5.2 Results and Discussion

5.2.1 General Strategy for Multivariate Analysis of Ligand Binding

Our multivariate method will reveal ligand binding from distinct perspectives. Each perspective serves as a sensor that contributes a signal to the overall profile of the ligand binding. The submolecular mechanical dissection method is ideal to provide different sensors. We used human telomeric sequence, 5’-(TTAGGG)₄TTA, to illustrate our method. Based on previous finding that the sequence assumes a hybrid-1 G-quadruplex structure, we introduced an alkyne group through the 3’ phosphate of each of the two Guanine nucleotides, which are located at the same lateral side of a G-quartet in the telomeric G-quadruplex (Figure 5.1). In total, three modified sequences were obtained that represent the modification of the top (G₄, G₂₂), middle (G₅, G₂₃), and bottom (G₆, G₂₄) G-quartets, respectively. These sequences were joined with two dsDNA handles through copper assisted cycloaddition (CuAAC) reaction (or click chemistry). Since the two dsDNA handles were labeled with digoxigenin and biotin, respectively, each DNA construct can be anchored to the two optically trapped beads coated with anti-digoxigenin antibody and streptavidin, respectively, through the affinity interactions. Due to the close distance to the attached handle, the 3’ modification of the phosphate in a specific guanine allowed mechanical unfolding via the G-quartet at the 5’ side (Figure 4.2). Three DNA constructs with dsDNA handles attached to the three different G-quartet planes were then evaluated separately for their mechanical
stabilities by performing force ramping experiments (see Materials and Methods). The tension in the DNA construct increased by moving one of the trapped beads away from another using a steerable mirror that guided one of the trapping lasers until structures in each DNA construct were mechanically unfolded (see Figure 5.2a and Materials and Methods for details).

5.2.2 Unfolding Force Patterns for G-quadruplexes Bound with Ligands through Known Interacting Sites

First, we used ligands with defined binding sites to human telomeric G-quadruplex as a training set. We chose telomestatin monomer (L2H2-6OTD monomer) and dimer (L2H2-6OTD dimer, Figure 5.1) derivatives to represent bindings to the top G-quartet (viewed from the 5' end) and the two external G-quartets, respectively. To ensure the binding of a ligand to the G-quadruplex, we incubated the modified DNA construct with ligand concentration about ten times higher than $K_d$ (10.8 nM for monomer and 8.3 nM for dimer). Since mechanical unfolding via the desired G-quartet was designed according to the hybrid-1 G-quadruplex conformation, it is critical that the structure was not changed after ligand binding. In fact, circular dichroism (CD) spectra were consistent with the hybrid-1 structure (Figure 4.5). In addition, we measured distances between the two modified phosphorus atoms between which a G-quadruplex construct was mechanically unfolded (see Figure 5.2 and Table 5.2). After comparing these values with those measured from the PDB structures (Table 5.3), we found the best matching conformation was indeed hybrid-1 structure (Figure 5.5, see Materials and Methods for
Figure 5.2 Histograms of change-in-contour-length (ΔL) obtained during unfolding of G-quadruplex through the top, middle, or bottom G-quartet, with or without specific ligands (see main text for concentrations) in a 10 mM Tris buffer supplemented with 100 mM KCl at pH 7.4. Solid curves represent Gaussian fitting. Monomer and Dimer depict the telomestatin L2H2-6OTD monomer and dimer derivatives, respectively. The values in each histogram represent ΔL (±standard deviation) in nanometer. The standard deviation of ΔL was determined from three sets of data. The expected ΔL for the unfolding of the human telomeric G-quadruplex was calculated by the equation ΔL = n × Lnt – x, here n is the number of nucleotides between the two attaching handles in the folded human telomeric G-quadruplex (n=18), Lnt is the contour length per nucleotide (Lnt=0.45 nm/nt), and x is the residue-to-residue distance of folded G-quadruplex (for the top, middle, and bottom G-quartet constructs that are attached through the 4th-22th, 5th-23th, 6th-24th residue pairs, respectively, x has the same value of 1.5 nm). The calculation yields the same ΔL = 6.6 nm for the unfolding of G-quadruplexes through the top, middle, or bottom G-quartet. See Table 5.1 for the number of events measured in each experiment.

details and Figure 5.4 and Table 5.4 for the comparison against a complete list of PDB G-quadruplexes). It is noteworthy that for the second best matching structure, hybrid-2, the unfolding via specific G-quartet remains the same as that for hybrid-1 (Figure 5.4). Such
Figure 5.3 Comparison based on root mean square deviation (RMSD) of the distances between the two phosphate handles measured from laser tweezers (Table 5.2) and those calculated from PDB structures (Table 5.3). Least values are obtained for hybrid-1 G-quadruplex structures (depicted by black arrows; see Table 5.4 for RMSD values). Note G-quadruplexes without ligand were analyzed by taking a total of 9 different geometries, which include 5 geometries from Tables 5.2&5.3 and 4 other geometries (G4-T14, G4-T20, T14-G24 and T8-T20) from literature. Results were consistent with previous investigations that showed the hybrid-1 conformation for the G-quadruplex bound by either of the two telomestatin derivatives.

After determination of the G-quadruplex structure, we evaluated mechanical stabilities (or unfolding forces) of free G-quadruplexes as well as those bound with each training ligand from the mechanical unfolding of each of the three G-quartet planes (Figure 5.5c). Analyses on the rupture events of ligand-free G-quadruplexes revealed that average unfolding forces through the top, middle, or bottom G-quartet were different (Figure 5.5 c&d). Unfolding
Table 5.2 Distances between the two modified phosphorus atoms measured from mechanical unfolding experiments.

<table>
<thead>
<tr>
<th>Residues</th>
<th>Distance (± standard deviation in nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Ligand</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Top (G4-G22)</td>
<td>1.1±0.03</td>
</tr>
<tr>
<td>(G5-G23)</td>
<td>1.6±0.05</td>
</tr>
<tr>
<td>(G6-G24)</td>
<td>1.6±0.05</td>
</tr>
<tr>
<td>5’-3’ (G4-G24)</td>
<td>0.39±0.01</td>
</tr>
<tr>
<td>L1-3’ (T8-G24)</td>
<td>1.58±0.01</td>
</tr>
</tbody>
</table>
Figure 5.4 Comparison of the hybrid-1 and hybrid-2 G-quadruplex structures. The alkyne-modified residue pairs, G4-G22, G5-G23, and G6-G24, stay in the same top, middle, and bottom G-quartets (viewed from the 5' end), respectively, in both structures.

through the middle quartet required less force than that via the top or bottom G-quartet, which shared similar rupture forces. As discussed previously, the discrepancy in the unfolding forces is likely due to the stabilization of the top and bottom G-quartets by greater loop interactions with respect to the middle, more shielded G-quartet.

Binding of the telomestatin monomer increased the mechanical stability of the G-quadruplex evaluated from all three G-quartets due to the ligand-stabilization effect. Interestingly, the increased rupture force was higher for the top quartet compared to that via the middle or bottom G-quartet (see Figure 5.5 c&d). This result is consistent with the NMR structure in which the telomestatin monomer derivative binds preferentially to the top G-quartet. All these
Table 5.3 Distances between the two modified phosphorus atoms measured from PDB structures.

<table>
<thead>
<tr>
<th>PDB Structure</th>
<th>Distance (± standard deviation in nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Top (G4-G22)</td>
</tr>
<tr>
<td>2HY9\textsuperscript{133} (Hybrid 1)</td>
<td>1.50±0.05</td>
</tr>
<tr>
<td>2GKU\textsuperscript{169} (Hybrid 1)</td>
<td>1.59±0.02</td>
</tr>
<tr>
<td>2JSM\textsuperscript{148} (Hybrid 1)</td>
<td>1.55±0.02</td>
</tr>
<tr>
<td>2JPZ\textsuperscript{149} (Hybrid 2)</td>
<td>1.57±0.07</td>
</tr>
<tr>
<td>2JSL\textsuperscript{148} (Hybrid 2)</td>
<td>1.66±0.03</td>
</tr>
<tr>
<td>1KF1\textsuperscript{150} (Parallel)</td>
<td>1.55</td>
</tr>
<tr>
<td>143D\textsuperscript{151} (Basket)</td>
<td>2.23±0.07</td>
</tr>
<tr>
<td>2KF8\textsuperscript{152} (Basket)</td>
<td>2.02±0.08</td>
</tr>
</tbody>
</table>
Figure 5.5 G-quadruplex with ligands of known binding modes. a) Force-extension (F-X) curves for the top, middle, or bottom G-quartet constructs in a 10 mM Tris buffer (pH 7.4, 100 mM K\(^+\)) with or without 100 nM telomestatin monomer or dimer derivatives. Size of a rupture event (change-in-contour-length, \(\Delta L\), in nm) is depicted in each inset. Scale bar represents 100 nm. b) Comparison in root mean square deviation of the distances between the two handle residues measured from the mechanical unfolding and those from PDB structures. Hybrid-1 structures show the best match (depicted by arrows; see Figure 5.3 and Table 5.4 for values). c) Rupture force histograms measured from F-X curves in a). Solid curves represent Gaussian fittings. Average rupture force (pN) for bound G-quadruplex is shown in each histogram. d) G-quadruplexes bound with L2H2-6OTD monomer (middle) and L2H2-6OTD dimer (right) are defined by a Cap model and a Clam model, respectively.
Figure 5.6 Circular dichroism (CD) spectra of 5 µM telomeric G-quadruplexes with 5 µM ligands in a 10 mM Tris (pH 7.4) buffer with 100 mM KCl. “WT” depicts wild type sequence. “Top”, “Middle”, and “Bottom” represent G-quadruplexes modified with the alkyne groups in the top, middle, and bottom G-quartets, respectively. “Monomer”, “Dimer”, “PDS”, and “Phen-DC3” depict CD spectra in the presence of 5 µM of (a) L2H2-6OTD monomer, (b) L2H2-6OTD dimer, (c) PDS, and (d) Phen-DC3, respectively. All spectra show CD signals (valleys at ~ 240 nm, peaks/shoulders at ~265 nm and ~290 nm, see gray strips) consistent with the hybrid-1 structure. In a few cases (Top PDS, Middle Phen-DC3, and Bottom Phen-DC3 in particular), a small peak at ~248 nm was observed. Similar peaks were also observed during the conversion of the basket to hybrid-1 G-quadruplexes. Notice (a) and (b) were redrawn according to reported data.

Observations led us to propose that the binding of the telomestatin monomer to the G-quadruplex follows a Cap model (Figure 5.5d, middle).
On the other hand, binding of a telomestatin dimer derivative involves both top and bottom G-quartets as evidenced from similar rupture forces for all three quartets (Figure 5.5c&d, right panel). The almost identical unfolding forces for all three G-quartets can be explained by the fact that binding of the dimer shields the loop interactions for the two external G-quartets, which leads to similar stacking interactions among all three G-quartet planes. Such a binding conformation for telomestatin dimer has been supported by previous investigations.\textsuperscript{91, 99, 134} Taken together, we ascribed the G-quadruplex binding of the telomestatin dimer to a Clam model (Figure 5.5d, right).

The assignment of the two binding modes allowed us to construct corresponding unfolding force profiles. Each profile consists of three unfolding forces associated with the unfolding geometries through the three G-quartets, respectively (black traces in Figure 5.5d). These profiles will serve as training patterns for the Cap (telomestatin monomer) and the Clam (telomestatin dimer) modes separately (Figure 5.5d). It is noteworthy that these binding modes represent G-quartet binding instead of loop or groove binding.

5.2.3 Classification of Binding Models for Ligands with Unknown Binding Sites to G-quadruplex

After establishing the binding models using the training ligands, next, we proceeded to classify molecules with unknown binding modes to the telomeric G-quadruplex according to these models. As a proof of concept, we classified two ligands, Phen-DC3\textsuperscript{97, 153-154} and pyridostatin (PDS).\textsuperscript{51, 81} CD spectra suggest that the hybrid-1 conformation exists for the telomeric G-quadruplex in the presence of Phen-DC3 or PDS (Figure 5.6). In addition,
using the RMSD-based single-molecule structural determination method described above,\textsuperscript{96, 98, 101, 143} we compared the ligand-bound G-quadruplex with the known PDB structures (see Figure 5.3 and Table 5.4 for details). Again, the hybrid-1 structure was found to be the best matching conformation for the Phen-DC3 or PDS bound G-quadruplex (Figure 5.7b). The same hybrid-1 conformation of the G-quadruplex bound with the training ligands (see above) or the classification ligands, therefore, allowed us to compare these two groups directly using the submolecular mechanical unfolding approach.

To confirm these binding model assignments, we compared the root mean square deviation (RMSD) of the rupture force profiles between the classification group and the training group. Similar method was reported to identify biomolecular structures,\textsuperscript{96, 101} as well as to determine the similarity in image patterns.\textsuperscript{155} For accurate evaluation, we normalized the average rupture force in each unfolding force profile to the same level so that comparison can be carried out among different groups (see Materials and Methods). As shown in Figure 5.8, the PDS is closest to the Clam model, while the Phen-DC3 displays the smallest difference from the Cap model. Recent fluorescence quenching measurements have suggested that PDS is indeed a dual G-quartet binder.
Figure 5.7 Classification of binding modes of G-quadruplex ligands. a) Force-extension (F-X) curves for the top, middle, or bottom G-quartet constructs in a 10 mM Tris buffer (100 mM KCl at pH 7.4) with or without 1 µM Phen-DC3 or PDS. Size of a rupture event (ΔL, in nm) is depicted in each inset. Scale bar represents 100 nm. b) Comparison in root mean square deviation of the distances between the two handle residues measured from the mechanical unfolding and those from PDB structures. Hybrid-1 structures show the best match (depicted by arrows for the least RMSD values; see Figure 5.3 and Table 5.4 for details). c) Rupture force histograms obtained from the corresponding F-X curves shown in a). Solid curves represent Gaussian fittings. Average rupture force (pN) for bound G-quadruplex is shown in each histogram. d) Bar diagram of average rupture force obtained from the histograms in c). Cartoons for the Cap and Clam models correspond to the Phen-DC3 and PDS bound G-quadruplexes, respectively. Error bars represent standard deviations from at least 3 measurements (see Table 5.5 for values).
Table 5.4 Root mean square deviation (± standard deviation in nm) between the distances obtained from mechanical unfolding experiments and those from PDB structures with or without various ligands. Least values were obtained for the hybrid-1 structures.

<table>
<thead>
<tr>
<th>Type</th>
<th>Without Ligand*</th>
<th>L2H2-6OTD (Monomer)</th>
<th>L2H2-6OTD (Dimer)</th>
<th>Pyridostatin (PDS)</th>
<th>Phen-DC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>2HY9\textsuperscript{133} (Hybrid 1)</td>
<td>0.58 (±0.17)</td>
<td>0.39 (±0.15)</td>
<td>0.33 (±0.05)</td>
<td>0.11 (±0.05)</td>
<td>0.50 (±0.18)</td>
</tr>
<tr>
<td>2GKU\textsuperscript{69} (Hybrid 1)</td>
<td>1.08 (±0.28)</td>
<td>0.42 (±0.12)</td>
<td>0.38 (±0.04)</td>
<td>0.30 (±0.08)</td>
<td>0.62 (±0.10)</td>
</tr>
<tr>
<td>2JSM\textsuperscript{148} (Hybrid 1)</td>
<td>2.15 (±0.25)</td>
<td>0.35 (±0.07)</td>
<td>0.39 (±0.04)</td>
<td>1.2 (±0.2)</td>
<td>1.2 (±0.1)</td>
</tr>
<tr>
<td>2JPZ\textsuperscript{149} (Hybrid 2)</td>
<td>0.84 (±0.19)</td>
<td>0.37 (±0.10)</td>
<td>0.41 (±0.08)</td>
<td>0.14 (±0.03)</td>
<td>0.56 (±0.13)</td>
</tr>
<tr>
<td>2JSL\textsuperscript{148} (Hybrid 2)</td>
<td>1.0 (±0.4)</td>
<td>0.37 (±0.12)</td>
<td>0.43 (±0.06)</td>
<td>0.27 (±0.07)</td>
<td>0.68 (±0.15)</td>
</tr>
<tr>
<td>1KF1\textsuperscript{150} (Parallel)</td>
<td>1.73 (±0.06)</td>
<td>0.40 (±0.08)</td>
<td>0.37 (±0.02)</td>
<td>0.30 (±0.05)</td>
<td>0.71 (±0.21)</td>
</tr>
<tr>
<td>143D\textsuperscript{151} (Basket)</td>
<td>1.2 (±0.5)</td>
<td>0.61 (±0.14)</td>
<td>0.90 (±0.23)</td>
<td>0.8 (±0.2)</td>
<td>1.1 (±0.2)</td>
</tr>
<tr>
<td>2KF8\textsuperscript{152} (Basket)</td>
<td>1.1 (±0.6)</td>
<td>0.6 (±0.2)</td>
<td>0.75 (±0.26)</td>
<td>0.7 (±0.3)</td>
<td>1.0 (±0.3)</td>
</tr>
</tbody>
</table>
*Note G-quadruplexes without ligand were analyzed by taking a total of 9 different geometries, which include 5 geometries from Tables 5.22&5.3 and 4 other geometries (G4-T14, G4-T20, T14-G24 and T8-T20) from literature ⁹⁶.

![Diagram showing Root mean square deviation (RMSD) of the rupture force patterns between Phen-DC3 or PDS bound G-quadruplex and those observed for the Cap or Clam model. The least RMSD values are depicted by arrows.](image)

**Figure 5.8** Root mean square deviation (RMSD) of the rupture force patterns between the Phen-DC3 or PDS bound G-quadruplex and those observed for the Cap or Clam model. The least RMSD values are depicted by arrows.

for c-kit G-quadruplex, whereas Phen-DC3 binds preferentially to the top G-quartet,¹⁵⁶ therefore, supporting our binding mode assignments. Another support for the assignment comes from the top binding of the Phen-DC3 to the parallel c-Myc G-quadruplex as revealed by NMR.¹⁵⁷ It is important to note that one molecule of L2H2-6OTD dimer is involved in calm binding mode whereas two PDS molecules maybe required to bind both terminal quartets.
Table 5.5 Average rupture force (± standard deviation) in piconewton (pN) for unfolding G-quadruplex through the top, middle, or bottom G-quartet with or without ligands.

<table>
<thead>
<tr>
<th></th>
<th>No Ligand</th>
<th>Monomer</th>
<th>Dimer</th>
<th>Phen-DC3</th>
<th>PDS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top</td>
<td>31±1</td>
<td>47±1</td>
<td>38±3</td>
<td>44±2</td>
<td>47±1</td>
</tr>
<tr>
<td>Middle</td>
<td>20±3</td>
<td>29±2</td>
<td>37±2</td>
<td>25±1</td>
<td>45±4</td>
</tr>
<tr>
<td>Bottom</td>
<td>31±1</td>
<td>34±2</td>
<td>36.8±0.4</td>
<td>29±3</td>
<td>43±1</td>
</tr>
</tbody>
</table>

Table 5.6 Root mean square deviation (± standard deviation) in piconewton (pN) of the rupture forces of the G-quadruplexes bound with the Phen-DC3, or PDS with respect to those of the Cap, or Clam mode in the training group.

<table>
<thead>
<tr>
<th></th>
<th>Cap</th>
<th>Clam</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phen-DC3</td>
<td>4±1</td>
<td>9±3</td>
</tr>
<tr>
<td>PDS</td>
<td>9±4</td>
<td>3.3±0.6</td>
</tr>
</tbody>
</table>

Compared to traditional structural determination methods such as X-ray and NMR that provide information from an ensemble average perspective, the mechanical dissection approach described here is single-molecule in nature. Therefore, the method is capable of revealing subpopulation information, which suggests the homogeneous sample preparation required in traditional method
is no longer a necessity. Although it is rather challenging for the mechanical dissection to reach the atomic resolution demonstrated in traditional approaches, the method allows investigating transition kinetics of individual species. By evaluating a structure from different unfolding/refolding angles, individual transition trajectory can be obtained.\textsuperscript{96} Notably, the submolecular mechanical dissection method does not employ mutations or use modifications in nucleobase or deoxyribose. Instead, the handles for mechanical manipulations are introduced through phosphate groups in the DNA backbone, which cause the least perturbation to the G-quartet formation, resulting little conformational change, even though the stability of the G-quadruplex could be affected.\textsuperscript{67, 95} From this perspective, the modified DNA construct is anticipated to assume native-like conformations. Comparison of the CD spectra between modified and native constructs provides strong support for this point (Figure 5.6).\textsuperscript{98} The versatility of the submolecular mechanical dissection (SMD) method is reflected by the fact that the attachment points for pulling handles can be conveniently varied throughout a particular structure. For example, by introduction of pulling handles at loop residues, the SMD can evaluate the ligand binding via loop interactions. Compared to fluorescence based single-molecule methods to characterize ligand-receptor pairs, probing transition kinetics from different trajectories is the inherent advantage in the SMD. Another unique advantage of the SMD lies in the fact that mechanical properties can be unveiled. Such information is highly significant as many crucial enzymes in cells, such as polymerases and helicases, are motor proteins whose mechanical properties determine their activities.\textsuperscript{158}

5.3 Conclusion

In summary, by combining supervised multivariate classification with the SMD approach at the sub-molecular level, we could determine the binding modes of Phen-DC3 and PDS, whose
interaction sites to telomeric G-quadruplex are not known. This method is quick and particularly suitable to reveal ligand-biomolecular interactions under conditions not amenable to current structural determination method such as X-ray and NMR.
Chapter VI: Conclusions and Perspective

Lipid droplets inside the cell are understood as functional organelles and their fundamental properties and functions are still largely unknown. The controlled particle collision and the position measurement of a trapping laser in the LIAT platform was used to synchronize various processes in the lipid droplet fusion. The very high temporal resolution in this method allowed us to directly observe docking, potential hemifusion, and full fusion processes of lipid droplets. It was found that the rate-limiting docking process involves interfacial water removal before fusion, while the actual physical fusion event is associated with the breaking of phospholipid coatings. These results provide yet unavailable insights into the process of lipid droplet fusion. The method developed here is easily expandable to investigate the fusion of other organelles, such as lipid vesicles, inside cells.

Human telomeric G-quadruplexes were dissected submolecularly, and it was found that loop interaction contributes more to the G-quadruplex stability than the G-quartet stacking. These results signal that in the design of small molecule G-quadruplex binding ligands, molecules that also interact with loops perhaps affect the structural stability more than those that just intercalate with terminal G-quartets. As stacking modes of telomestatin derivatives can be well-differentiated at the submolecular level, we used this dissection method to probe ligand binding modes in the G-quadruplex. Combining supervised multivariate classification with the dissection approach at the sub-molecular level, we could determine the binding modes of Phen-DC3 and PDS, whose interaction sites to telomeric G-quadruplexes are unknown. This method is simple, quick and suitable to find ligand-biomolecular interactions close to physiological conditions which is not accessible to current structural determination method such as X-ray and NMR.
References


   ILPR G-Quadruplexes Formed in Seconds Demonstrate High Mechanical


88. Yangyuoru, P. M.; Dhakal, S.; Yu, Z.; Koirala, D.; Mwongela, S. M.; Mao, H., Single-
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