Engineering G-quadruplex DNA : a combined computational and experimental study

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DNA: A COMBINED
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A thesis submitted to the Nanyang Technological University in partial
fulfillment of the requirement for the degree of Doctor of Philosophy

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Abbreviations

93-del	G-quadruplex-forming sequence with anti-HIV activity
A	Adenine
AS1411	G-quadruplex-forming sequence with anti-cancer activity
C	Cytosine
CD	Circular dichroism
CT	Charge transfer
D₂O	Deuterium oxide
DFT	Density functional theory
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
D.S.S.	4,4-dimethyl-4-silapentane-1-sulfonic acid
ET	Electron transfer
FCD	Fragment charge difference
FRET	Förster resonance energy transfer
G	Guanine
G-tetrad	Guanine tetrad
G-wire	Long molecular wire arranged of stacked G-tetrads
AmG	8-amino-guanosine
BenG	N2-benzyl-guanine
BrG	8-bromo-guanine
F²G	2'-F-guanosine
FANA²G	2'-F-ANA-guanosine
HexG	N2-6-amino-hexyl-guanine
LNA²G	Locked nucleic acid modified guanosine
Met²G	N2-methyl-guanosine
Omet²G	8-O-methyl-guanine
Oxo²G	8-oxo-guanine
HIV	Human immunodeficiency virus
HMBC	Heteronuclear multiple bond correlation
HSQC	Heteronuclear single quantum correlation
HT	Hole transfer
KPi	Potassium phosphate
LNA	Locked nucleic acid
MD	Molecular dynamics
MM	Molecular mechanics
MP2	2 nd Order Møller-Plesset perturbation theory
NMR	Nuclear magnetic resonance
NOE	Nuclear overhauser effect
NOESY	Nuclear overhauser effect spectroscopy
NT	Nucleotide
PDB	Protein data bank
PNA	Peptide nucleic acid
PPM	parts per million
QM	Quantum mechanics
RMSD	Root-mean-square deviation
RNA	Ribonucleic acid
SD	Standard deviation

SELEX	Systematic evolution of ligands by exponential enrichment
T	Thymine
T_m	Melting temperature
TBA	Thrombin-binding aptamer
TERRA	Telomeric repeat-containing RNA
TOCSY	Total correlation spectroscopy
UNA	Unlocked nucleic acid
UTR	Untranslated region
UV	Ultraviolet
V	Electronic coupling

Abstract

G-quadruplex nucleic acids are four-stranded structures that arise from guanine-rich sequences. G-quadruplexes have received a great deal of research attention for their biological roles, therapeutic potential, and roles in nanotechnology. The research of this thesis is dedicated to the engineering of G-quadruplex DNA structures. This is done through a mixture of experimental and computational approaches. We examine some of the fundamental properties that govern G-quadruplex structure and their high-order assemblies. We also investigate the application of alternative nucleic acid chemistries within G-quadruplex structures. These include modifications to the base and the sugar moiety of the deoxyribose-phosphate backbone. We demonstrate how these chemical tools may be used to enhance the properties G-quadruplexes and apply these tools in our efforts to engineer G-quadruplex molecules.

The stacking of G-tetrads in the core of G-quadruplexes is a defining feature of these nucleic acid structures. Stacked G-tetrads can adopt a variety of different geometries. We perform a survey and characterization of experimental G-tetrad stacking geometries published in the literature, identifying unique G-tetrad stacking geometries with the core and at the interface of stacked G-quadruplexes. QM computational approaches are used to calculate the G-tetrad energy landscapes, providing an understanding of the energy differences between different G-tetrad stacking orientations. All atom MD simulations of stacked G-quadruplexes provide an understanding for the preferential orientation of stacked G-quadruplexes observed in the literature and highlight important consideration for the engineering of stacked G-quadruplex systems.

Long strands of stacked G-tetrads offer a unique avenue for the generation of conductive

wires on the nanoscale (G-wires). Currently, there is little understanding of how the orientation of stacked G-tetrads affects the migration of charge through the G-tetrad core. We characterize the ground state electronic properties of different G-tetrad stacking arrangements. Large samples of molecular geometries are extracted from MD simulations. We demonstrate that electron hole delocalization and electron transfer rates vary significantly for different G-tetrad stacking geometries.

Modification of DNA by alternative chemistries can enhance the thermal stability of oligonucleotides as well as their resistance to enzyme degradation. We examine the guanine analog containing a locked nucleic acid (LNA) chemical modification (^{LNA}G) in the context of G-quadruplex. The formation of G-quadruplex and the thermal stability of resulting structures are characterized. We question old assumptions of effective ^{LNA}G substitution and report on novel limitations of ^{LNA}G incorporations into G-quadruplex DNA. To explore the structural effects of ^{LNA}G substitutions, we solve the high-resolution NMR-solution structure of a (3+1) G-quadruplex containing a single ^{LNA}G modification.

In a similar manner, we study the effects of 2'-deoxy-2'-fluoro-riboguanosine and 2'-deoxy-2'-fluoro-arabinoguanosine modification into G-quadruplex. Receiving less research attention than LNA, we seek to define the rules which govern effective incorporation of these nucleotides via a systematic study on multiple G-quadruplex platforms. We also explore the ability to modulate G-quadruplex folding topology based on the observed glycosidic preference of these modified nucleotides and their rational substitution into G-quadruplex forming sequences.

Modification of the guanine base at the C8-position has been previously demonstrated to favor a syn-conformation. We examine the site-specific incorporation of 8-bromo-guanine,

8-O-methyl-guanine, 8-amino-guanine, and 8-oxo-guanine. We demonstrate the site-specific and chemical-specific nature of the effects these modifications have structure and stability. We report on the novel effects of these modifications on NMR chemical shifts, and relate these observations to changes in G-tetrad hydrogen bond geometry through QM studies.

Modification at the N2-position of the guanine base has received little research attention to date. We show that such modifications generally stabilize the G-quadruplex to a high degree. The projection of N2-modification into the groove represents a novel way to functionalize the G-quadruplex in a spatially-specific manner. We design a novel and highly stable “Ball-with-hair” G-quadruplex-based nanoparticle for general use as functionalizable and thermally stable nanoparticle scaffold.

The work presented here will be valuable to the molecular design of novel G-quadruplex structures. It will also enable the engineering and optimization of existing G-quadruplexes scaffolds.

Chapter 1:

Introduction to G-quadruplex Nucleic Acids

1.1 Discovery and Biological Importance

In 1953, the interpretation of X-ray crystallographic data by Watson and Crick revealed the first correct model of “double helix” DNA (1-3). However, in 1910, long before the discovery of the double helix, it was observed that guanine mono-phosphate was capable of forming gels (4). This curious observation was later explained in 1962 by Gellert and colleagues when crystallographic structures of these aggregates demonstrated the formation guanine-tetrads (5), later realized to be the building block of the G-quadruplex structures. Since the first crystallographic and NMR solution structures of G-quadruplex DNA in the early 1990’s (6-8), roughly 150 atomic-resolution structures have collectively demonstrated the diversity of this unique class of nucleic acids. The interest in G-quadruplex structures has been largely fueled by their various suggested roles in biological processes (9,10). The identification of proteins that recognize and unwind G-quadruplex structures along with the recent visualization of G-quadruplexes within human cells highlights the growing consensus on the biological importance of these nucleic acid structures (11-13).

Figure 1-1: Schematic of the Watson-crick double helix proposed in 1953. Sourced from (1)

The human telomere is a region of the genome which acts as a protective cap at the end of chromosomes. It is characterized by tandem repeats of the sequence $(TTAGGG)_n$ and contains a single stranded 3'-end overhang (10,14). Due to an inability of DNA polymerase to completely replicate the telomere, telomeric DNA shortens after each cell division (15,16). The enzyme telomerase counter-acts this shortening by extending the telomere (17-19), but it is not normally activated in somatic cells (20). It was hypothesized that the formation of G-quadruplex in this G-rich region may play a role in telomere stability and maintaining genome integrity. Human telomeric sequences have been shown to form a variety of G-quadruplexes in crystal and solution environments (6,21-33).

Gene promoter regions of the human genome also contain G-rich sequences capable of forming G-quadruplexes. In fact, bioinformatics surveys of the human genome have shown that over 40% of gene promoter regions are thought to contain G-quadruplex

forming sequences (34). This number is significantly higher for the promoter regions of oncogenes (35), which are genes whose mutation or over expression are associated with cancer. Alternatively, this number is much lower for tumor suppressor genes (36). G-quadruplex structures formed by numerous oncogenic sequences in vitro have been solved to date (37).

G-quadruplex-forming sequences are also prevalent in the 5'-untranslated regions (UTRs) of messenger RNAs (10,38). These non-coding regions play important regulatory roles in translation. Bioinformatics studies of the human genome have shown these regions to contain a high number of G-quadruplex forming motifs compared to other transcribed areas of genome (39), including numerous proto-oncogenes. The formation of G-quadruplex in 5'-UTRs can inhibit (40,41) and in some cases enhance (42) translation.

The realization that G-quadruplex formation occurs in genomic regions important to cancer proliferation has sparked interest in targeting these structures as an anti-cancer strategy. The enzyme telomerase responsible for telomere elongation is over expressed in a majority of cancers (43). As G-quadruplexes have been shown to inhibit this extension (44), the induction of telomeric G-quadruplex formation by small molecules is one such anti-cancer strategy (45) (Fig 1-2A). Another feature of many cancers is the over-expression of oncogenes. The induced formation of G-quadruplex in oncogenic promoter regions, to inhibit oncogene expression, represents another anti-cancer strategy involving the drug-targeting of biologically important G-quadruplexes (37) (Fig 1-2B). Considering these strategies, many small molecules have been identified to target G-quadruplexes (46). While many of these compounds demonstrate high binding affinity to G-quadruplex, only some demonstrate selectivity over duplex DNA and no compounds have been identified that are highly selective among different G-quadruplexes forming sequences.

Figure 1-2: Anti-cancer strategies targeting G-quadruplex DNA with small molecules: **(A)** Telomerase (light blue), over expressed in the majority of cancers, elongates the telomere creating cell immortality. The induction of G-quadruplex formation in the single stranded telomeric overhang via small molecules (green) blocks telomere extension by telomerase and is a potential anti-cancer strategy. Figure adapted from (37). **(B)** The formation of G-quadruplex and i-motif (C-quadruplex) within the promoter regions is suspected to occur under super-coiled DNA during the transcription process. The stabilization of G-quadruplex structures with small molecules (green) blocks transcription of over expressed oncogenes as a potential anti-cancer strategy. Figure adapted from (37).

In addition to their roles in telomere maintenance and gene regulation as discussed above, G-quadruplexes are suspected of playing other important biological roles (47). G-rich RNA which are transcriptional products of telomeric and sub-telomeric DNA are capable of forming G-quadruplex structures (48,49) and are thought to play protective and regulatory roles in the telomere (50). Additionally, the formation of DNA G-quadruplexes in non-coding regions of the genome has been shown to affect replication and recombination (51,52), inducing genomic instability and gross chromosomal rearrangement in yeast (52,53).

1.2 Structure

1.2.1 Guanine-tetrads and the G-quadruplex core

The guanine-tetrad is a fundamental building block of the G-quadruplex architecture. Also termed G-tetrads, these roughly planar assemblies are found in the interior of G-quadruplex structures. G-tetrads arise when four guanine bases form Hoogsteen hydrogen bonds in a cyclical pattern (Fig 1-3). During G-tetrad formation, the N2-H (amino proton) and N1-H (imino proton) of one guanine base bonds with the N7 and O6 position, respectively, of its neighbor. This association usually occurs in the presence of cations that stabilize G-tetrad formation through coordination with the O6-position of the guanine bases (54). The formation of G-quadruplex is sensitive to ion concentration and chemistry (55-57). Different ions types stabilize G-quadruplex formation to different extents. For example, guanine-rich sequences that readily assemble in K^+ or Na^+ solution are often observed to not form G-quadruplexes in the presence of Li^+ (58). The size difference of various ions can also influence the mobility and coordination sites within the G-tetrad core.

Within the core of a G-quadruplex structure, multiple G-tetrads stack upon one another, with the guanine base from one G-tetrad stacked with a guanine from a neighboring tetrad. In most cases, guanines that are stacking partners are also consecutive bases within the G-quadruplex forming sequence. For example the first and second guanine of the sequence (GGGTTA)₄ will most likely stack upon one another in neighboring G-tetrads. However, in some more complex structures this is not the case.

The geometry of stacked G-tetrads can vary significantly, affecting both the stability of

Figure 1-3: Schematic of a guanine tetrad: Hoogsteen hydrogen bond (red) and ion coordination (grey) patterns are shown. Magnified view of guanine highlights common atomic naming convention.

the G-tetrad stack and the geometry at the exterior of the molecules that interacts with solvent (59). The cyclic nature of the hydrogen bond pattern in a G-tetrad gives rise to a specific donor-to-accepter directionality and can serve as a convenient way for defining the geometry of stacked G-tetrads (Fig 1-4A). Based on this definition of polarity, two neighboring G-tetrads can stack with their polarities in the same direction, ‘same-polarity stacking’, or opposite direction, ‘opposite-polarity stacking’. In an alternative nomenclature, the donor-to-accepter directionality defines a G-tetrad face, with positive out-of-plane projection being the “head” and negative being the “tail” according to right-handed vector notation (60). G-tetrads may then be stacked in a “Head-to-Head”, “Tail-to-Tail” or “Head-to-Tail” manner.

While, in principle, stacked G-tetrads may adopt many different geometries when rotated

Figure 1-4: (A) Hydrogen-bond polarity of the guanine-tetrad as defined by the N1-H→O6 or N2-H→N7 direction. (B) Schematic examples of the 'same-polarity' and 'opposite-polarity' orientation of two stacked guanine-tetrads

with respect to each other, only specific stacking conformations are observed experimentally. This is due to covalent linkage between the guanine base and the G-tetrad backbone. Three separate G-tetrad stacking geometries are observed within the G-tetrad core of G-quadruplexes (61). The *Partial 5/6-ring* geometry is adopted when G-tetrads stack in a same-polarity manner, with guanine bases exhibiting a partial overlap of a 5-member ring with a 6-member ring (Fig 1-5A). The *5-ring* and the *Partial 6-ring* stacking geometries can arise when G-tetrads stack in an opposite polarity manner, with stacked guanines displaying overlap of their 5-member rings and a partial overlap of their 6-member rings respectively (Fig 1-5B-C). The geometries adopted within the G-tetrad core of the G-quadruplex are intrinsically linked to the glycosidic conformation of the base (discussed below).

Figure 1-5: G-tetrad stacking modes: (A-C) G-tetrad stacking geometries found within the core of G-quadruplexes. Guanines bases of different polarities are colored syn and magenta in relation to their glycosidic conformation. (See “1.2.2 Strand Directionality” section). (D-G) G-tetrad stacking geometries observed at the interface of higher-order G-quadruplex assemblies

The stacking of G-tetrads can also occur in higher order aggregates of G-quadruplex. Two G-quadruplex monomer units are sometimes observed to stack upon one another at their G-tetrad interface. This dimerization can result in unique G-tetrad stacking geometries at the stacking interface, some of which are not found within the core of covalently constrained G-tetrads (Fig 1-5D-G) (21,62-64). These geometries are also named after the relative overlap of their aromatic rings. Some of these geometries are also observed in unique conformations of dimeric G-quadruplexes (65).

1.2.2 Strand directionality

In a strand of DNA, guanine bases are covalently attached at the N9-position of the base to the ribose sugar of the phosphate-sugar backbone. In G-quadruplexes, guanine bases that participate in G-tetrad formation usually adopt two different glycosidic conformations with respect to sugar as defined by the dihedral angle O4'–C1'–N9–C4 (Fig 1-6A). The conformational ranges adopted by the base are a defining characteristic

Figure 1-6: The G-quadruplex strand and guanine base glycosidic conformation: (A) Atomic naming convention for guanosine nucleotides. The glycosidic conformation is described by the O4'-C1'-N9-C4 dihedral angle as indicated. Examples of an (B) *anti* and (C) *syn* glycosidic base conformation are presented. The strand directionality of the G-quadruplex stem is defined in the 5'→3' direction. Backbone and sugar atoms are generally represented as grey with the exceptions of phosphorous (orange) and the O4' of the sugar (red). Guanine base colors correspond to an *anti* (cyan) or *syn* (magenta) conformation.

of the overall G-quadruplex structure, and are usually found within an “*anti*” $-120^{\circ} \rightarrow 180^{\circ}$ or “*syn*” $0^{\circ} \rightarrow 90^{\circ}$ conformational range (Fig 1-6B-C) (66). The asymmetry of the sugar-phosphate backbone can be used to define a strand directionality. The directionality from C5' to the C3' atom of the backbone is a commonly regarded reference for positive strand directionality, often simply described as “5'→3'” (Fig 1-6).

The directionality of the strands within a G-quadruplex can vary in orientation (Fig 1-7). When all strands point in the same direction, the G-quadruplex folding topology is referred to as being in a “parallel” or a “(4+0)” orientation. The “(3+1)” or “hybrid” orientation is formed when one strand is oriented in the opposite-direction of the other

Figure 1-7: Strand directionality within the G-quadruplex core: (A) Parallel “(4+0)” strand orientation with all strands point in the same direction. (B) Hybrid “(3+1)” folding with three strands in one direction and one in the opposite. (C) Anti-parallel “(2+2)” orientation, with 2 strands pointing in each direction. Guanines are colored based on their *anti* (cyan) or *syn* (magenta) glycosidic conformation. The relative glycosidic conformation of guanines within a G-tetrad are related to the strand directionality.

three strands. In a similar manner, an “anti-parallel” or “(2+2)” orientation defines a G-quadruplex formed by two strands pointing in one direction, and two in the other. In most G-quadruplex structures, the glycosidic conformations of guanines within a single G-tetrad are directly related to the relative strand-orientations of the G-tetrad core. When all strands point in the same direction, all guanines within a G-tetrad adopt the same glycosidic conformation. The re-orientation of one or two strand leads to a differing glycosidic conformations on these strands (Fig 1-7).

1.2.3 Grooves

Similar to double helix DNA, the sugar-phosphate backbone strands that connect guanines of the G-tetrads give rise to grooves. These grooves, or concave regions between

Figure 1-8: Groove widths of G-quadruplex: Groove widths are commonly referred to as (A) Narrow, (B) Medium or (C) Wide. Grooves of different widths arise from various glycosidic conformations of bases within the G-tetrad. (Top) guanine bases are colored based on their *anti* (cyan) or *syn* (magenta) glycosidic conformation. Phosphorous (Orange) and O4' atoms (red) are colored for clarity. Strand directionality is referenced as into (X) or out of (•) the page. Groove widths for the presented strand orientation are abbreviated as Narrow (N), Medium (M), and Wide (W). (Bottom) Illustrative examples of grooves widths: Surface representations are shown with a qualitative measure of groove width indicated (orange dotted line).

the sugar-phosphate backbones, can vary in dimensions depending on the nucleic acid conformation. In G-quadruplex DNA, The four strands that make up the stem of the G-quadruplex give rise to four grooves, the dimensions of which depend on the directionality of the strands (Fig 1-8) (67,68). If all of the strands in the stem are oriented in the same direction as in a (4+0) parallel structure (Fig 1-7A) then four grooves of “Medium” width will be formed. The orientation of one strand in an opposite direction such as in the (3+1) hybrid (Fig 1-7B) will generate a narrow and a medium groove (Fig 1-8). G-quadruplex grooves are an important feature of the G-quadruplex structure as small-molecules have been identified which interact with G-quadruplex grooves (46,69,70).

1.2.4 Stoichiometry

The four strands of a G-quadruplex may assemble from different parts of a single guanine-rich G-quadruplex forming sequence, or from the conjunction of multiple sequences. The stoichiometry of a G-quadruplex, or the number of sequences from which it is made, may be unimolecular, bimolecular, or tetrameric (Fig 1-9).

Figure 1-9: Stoichiometry of G-quadruplex DNA: G-quadruplexes can form from (A) a single strand of DNA, (B) two strands or (C) four strands.

1.2.5 Loops

The assembly of guanine rich regions from less than four strands leads to segments nucleic acid that connect strands but do not participate in G-tetrad formation. These segments, called loops, can vary in their length, base chemistry and geometry. There are three commonly occurring loop types, which are classified by the directionality and orientation of the strands they connect (Fig 1-10). A “diagonal” loop connects two strands oriented in opposite directions and located diagonally across from one another in the G-quadruplex. An “edge-wise” loop connects two neighboring strands oriented in opposite directions, while a “propeller” or “double-chain-reversal” loop connects two neighboring strands oriented in the same direction.

Figure 1-10: Commonly occurring loops of G-quadruplex DNA: (A) Diagonal loop (B) Edge-wise loop (C) Propeller loop. Figure adapted from (67).

Loops are a dynamic structural feature of G-quadruplexes (71,72). The geometries adopted by loops vary depending on loop type, length and loop sequence. There are many favorable interactions that can arise from interactions within the loops or between the loops and neighboring structural elements. For example, bases within loops have been observed to stack upon another (49,73) and to hydrogen bond to the edge of a G-tetrad leading to more elaborate planer assemblies such as pentads (74,75).

1.3 Therapeutics and Nanotechnology

The unique geometry and chemical nature of the G-quadruplex scaffold has led to the identification of numerous engineered G-quadruplexes that demonstrate potential use as therapeutics. The discovery of biologically active G-quadruplexes often starts by screening a large library of nucleic acid sequences against a target protein via a SELEX type procedure (76-78). Sequences identified to bind to the protein target with high affinity, termed aptamers, are often further refined by rational engineering of structure.

One of the earliest and most well-studied biologically active G-quadruplexes is the Thrombin-binding aptamer (TBA). The TBA, formed by the sequence $d[G_2T_2G_2TGTG_2T_2G_2]$, was first identified to be a single stranded binder of the thrombin protease in nanomolar affinity, inhibiting blood coagulation (79). This sequence was later shown to adopt a G-quadruplex structure in X-ray crystallographic and NMR solution studies. (Fig 1-11A) (80,81) Numerous studies involving the optimization and chemical modification of the TBA have demonstrated its potential as a sensing agent (82).

Figure 1-11: Scaffolds identified with pharmacological properties: Structural schematics of the (A) Thrombin binding aptamer displaying anti-coagulant activity (B) T30923 displaying anti-cancer activity and (C) 93-del demonstrating anti-HIV1 Integrase activity (74,80,83).

A series of engineered G-quadruplex structures have been identified that with anti-cancer properties (84,85). The oligonucleotide T30923 and its sequence variants display inhibition of the Stat3 protein, involved in oncogenic signaling in many cancer types, at ~10-50 μ M affinity. The T30923 sequence has been shown to adopt a dimeric G-quadruplex molecule in solution (83) (Fig 1-11B). Another promising anti-cancer oligonucleotide is the AS1411 sequence which demonstrated anti-cancer effects in numerous cell lines (85). The inhibitory properties of AS1411 are dependent upon G-quadruplex formation (86-88), and it is believed that AS1411 binds to the membrane protein nucleolin (85). However, the exact mechanism of action is not yet clear. The AS1411 sequence is one of the most pharmacologically successful G-quadruplex drugs, currently in Phase II clinical trials.

Numerous G-quadruplex forming sequences have also been identified which demonstrate anti-HIV activity (78). The tetrameric G-quadruplex formed by d(T₂G₄T₂) was one of the first G-quadruplex forming sequences identified with anti-HIV properties (89), binding the HIV envelope protein gp120, a protein crucial to viron-cell membrane fusion, at sub- μ M concentrations. Another G-quadruplex which displays anti-HIV activity is the 93-del molecule formed from the sequence d(G₄TG₃AG₂AG₃T) (90). The 93-del oligonucleotide has been shown to inhibit HIV-I Integrase, which is a protein crucial to incorporation of the viral genome into host cells. This oligonucleotide adopts an interlocked-dimeric G-quadruplex structure (Fig 1-11C) (74). Other G-quadruplex forming sequences of note have been identified to bind to biological targets, but demonstrate less selectivity and pharmaceutical potential than those discussed above (78).

In addition to their role as therapeutic agents, G-quadruplex structures have demonstrated their usefulness in the field of nanotechnology. The self-assembling of guanine-

derivatives can be used to generate nanopores and grow liquid crystals (54). The G-quadruplex-architecture has also been used to create long wires (G-wires) (91,92). G-wires offer a potential approach to the generation of conductive nanoscale circuitry.

The G-quadruplex architecture is also at the heart of many sensing applications, including sensing of proteins, small molecules, ions, and oligonucleotides (93,94). Some of these sensors are based on G-quadruplex DNA that possesses catalytic activity. These G-quadruplex based deoxyribozymes (DNAzymes) can bind to a target molecule which modulates their catalytic behavior (95-98). One class of structures that has received particular research interest is G-quadruplex based DNAzymes that bind hemin and exhibit peroxidase-like activity (99-102).

1.4 Alternative Nucleic Acid Chemistry

While many G-quadruplex forming molecules have been identified that demonstrate therapeutic potential, native nucleic acid chemistry has limitations. Primarily, native DNA is readily broken down by nuclease within the human body. This is true for G-quadruplex forming DNA as well. The incorporation of alternative nucleic acid chemistries into G-quadruplexes can reduce the effects of enzyme degradation and prolong the lifetime of pharmacologically active G-quadruplex molecules (103-107). Alternative nucleic acid chemistries have many other advantages as well. Incorporation of modified-nucleotide chemistries can stabilize G-quadruplex structure (107). Increasing the stability of pharmacologically active G-quadruplex molecules can lead to increased activity at body temperature simply by increasing the amount of DNA that is forming the desired structure. Incorporation of alternative nucleic acid chemistry can also lead to improved cellular uptake (104).

One approach to modification of nucleic acid is functionalization of the base. In fact, nucleic acid bases often naturally encounter lesions within the cell due to oxidative damage and natural biological processes (108-110). Within the context of G-quadruplexes, modification can be made to bases within the loops or to guanine bases within the G-tetrad core (107). The altering of guanine base chemistry within the G-tetrad core can have profoundly negative effects on G-quadruplex formation if such functionalization disrupts G-tetrad hydrogen bonding (111,112).

There are many locations on the guanine base that may be functionalized or chemically altered (Fig 1-6A). The C8-position is one of the most studied types of guanine base modifications in the context of G-quadruplexes (25,111,113-117). Normally containing a

hydrogen atom, the C8 position protrudes outward from the core of the G-quadruplex into the groove. While most C8-modifications can be made without hindering G-tetrad formation, some lesions such as 8-oxo-guanine disrupt hydrogen bonding of the G-tetrad via protonation at the N7 position. The proximity of the C8-position to the deoxyribose-phosphate backbone makes modification at this position sensitive to the glycosidic conformation of the guanine base (Fig 1-6). Substitutions that are bulkier in nature tend to prefer a *syn* conformation over an *anti* orientation of the base (25,113,118). As the folding topology of G-quadruplex is intrinsically related to glycosidic conformation, C8-modifications can be used to drive structural changes in G-quadruplex DNA. However, due to this sensitivity, careful use of C8-modifications is required if one desires to maintain to the same global structure following C8-guanine substitution.

Modification at the O6 and N1 positions are very limited as these positions are crucial to Hoogsteen bonding and G-tetrad formation. While simple adducts will generally destabilize G-quadruplex formation (111,112), bases that contain the electronegative O6 and N1 substituted with other atoms suitable for Hydrogen-bond formation can be used (111). Careful substitution at positions that hydrogen bond can even allow for the alteration of hydrogen bonding network within the G-tetrad (119).

The N2 position holds an amino group also important for the hydrogen bonding of the G-tetrad. Complete substitution of this group with other chemistries is generally deleterious (111). The replacement of the amino group with a simple hydrogen atom, as in inosine, still allows for hydrogen bonding, but usually at a cost of thermal stability (111,120,121). However, replacement of a single hydrogen atom in the amino group with alkyl and alkyl-linked functional groups does not necessarily disrupt G-tetrad formation (122,123). Similar to C8, the N2 position projects out into the grooves of the G-quadruplex and is

located at a further distance from the deoxyribose-phosphate backbone.

Another approach to modification of G-quadruplex DNA is alteration of the sugar moiety of the deoxyribose-phosphate backbone. This can be particularly helpful to prevent against degradation of the nucleic acid by enzymes (103-106,124). Within the context of G-quadruplex, such modifications can affect the puckering of the sugar moiety, which can affect the preferred glycosidic conformation of the base (106,124,125). Thus, sugar-modified chemistries can also be used to modulate the adopted conformations of G-quadruplex forming sequences. Similar to the incorporation of base-modifications, it is important to consider the structural environments when substituting G-tetrad forming guanines with sugar-modified nucleotides.

One nucleotide analog that has received particular attention is Locked nucleic Acid (LNA). While DNA usually adopts a 2'-endo sugar conformation, LNA contains an O-CH₂ bridge, linking the C2' and C4' atoms, which forces the sugar moiety into a C3'-endo orientation (Fig 1-12B). LNA-modified guanines generally stabilize structure when substituted into an *anti* conformation (65,126,127). Different locked sugar chemistries have also been synthesized, but have received less research attention in the context of G-quadruplex (124). Conversely to LNA, unlocked nucleic acid (UNA) contains a broken sugar ring with detachment between the C2' and C3' atoms (Fig 1-12B). This chemistry has mixed effectiveness when substituted into the loops and is very destabilizing when substituted into a G-tetrad (128).

Other modified chemistries that have received slightly less attention than LNA are 2'-deoxy-2'-fluoro-ribo-guanosine (2'-F) and 2'-deoxy-2'-fluoro-arabino-guanosine (2'-F-ANA) modifications. The substitution of a fluorine atom with one of the two C2' hydrogen atoms grants nuclease resistance to 2'-F- and 2'-F-ANA-containing

Figure 1-12: (A) Schematic of sugar pucker nomenclature. (B-D) Chemical schematics of nucleotide analogs containing (B) modified sugar, (C) modified phosphate group, (D) peptidic backbone. Areas of deviation from natural DNA are highlighted in light blue.

oligonucleotides (103,105,106). In some respects, 2'-F modified nucleotides mimic RNA nucleotides, which adopt a different preferential sugar pucker (129).

Modification to the phosphate group has also been examined in the context of G-quadruplex DNA. The substitution of one of the phosphate oxygen atoms with a sulfur atom (Fig 1-12C) has a mix of results on the formation and thermal stability of modified G-quadruplexes, with most favorable substitutions being observed within the loops (130,131). In some cases, drastically different analogs have been investigated in G-quadruplex DNA which lack a ribose sugar or phosphate group. Peptide nucleic acid (PNA) is one such analog, which links bases via a peptidic backbone (Fig 1-12D) (132-134). A 'click chemistry approach' has also been previously used to build G-quadruplex structures, replacing the phosphate group with a triazole linker (135).

1.5 Research Objectives

The over-arching goal of the research presented in this Ph.D. thesis is to investigate G-quadruplex structural features and alternative nucleic acid chemistries that enable the design of engineered G-quadruplex structures. These goals can be broken into two main categories.

1. *Investigation of the physical interactions in the G-tetrad core of G-quadruplexes*

Chapter 2: We investigate how G-tetrad stacking geometry influences structure.

As a main building block of G-quadruplex structures, we investigate the roles of G-tetrad stacking in the context of monomeric G-quadruplexes with special interest paid to the roles of stacking at the interface of high-order G-quadruplex assemblies.

Chapter 3: The role of G-tetrad stacking geometry on the electronic properties of the G-tetrad core is considered. Results from this study highlight new structural considerations for the engineering of conductive G-quadruplex-based nanowires.

2. *Investigation of alternative nucleic acid chemistries for the design of G-quadruplexes*

Chapter 4: Conformationally-restricted locked nucleic acid (LNA) is a useful nucleotide analog that has been previously shown to demonstrate beneficial properties when incorporated into G-quadruplex. We investigate the effects of LNA-modified guanosine on G-quadruplex structure and stability, exploring a variety of structural environments.

Chapter 5: In a similar manner to LNA, we investigate 2'-F-guanosine and 2'-F-ANA-guanosine. We seek to broaden the understanding of the appropriate

structural environments for incorporation of these nucleotides into G-quadruplexes.

Chapter 6: Base modification at the C8-position has been previously observed to favor a *syn* conformation. We investigate 8-bromoguanine, 8-aminoguanine, 8-oxoguanine, and previously unexplored 8-O-methylguanine on G-quadruplex structure and stability as well as their effect on G-tetrad geometry and NMR chemical shifts.

Chapter 7: Modification at the N2-position of the guanine base is investigated for N2-methyl-guanine, N2-6-amino-hexyl-guanine, and N2-benzyl-guanine nucleotide analogs. We also undertake the design of a highly-stable G-quadruplex nanoparticle using a novel approach to G-quadruplex functionalization through N2-modifications.

Chapter 2:

Fundamentals of G-quadruplexes: Base Stacking and High-Order Assemblies

This chapter was largely adapted from the authors work: Lech, C.J., Heddi, B., Phan, A.T. (2013) Guanine base stacking in G-quadruplex Nucleic Acids. *Nucleic Acids Research*, 41 (3), 2034-2046.

Author Contributions: Survey of the Protein Databank and characterization of experimental structures was carried out by C.J.L.; QM calculations of single and stacked tetrads were undertaken by C.J.L.; MD simulations of stacked G-quadruplexes were performed by C.J.L. and B.H.; The MM parameterization of guanines bases and MM landscape computations were performed by B.H. and C.J.L.; Data analysis was done by C.J.L. and B.H., Experimental and computational approaches were designed by all authors; All authors contributed to the writing of above the manuscript from which this chapter is based.

2.1 Introduction:

Study of the physical interactions that govern G-quadruplex formation and their high-ordered assemblies is important to understanding the structure and function of biologically relevant G-quadruplexes and facilitates the engineering of aptamer molecules. G-quadruplexes are stabilized by a number of physical interactions. Like more canonical nucleic acid structures, the π - π stacking of aromatic bases is central to the formation of G-quadruplexes. Consequently, a great deal of research has gone into understanding the relative energies of stacked nucleic acid bases in varying geometries (136-141), including G-tetrad geometries found in G-quadruplexes (142-144).

Besides playing a fundamental role in G-quadruplex formation, guanine base stacking plays an important role in higher-ordered G-quadruplex assemblies. Individual G-

quadruplex units have the capacity to stack upon one another at their G-tetrad interfaces as seen in both X-ray crystallographic (7,21,62-64,145-150) and NMR solution structures (74,75,83,151-154). Unlike in the G-quadruplex core, base stacking geometries at free stacking interfaces are not constrained by covalent linkage through the backbone. Understanding the roles of base stacking at the interface of stacked G-quadruplexes is particularly important considering that high-order assemblies of telomeric G-quadruplex nucleic acid have been observed to form *in vitro* from naturally occurring telomeric DNA (21) and telomeric repeat-containing RNA (TERRA) (155,156) and that a number of G-quadruplex-based aptamers are formed from stacked G-quadruplexes (74,83,157). With this in mind, we set out to quantitatively characterize the base stacking geometries of G-quadruplexes. We report on the results of an extensive structural survey of G-quadruplex structures published in the Protein Data bank (PDB).

We also investigate base stacking on a fundamental level, examining how G-tetrad stacking energy differs with varying geometry. Previous quantum mechanical (QM) computational studies have looked at the relative stacking energies between some optimized geometries of stacked G-tetrads coordinated with central cations (142-144). The present study expands on previous works by generating comprehensive QM energy landscapes with a large basis set at the MP2 level of theory for both same-polarity and opposite-polarity stacked tetrads by systematically varying the separation and relative rotation of stacked K^+ coordinated tetrads (Fig 2-1 and Appendix II – Protocols – Chapter 2). Comparison of our experimental survey results and QM energy calculations allows us to understand the relative energies of stacked G-tetrad geometries, both within a single G-quadruplex and at the interface of stacked G-quadruplexes.

We also set out to investigate the role of base stacking in high-order assemblies of G-quadruplex

Figure 2-1. G-tetrad stacking types: **(A)** G-tetrads have a polarity as defined by direction of the Hoogsteen bond donor-to-acceptor pattern. Tetrad stacking can be primarily described as **(B)** opposite-polarity or **(C)** same-polarity stacking. Model stacked G-tetrad geometries used in this study varied by **(D)** relative rotation angle (θ_{tet}) and **(E)** G-tetrad separation distance (d_{tet}).

druplexes. Molecular dynamics (MD) has been used in past work to investigate the energy differences of G-quadruplexes with varying structural features (59,158,159). In this work we evaluate the ability of an AMBER molecular mechanics (MM) description of energy to accurately represent the base stacking energy of coordinated guanine-tetrads in comparison to QM calculations. MM computed energy differences between base stacking modes are discussed with special attention paid to their implications towards high-order G-quadruplex assemblies. MD simulations are carried out to investigate the observed preference in our survey of G-quadruplexes to stack predominantly in a 5'–5'

manner, with the G-tetrad stacking interface occurring between the 5'-end of individual G-quadruplex units. MD simulations provide some explanation for this observation in terms of different accessible base stacking geometries between 3'-3' and 5'-5' stacked G-quadruplexes.

2.2 Nomenclature:

The stacking of adjacent G-tetrads can be described by the relative polarity of the Hoogsteen hydrogen-bond pattern (Fig. 2-1). Within a single G-tetrad, the hydrogen-bond polarity is defined in the direction of hydrogen-bond donor to acceptor, from N2-H to N7 and from N1-H to O6. Two neighboring G-tetrads can therefore be described as belonging to one of two classes of stacking: 1) “same-polarity” if the polarities are in the same direction (Fig. 2-1C) or 2) “opposite-polarity” if in the opposite direction (Fig. 2-1B). The possible geometries of stacked guanine bases are fundamentally different between same- and opposite-polarity stacked G-tetrads. Nomenclature of commonly occurring base stacking modes from experiment are based upon a description of the aromatic-ring overlap of stacked guanines. For example, stacking geometry characterized by overlap of the 5-member rings of guanine bases is termed *5-ring* stacking. Within the G-quadruplex core, the geometries of stacked guanines are related to glycosidic conformation of the bases (61) and are therefore sometimes referred to in these terms. For example, the *Syn/Anti* nomenclature describes the base stacking geometry of the 5'-*Syn*→*Anti*-3' dinucleotide step.

In this paper we refer to stacked guanine bases as belonging to one of two classes of stacking termed same-polarity and opposite-polarity. In literature, it is common for these classes to be further divided and referred to as head-to-tail or tail-to-head belonging to same-polarity stacking and head-to-head or tail-to-tail belonging to opposite-polarity stacking (160). In this notation, head and tail refer to the different faces of a guanine base. In the isolated planar G-tetrad models used in our QM studies there is no difference between head-to-head and tail-to-tail models or the head-to-tail and tail-to-head models of similar separation and relative rotation. For this reason, we classify stacking primarily as

belonging to either the same-polarity or opposite-polarity type.

The G-tetrad models used for both energy calculations and structural characterization are symmetric planar guanine assemblies. We allow the relative rotation angle, termed θ_{tet} (Fig. 2-1D), and the separation distance d_{tet} defined to be the distance between parallel planar tetrads (Fig. 2-1E), to vary in our models. Due to the nature of G-tetrads and the symmetry of our models, the geometries of stacked guanines repeat as θ_{tet} varies over 90° intervals. In the case of opposite-polarity stacked tetrads, the θ_{tet} angle between stacked tetrads is defined to be the rotation of one tetrad, in the direction of its hydrogen-bond polarity, with respect to the other through the central axis perpendicular to the tetrad plane. In the case of same-polarity stacked tetrads, the θ_{tet} angle is defined arbitrarily to be the rotation of the tetrad containing the 5'-most guanine being considered in the direction of its hydrogen-bond polarity. The θ_{tet} angle is defined to be zero when O6 atoms of stacked tetrads are on top of each other such that the inter-tetrad O6-O6 distance of stacked guanines is at its minimum and is equal to d_{tet} , the separation between G-tetrads.

The stacking of individual G-quadruplexes at a G-tetrad interface is most readily observed for parallel-stranded G-quadruplexes. This G-quadruplex folding topology gives rise to well-defined 3'- and 5'-ends as determined by the 5'-to-3' directionality of the strands in the G-tetrad core. The 5'-5' notation, for example, is used to describe the stacking of two individual G-quadruplexes at the tetrad interface on their 5'-ends.

2.3 Theory:

The QM-computed interaction energy, termed “stacking energy” or ΔE , represents the gas-phase energy difference between a complex of stacked K^+ -coordinated G-tetrads and isolated fragments as shown in eq. 1.

$$\Delta E = E^{complex} - E^{tetrad\ 1} - E^{tetrad\ 2} - E^{ion} \quad (1)$$

The counterpoise procedure is used to correct for basis set superposition errors (161). Here, $E^{complex}$ is the total energy of the complex, while $E^{tetrad1}$, $E^{tetrad2}$ and E^{ion} are the energies of individual tetrads and a central coordinating ion respectively. For QM-computed energy landscapes, ΔE^{norm} represents the normalized stacking energy, determined relative to the minimum ΔE observed across both same- and opposite-polarity landscapes. For higher-level MP2/6-311+G(2d,2p) calculations, ΔE^{norm} was determined relative to the lowest ΔE across the geometries computed. For MM-computed energy landscapes, E^{Amber} represents the total MM description of system energy for a given geometry, normalized relative to the minimum across both same- and opposite-polarity landscapes. Let us note how interaction energy differs from the free energy of a system. Interaction energy, or stacking energy, is the energy associated with molecules interacting in gas phase. This energy is calculated to describe how favorable the geometry of a molecular ensemble is compared to sum of each molecule in isolation. These calculations are limited to a quantum description of interactions in the absence of solvent. In fully represented and solvated systems, other interactions such as hydrophobic interactions and the polarization of solute molecules by solvent influence the total free energy of the system.

2.4 Results and Discussion:

2.4.1: Structural survey and characterization: Guanine stacking within the G-tetrad core

A structural survey of the PDB was performed in order to characterize the guanine base stacking geometries observed between tetrads within the G-tetrad core of crystallographic G-quadruplex structures (Appendix III Table A3-1). Individual G-quadruplex units were found to have three common stacking geometries within the G-tetrad core (Fig. 2-2), which are intrinsically related to the glycosidic conformation of the bases: 1) *Partial 5/6-ring* stacking (Fig. 2-2A) of the *Anti/Anti* step is formed from same-polarity stacked tetrads with a partial overlap of the 5-member ring of one guanine with the 6-member ring of another; 2) *5-ring* stacking (Fig. 2-2B) of the *Syn/Anti* step is a result of opposite-polarity stacking with an overlap of the 5-member rings of stacked guanines; 3) *Partial 6-ring* stacking (Fig. 2-2C) of the *Anti/Syn* step is also formed by opposite-polarity stacked tetrads and exhibits a partial overlap of the 6-member rings of stacked guanines.

To quantify the stacking geometries of different core guanine-guanine steps, stacked gua-

Figure 2-2. Illustrative examples of core base stacking modes from the structural survey. (A) ‘*Partial 5/6-ring*’ stacking of the ‘*Anti/Anti*’ step, (B) ‘*5-ring*’ stacking of the ‘*Syn/Anti*’ step and (C) ‘*Partial 6-ring stacking*’ of the ‘*Anti/Syn*’ as observed in crystallographic structures. ‘*Syn*’ and ‘*Anti*’ glycosidic conformations are depicted as magenta and cyan, respectively. Stacking modes are labeled along with the θ_{tet} rotation value and the PDB ID code of the experimental structure from which the geometries are taken.

nines from the tetrads of experimentally determined structures were compared to a library of stacked guanines from model G-tetrads varying over relative rotation (θ_{tet}) and separation (d_{tet}) space (Fig. 2-1). Values of these parameters were assigned to experimental pairs of stacked guanines via lowest RMSD alignment with model tetrads (See Appendix II – Protocols – Chapter 2 – Fig A2-1). The results of our characterization of experimental guanine base stacking geometries are shown in Figure 2-3 and Table 2-1. Expectedly, there exist discrete regions of θ_{tet} for the *Anti/Anti*, *Anti/Syn* and *Syn/Anti* steps (Fig. 2-3A). Unexpectedly, the average d_{tet} was found to vary among stacking modes (Fig. 2-3B and Table 2-1). Average d_{tet} values of 3.39, 3.47, and 3.55 Å observed

Figure 2-3: Histograms representing the (A) relative rotation (θ_{tet}) and (B) separation (d_{tet}) values observed for stacked guanines within the G-quadruplex core of cataloged crystallographic G-quadruplexes. Counts are binned every 1° (θ_{tet}) and 0.02 Å (d_{tet}). Separation histograms are fitted with Gaussian distributions for visualization purposes. For a detailed explanation of how geometrical parameters are extracted from experimental structures see Appendix II – Protocols – Chapter 2.

Table 2-1. Statistics of base stacking modes as observed in experimentally determined structures.

Stacking Mode	Count ^a	X-ray				Count ^c	NMR ^b			
		θ_{tet} (°)		d_{tet} (Å)			θ_{tet} (°)		d_{tet} (Å)	
		Avg	1 st s.d. ^d	Avg	1 st s.d. ^d		Avg	1 st s.d. ^d	Avg	1 st s.d. ^d
<i>Core</i>										
Partial 5/6-ring (<i>Anti</i> / <i>Anti</i>)	310	61.5	3.3	3.39	0.10	23	61.1	5.5	3.28	0.17
5-ring (<i>Syn</i> / <i>Anti</i>)	245	83.2	2.4	3.47	0.11	44	84.5	4.7	3.54	0.29
Partial 6-ring (<i>Anti</i> / <i>Syn</i>)	118	29.2	1.4	3.55	0.08	8	30.7	8.2	3.57	0.29
<i>Interface</i>										
Partial 6-ring	4	26.2	-	3.50	-	-	-	-	-	-
6-ring	4	41.6	-	3.41	-	-	-	-	-	-
5/6-ring	3	56.5	-	3.41	-	15 ^e	61.8	-	3.43	-
5-ring	33	83.2	1.4	3.52	0.08	5 ^e	81.4	-	3.11	-

^a The number of observed base stacking geometries that are defined uniquely within the unit cell or within the interface of symmetry mates.

^b A selection of available NMR structures (Appendix III - Table A3-2) were cataloged in order to determine if there exist fundamental differences between the base stacking modes exhibited in X-ray and NMR structures.

^c For NMR structures, the base stacking geometry of a single pair of stacked guanines is an average across all of the models present in a given PDB file.

^d Standard deviations values highlight the ensemble spread of our characterized geometries used to determine θ_{tet} and d_{tet} . They do not reflect the uncertainty in experimental measurements.

^e the interface geometries of some NMR structures (PDB I.D. 1MY9 and 2RQJ) were observed to contain mixed base stacking modes at their stacking interface.

for the *Anti/Anti*, *Syn/Anti* and *Anti/Syn* steps respectively. However, regions of d_{tet} (Fig. 3B) for different base stacking modes are highly overlapped.

In order to account for a possible bias of experimental conditions on our statistical results of base stacking geometries, sub-classes of G-quadruplex structures were also analyzed. When appropriate, crystallographic structures were further classified by their varying structural features (Table 2-2), including whether the oligonucleotide chemistry was DNA or RNA, the type of coordinating ion, the presence of a drug molecule, strand directionality, and strand stoichiometry. No significant differences are observed among the features investigated in this work. To see if our statistical findings were specific to crystallographic structures, a set of NMR structures were also examined (Table 2-1 and Appendix III - Table A3-2). Similar average values of d_{tet} and θ_{tet} were observed for the

Table 2-2. Sub-classification of stacked guanine geometries within the G-tetrad core of cataloged crystallographic G-quadruplex structures

Classification	Count	Rotation (°)		Separation (Å)		Associated Tetrad Energy ^a (kcal/mol)
		Mean	s.d	Mean	s.d	
Telomeric – Anti → Anti	68	62.07	3.57	3.376	0.100	5.73
- DNA	56	62.13	3.35	3.370	0.100	5.70
- RNA	12	61.78	4.62	3.402	0.088	5.87
- Drug	44	62.70	3.51	3.373	0.107	5.77
- No Drug	24	60.91	3.44	3.380	0.082	5.65
- Intramolecular	24	62.11	1.97	3.381	0.070	5.76
- Bimolecular	44	62.04	4.21	3.377	0.111	5.71
Oxythricha – Syn → Anti	236	83.25	2.41	3.462	0.109	8.55
- Drug	88	83.78	2.18	3.465	0.096	8.62
- No Drug	148	82.94	2.49	3.460	0.117	8.51
- K ⁺ Salt	220	83.29	2.39	3.460	0.108	8.53
- Tl ⁺ Salt	16	82.76	2.71	3.491	0.125	8.84
- Diagonal Loop	208	83.29	2.42	3.461	0.112	8.55
- Edgewise Loop	28	82.97	2.38	3.465	0.090	8.56
- ↑•↑•↓•↓ Strand Direction	16	83.52	2.53	3.456	0.099	8.51
- ↑•↓•↑•↓ Strand Direction	12	82.25	2.02	3.477	0.078	8.40
Oxythricha – Anti → Syn	118	29.23	1.40	3.549	0.076	11.53
- Drug	44	29.14	1.19	3.565	0.076	11.90
- No Drug	74	29.28	1.51	3.540	0.075	11.31
- K ⁺ Salt	110	29.23	1.37	3.548	0.078	11.50
- Tl ⁺ Salt	8	29.19	1.91	3.568	0.035	11.95
- Diagonal Loop	104	29.23	1.39	3.552	0.073	11.59
- Edgewise Loop	14	29.21	1.55	3.531	0.096	11.13
- ↑•↑•↓•↓ Strand Direction	8	28.49	1.29	3.493	0.101	10.61
- ↑•↓•↑•↓ Strand Direction	6	30.17	1.43	3.582	0.065	9.98
Tetramer – Anti → Anti	242	61.32	3.20	3.391	0.096	5.75
- DNA	216	61.11	3.09	3.390	0.095	5.73
- RNA	26	63.08	3.62	3.404	0.103	5.99
- K ⁺ Salt	9	65.09	1.47	3.507	0.055	7.85
- Na ⁺ Salt	165	60.99	3.13	3.381	0.082	5.66
- Ca ⁺² Salt	8	60.26	1.67	3.339	0.049	5.34
- Tl ⁺ Salt	44	61.76	3.07	3.432	0.129	6.39
- Sr ⁺² Salt	8	63.16	1.21	3.310	0.049	5.52
- Ba ⁺² Salt	4	65.69	0.53	3.459	0.049	6.96
- No Salt	4	55.45	0.41	3.306	0.017	4.47

^a Associated Tetrad energy is interpolated from MP2/6-31G*(0.25) landscapes based off average rotation and separation values for different sub-classes

Anti/Anti, *Syn/Anti*, and *Anti/Syn* core stacking modes. It is important to state that there was a notable occurrence of *Anti/Syn* geometries surveyed from crystal structures in which the stacked guanines were non-parallel, containing varying pitch angles about the

C2-C8 axis of an individual guanine. While our study is limited to understanding the effects of θ_{tet} and d_{tet} on stacking energy, it would be interesting to study the effects of other degrees of freedom on stacked coordinated G-tetrads.

The statistical information in Table 2-1 offers insight into the differences between core guanine base stacking geometries. While different average θ_{tet} angles are fundamental to unique stacking modes, perhaps unintuitive is the observed differences in average d_{tet} of stacked guanines. Another feature of note is that the variation in the 1st standard deviation of θ_{tet} with *Anti/Anti* > *Syn/Anti* > *Anti/Syn*. Notably, stacked guanines within the core of NMR structures demonstrate much larger 1st standard deviation values suggesting the geometries of stacked core guanines are more variable in NMR solution structures compared to crystallographic structures. This variation may reflect the different nature of crystal and solution environments or limitations of NMR-based models in providing the atomic precision necessary for a rigorous discussion of base stacking geometry. Considering the limitations in accuracy of experimentally determined structures, we must be cautious in our interpretation of statistical results dealing with small structural differences such as the average d_{tet} values for core stacking modes.

To better interpret the statistical data of Table 2-1, let us discuss the structural motifs included in this study. The *Anti/Anti* step is abundant in crystallographic structures of G-quadruplex telomeric sequences (21,64,150,156,162,163) containing the T₂AG₃ motif and its variants, as well as structures of tetrameric G-quadruplexes (7,62,145-149,164-168). The *Syn/Anti* and *Anti/Syn* steps are abundant in G-quadruplexes formed from *Oxytricha* telomeric sequences (169-174) containing the G₄T₄G₄ motif and its variants. Additionally, *Syn/Anti* steps are observed in some bulged RNA tetramer G-quadruplexes (165,175). The *Syn/Syn* stacking mode is observed in some NMR structures of unique folding topologies

(28,61,176-178). Similar to *Anti/Anti* steps, *Syn/Syn* steps also exhibit a *Partial-5/6ring* stacking geometry. The *Syn/Syn* step is not discussed here in length, as it has not been observed in crystallographic structures of G-quadruplex which to date are less diverse than NMR structures in terms of sequence and folding topology.

2.4.2: Structural survey and characterization: Guanine stacking at the G-tetrad interface of stacked G-quadruplexes

In our survey of the PDB we also cataloged the base stacking geometries at the interface of stacked G-quadruplexes. The G-tetrad stacking interfaces of stacked G-quadruplex arise due to dimerization of individual G-quadruplex units in solution or in a crystal packing environment. We include both crystallographic and NMR structures in our survey due to the limited number of stacked G-quadruplex complexes published to date. Experimentally observed interface geometries may be classified into four modes (Fig. 2-4). These stacking geometries are termed *Partial 6-ring* (Fig. 2-4A), *6-ring* (Fig. 2-4B), *5/6-ring* (Fig. 2-4C) and *5-ring* (Fig. 2-4D) based on the description of aromatic ring overlap observed between pairs of stacked guanine bases. We note that all interface stacking exhibits opposite-polarity stacking. Additionally, almost all parallel G-quadruplexes that exhibit interface stacking do so at their 5'-end. The preference for 5'-5' interface stacking is examined further in subsequent sections.

The *6-ring*, *5/6-ring*, and *5-ring* stacking modes are observed at the interfaces of crystallographic tetrameric (7,62,145-149) and human telomeric (21,64) G-quadruplex structures (Appendix III - Table A3-1 and A3-2). A single instance of *Partial 6-ring* stacking is observed in an G-quadruplex structure formed by the sequence d[G₄^{Br}UT₂G₄]₂ demonstrating 2+2 (↑•↑•↓•↓) strand directionality (63). Interface stacked G-tetrad geometries

Figure 2-4. Illustrative examples of G-tetrad base stacking modes at the interface of stacked G-quadruplexes as identified in our structural survey. (A) ‘Partial 6-ring’, (B) ‘6-ring’, (C) ‘5/6-ring’ and (D) ‘5-ring’ as observed in crystallographic structures. Stacking modes are labeled along with the θ_{tet} rotational value and the PDB ID code of the experimental structure from which the G-tetrads are taken.

ries in crystallographic structures exhibited mostly *5-ring* stacking (Table 2-1), largely due to the high number tetrameric structures formed from the d[TG_4T] sequence which predominantly demonstrated *5-ring* stacking (Fig. 2-4D).

NMR structures containing G-quadruplex interfaces were mostly propeller-type and demonstrated either the *5/6-ring* or the *5-ring* stacking geometries (Fig. 2-C,D). It is hard to draw any conclusions regarding different interface stacking preferences of NMR and crystallographic structures due to the differences in the types of G-quadruplexes studied. Interface base stacking geometries are sometimes found within interlocked G-quadruplexes such as the *Partial 6-ring* stacking of the interlocked V4 folding topology (65). Interlocked structures (65,74,75) are dimeric G-quadruplexes without a free-stacking interface, consisting of two stacked G-quadruplex units each contributing guanines to G-tetrad core of the other. These unique geometries are not considered in our statistics.

2.4.3: QM computational studies: Energy landscapes of K⁺ coordinated stacked G-tetrads

We employed a series of QM computations to calculate stacking energies (see section 2.2: Nomenclature), of many stacked G-tetrad geometries coordinated by a centralized K⁺ ion. The θ_{tet} and d_{tet} values of stacked tetrads were systematically varied to generate energy landscapes of same-polarity and opposite-polarity stacked tetrads at the MP2/6-31G*(0.25) level (Fig. 2-5). Slices of energy landscapes at a d_{tet} value of 3.3 Å illustrates the θ_{tet} energy dependence of same- and opposite-polarity stacked tetrads (Fig. 2-5A).

The θ_{tet} energy profile of same-polarity stacking is characterized by highly unfavorable stacking in the area of low θ_{tet} (<15° and >75°) and a broad energy minimum elsewhere (15°-75°) with a global minimum at 45° and two shoulders of comparable energy at 15° and 75° separated by a small energy barrier of roughly 2.5 kcal/mol. The energy landscape of the same-polarity stacked tetrad is presented (Fig. 2-5B), superimposed with regions indicating average geometries of base stacking modes cataloged from crystal structures (Table 2-1). There exists a broad low-energy basin within the d_{tet} range of 3.2 Å to 3.4 Å and 15° to 75°. The *Anti/Anti* core stacking geometry (Fig. 2-2A) is observed to fall well within this energy basin (Fig. 2-5B) in close proximity to the landscapes low energy states. The broad low energy basin of the same-polarity energy landscape may in part explain the relatively large 1st SD of θ_{tet} exhibited by the *Anti/Anti* same-polarity stacking mode (Table 2-1). It is interesting to note that the most favorable same-polarity stacking occurs at rotation 45° when there is little aromatic ring overlap of stacked bases.

In contrast, the θ_{tet} energy profile of opposite-polarity stacking is characterized by a deep global minimum around 55°-60° and local minima at 35°-45° and 85°-5° of approximate-

Figure 2-5: QM energy landscapes. (A) The rotational (θ_{tet}) energy profile of same-polarity and opposite-polarity stacked G-tetrads at a d_{tet} value of 3.3 Å separation. Energy landscapes of (B) same-polarity and (C) opposite-polarity stacked tetrads are presented with stacking modes as determined from cataloged crystallographic structures superimposed. Average core G-tetrad stacking modes are shown with average geometries indicated by a black dot and gray outlined regions having dimensions of the first SD for θ_{tet} and d_{tet} values. Average G-tetrad stacking modes at the interface of stacked G-quadruplexes are presented, color-coded as shown in Figure 2-4: ‘Partial 6-ring’ (yellow), ‘6-ring’ (purple), ‘5/6-ring’ (green) and ‘5-ring’ (orange).

ly 6 and 8 kcal/mol respectively (Fig. 2-5A). There is a sizable energy barrier of 4 kcal/mol separating the 85°-5° and 35°-45° minima. The energy landscape of opposite-polarity stacked tetrads (Fig. 2-5C) is a diverse landscape characterized by narrow energy

minima. The *Anti/Syn Partial 6-ring* and *Syn/Anti 5-ring* core stacking modes as well as interface stacking modes can be seen superimposed upon the opposite-polarity stacking landscape (Fig. 2-5C). Interestingly, the core G-tetrad stacking modes do not fall within calculated minima. Alternatively, the *5/6-ring* interface stacking mode occupies the global minimum (55° - 60°) with the *6-ring* interface stacking mode being found in the neighboring energy minimum (35° - 45°). It is very interesting that tetrad stacking at the interface of stacked G-quadruplexes, which is not constrained by a backbone, more readily occupies the energy minima of the QM computed energy landscapes. We note that the most favorable interface stacking geometry, the *5/6-ring* mode, is found in both crystallographic and NMR structures.

The relative stacking energies of average G-tetrad stacking geometries from experimentally determined structures were interpolated from the energy landscape and are presented in Table 2-3. Additionally, stacking energies were computed at a higher level of theory, MP2/6-311+G(2d,2p), for stacked G-tetrads that mimic the average experimental stacking geometries observed in our survey (Table 2-3). ΔE^{norm} stacking energies of core

Table 2-3. Calculated tetrad stacking energies (kcal/mol) of experimentally observed stacking modes

Stacking Mode	MP2 6-31G*(0.25) ^a		MP2 6-311+G(2d,2p)		AMBER
	ΔE	ΔE^{Norm}	ΔE	ΔE^{Norm}	E ^{Amber}
<i>Core</i>					
Partial 5/6-ring	-153.56	5.74	-157.37	4.21	2.07
5-ring	-150.74	8.56	-153.77	7.81	3.64
Partial 6-ring	-147.71	11.59	-149.70	11.88	10.09
<i>Interface</i>					
Partial 6-ring	-147.78	11.52	-149.90	11.69	9.24
6-ring	-153.72	5.58	-156.71	4.88	5.27
5/6-ring	-157.66	1.64	-161.58	0	2.29
5-ring	-149.99	9.31	-152.45	9.14	4.76

Rotation and separation coordinates corresponding to these energies are the averages presented in Table 1.

^a Energies determined by interpolation from available data on the energy landscapes of the same- and opposite-polarity stacked tetrads

tetrad stacking geometries were found to be ranked *Anti/Anti Partial 5/6-ring* < *Syn/Anti 5-ring* < *Anti/Syn Partial 6-ring* with values of 4.21, 7.81 and 11.88 kcal/mol respectively. For interface tetrad stacking geometries, ΔE^{norm} is ranked as follows: *5/6-ring* < *6-ring* < *5-ring* < *Partial 6-ring* with values of 0.00, 4.88, 9.14 and 11.69 kcal/mol respectively. The stacking energies interpolated from MP2/6-31G*(0.25) calculations of average experimental geometries are in agreement with higher level MP2/6-311+G(2d,2p) calculations (Table 2-3). It is important to remember that relative energies discussed here are for entire stacked tetrads, not a single pair of stacked guanines.

Since a large number of cataloged structures contain Na^+ ions instead of K^+ ions, the effects of Na^+ on the energy profiles of stacked tetrads was also investigated (Fig 2-6). Models were run with a central K^+ , a central Na^+ , two Na^+ ions in the tetrad plane, and no ion present. The rotational energy profiles for all of these models are very similar. However, ion type does affect the separation energy profile. When K^+ is substituted with Na^+ , the energy minimum is lowered. The use of two Na^+ ions is more similar to the curve of K^+ , except slightly flattened. Interestingly, the use of two Na^+ ions, one in the center of each tetrad, leads to an energy profile that is almost identical to model with no coordinating ion. In the presented Na^+ -containing crystal structures, ions are found in a range between the two extremes explored in these models with the 2 Na^+ motif being slightly more observed. With this in mind we include all structures in the statistics used to derive relative experimental base stacking energies (Table 2-3).

One should use caution when comparing quantitative relative energies from gas phase energy calculations of isolated tetrads with experimentally observed tetrad stacking geometries. For reasons discussed above (see Section 2.3: Theory), the energy dependen-

Figure 2-6: Effect of ion type on QM energy profiles of stacked G-tetrads containing central K⁺ (○), central Na⁺ (□), and two Na⁺, one in the center of each tetrad (Δ): **(A)** Separation profile of 60° same-polarity stacked tetrads. Similar energy profiles are observed for the K⁺ and two-Na⁺ containing models. **(B)** Rotational energy profile of same-polarity stacked tetrads at 3.4 Å separation. **(C)** Rotational energy profile of opposite-polarity stacked tetrads at 3.4 Å separation. Energy profiles have been individually normalized with their individual minima Interaction energy taken to be zero.

ce of tetrad geometries calculated in gas phase can be altered by a water environment. For example, interactions of tetrad edges with ions and water are energetic contributions that can differ with varying tetrad geometries (150,156,162,163). When considering the accuracy of experimental structures, atomic coordinate precision can be on the order of

the geometrical differences examined in computational models. For these reason, we only compare QM calculations with results from our survey of crystallographic structures. These structures are expected to be generally better resolved than NMR. Despite these uncertainties, the results of our structural survey of crystallographic structures, particularly interface stacking geometries, are in good agreement with our computational studies suggesting that computed energy landscapes provide a good general description of preferable G-tetrad stacking geometries in G-quadruplex systems. Results from fully represented and solvated MD simulations of G-quadruplexes (discuss below) further support these findings.

Previous quantum chemical studies of G-tetrad stacking have also examined some of the base stacking geometries discussed in this study (142-144). A study by Gu et al. (143) in 2002 examined two geometries of stacked G-tetrad complexes with different coordinated cations. Initial geometries were similar to those found in crystallographic structures and then optimized at the Hartree-Fock (HF) level of theory. Optimized tetrad geometries of 5/6-ring stacking were observed for an opposite-polarity model and an optimized θ_{tet} of $\sim 45^\circ$ observed for a same polarity model. While far from their starting structures geometries, optimized tetrads in this study are in agreement with the two global minima observed in our study. In 2005, Meyer et al. (144) examined such tetrads in a similar manner using Density Functional Theory (DFT). Three optimized geometries were obtained based on model tetrads of different symmetries. These included the two geometries discussed above and a third corresponding to the global maximum of our same polarity landscape with a θ_{tet} of $\sim 0^\circ$. These past studies have examined G-tetrad stacking geometries for same- and opposite-polarity stacked tetrads and give an idea of the energy differences between them. The current study expands on previous works by a

systematic assessment of stacking energy landscapes at a high level of theory, MP2/6-31G*(0.25), and couples this investigation with a structural survey of experimental G-tetrad stacking geometries.

2.4.4: Molecular Mechanics energy landscapes of stacked G-tetrads

In addition to our QM investigation of G-tetrad stacking, we decided to assess the accuracy of a commonly used molecular mechanics (MM) force field in reproducing QM G-tetrad stacking landscapes. Knowledge of how well G-tetrad stacking interactions are represented in MD simulations is important to the structural determination and modeling G-quadruplexes.

Energy landscapes were computed for K^+ coordinated stacked G-tetrads using the ff94 AMBER force field (179) description of intermolecular Van der waals and electrostatic terms which have remain unchanged among commonly used revisions to this force field for nucleic acid simulations. The results of these studies are shown in Figure 2-7. The shape of the MM same-polarity energy landscape (Fig 2-7B) is very similar to that of the QM landscape, with a similar broad energy basin observed. The low θ_{tet} regions of the same-polarity energy landscape are again observed to be unfavorable. The opposite-polarity stacking landscape computed via the AMBER force field (Fig. 2-7C) is also generally similar in shape to that of the QM landscape except with smaller and less defined energy barriers. More specifically, the energy barriers between the *5/6-ring* and *5-ring* as well as the *Partial 6-ring* and the *6-ring* base stacking modes are practically negligible.

The differences between the QM and the MM energy landscapes lead to some notable dif-

Figure 2-7: MM energy landscapes. (A) Rotational energy profile of same-polarity and opposite-polarity stacked tetrads at a d_{tet} value of $3.3 \text{ \AA}$ separation. (B) MM energy landscapes of same-polarity and (C) opposite-polarity stacked tetrads.

ferences in the relative energies of various stacking modes (Table 2-3), particularly for the *Partial 5/6-ring* and *5-ring* modes of the core and the *5/6-ring* and *5-ring* modes of the interface. The less-defined energy minima of the opposite-polarity energy landscape computed via AMBER should be considered when using such techniques in modeling G-quadruplex structures. Generally speaking however, the AMBER force field shows a good agreement with QM computations, and favors the *5/6-ring* and *5-ring* Interface stacking

modes, which are seen occupied by all NMR-based experimental structures and most crystallographic structures. This agreement is interesting considering that the description of intermolecular interactions in the AMBER force field is limited to Van der Waals and electrostatic interactions.

Recently, studies have employed free energy analysis of MD simulations to explore the effects of varying structural features on the energy of G-quadruplex systems (59,159). Studies by Cang et al. examined energy differences of different di-nucleotide steps in the context of G-quadruplex DNA (59). A difference of 15 kcal/mol, calculated using MM-PBSA free energy analysis of MD simulations, is observed between 2-layered G-quadruplexes containing all *Syn/Anti* steps and one containing less favorable *Anti/Anti* steps. Our ranking of *Anti/Anti Partial 5/6-ring* < *Syn/Anti 5-ring* < *Anti/Syn Partial 6-ring* for core base stacking geometries does not directly contrast these results, as our work seeks to understand the energy contribution of base stacking which is only one component driving the formation of G-quadruplex structures. In a fully represented and solvated system, it is apparent that many other factors play a role. It is interesting to note that a comparison of QM and MM energy landscapes suggest a relative energy difference of 3.4 and 1.6 kcal/mol, respectively, between the more favorable *Anti/Anti Partial 5/6-ring* and the *Syn/Anti 5-ring* stacking geometries suggesting some discrepancy between the QM computed landscapes and the MM description of G-tetrad stacking energy. Nevertheless, the MM energy landscapes reproduce the general trends observed in QM energy landscapes suggesting a fairly accurate description of the relative stacking energy between stacked G-tetrad geometries.

2.4.5: Investigating 3'-3' and 5'-5' G-quadruplex stacking using Molecular Dynamics

Individual G-quadruplex blocks are sometimes observed to stack upon each other at the interface of their outermost exposed G-tetrads. Parallel-stranded G-quadruplexes contain well defined 3'- and 5'-ends giving rise to four possible stacking combinations (180), 5'-5', 3'-3', 5'-3' and 3'-5'. Stacking that is 5'-5' or 3'-3' arises from opposite-polarity tetrad stacking, while 5'-3' and 3'-5' arises from same-polarity tetrad stacking. No instances of 5'-3' stacking at the G-tetrad interface of G-quadruplexes have been reported, although unpublished data from our laboratory suggest that 5'-3' stacking can occur at the interface of two G-quadruplexes connected by a linker. In our survey, G-quadruplex stacking interfaces are almost entirely observed to be 5'-5' in nature. In tetramer G-quadruplex forming sequences, stacking is observed in a 5'-5' manner despite the presence of 3' and 5' flanking ends for most sequences (7,62,145,146,148,149). Of sequences with well defined free stacking interfaces, the only exception observed to stack in 3'-3' manner is a bimolecular telomeric G-quadruplex whose 5' end is occupied by small-molecule binding (64). NMR studies of the 5'-TTAGGG sequence demonstrate that when the 5'-end of a tetrameric G-quadruplex is occupied by a sizable flanking sequence, 3'-3' stacking still readily occurs (181). These studies suggest that 3'-3' stacking is possible, but less favorable compared to the more readily observed 5'-5' stacking. It is also interesting to note that some instances have been observed of drug-mediated 5'-5' stacking (163).

In order to shed light on the observed 5'-5' stacking preference, a series of MD simulations was performed. Using a variant from an NMR based model (182), we simulated the stacking of two propeller structures formed by the sequence d[G₃(TG₃)₃].

Individual G-quadruplex units were manually oriented at their 3'–3' or 5'–5' ends in multiple starting geometries. The θ_{tet} of interface stacked guanines was determined along the trajectories in order to understand the base stacking modes occupied by 3'–3' and 5'–5' stacked G-quadruplexes (Fig. 2-8A,B).

For all trajectories, the θ_{tet} values of tetrads at the interface of the stacked propeller G-quadruplexes equilibrate within the first few nanoseconds of simulation. More interestingly, each of the three trajectories run for the 3'–3' and 5'–5' models equilibrate to a single θ_{tet} value, regardless of their initial θ_{tet} value. What is perhaps most interesting is that the 3'–3' and 5'–5' stacking models equilibrate to very different interface geometries. The interface geometries of the 3'–3' model equilibrate to θ_{tet} value of roughly 40°-45° corresponding to the *6-ring* stacking mode (Fig. 2-4B), as seen experimentally (147), while the 5'–5' model equilibrates to 60°-65° similar to *5/6-ring* stacking (Fig. 2-4C). Longer MD simulations of 100 ns were computed for select trajectories to investigate if these stacking geometries are maintained over a longer time scale (Fig. 2-8C). Both the 3'–3' and the 5'–5' models remain folded and stacked throughout the 100 ns trajectories. The 3'–3' stacked G-quadruplexes are observed to periodically shift from the more favorable of *6-ring* stacking to a *Partial 6-ring* stacking geometry with a θ_{tet} of roughly 30°. The 5'–5' model remains in an equilibrium interface geometry exhibiting *5/6-ring* stacking over the entire 100 ns trajectory.

Structural analysis suggests that the 3'–3' model cannot occupy the interfacial *5/6-ring* stacking mode, as this would result in a clash between the sugars of the stacked guanines (Fig. 2-9). Conversely, the *5/6-ring* stacking of the 5'–5' model is easily achieved without unfavorable backbone interactions (Fig. 2-9A). Calculations at the MP2/6-311+G(2d,2p)

Figure 2-8. MD investigation of G-quadruplex G-tetrad stacking interfaces: 5-ns trajectories of stacked propeller G-quadruplexes formed by the sequences d[(G₃T)₃G₃] were run for (A) 5'-5' and (B) 3'-3' stacked G-quadruplexes. The trajectories of three different starting geometries of G-quadruplex stacking arrangements (Interface θ_{tet} varying by $\sim 30^\circ$) are shown. (C) Long 100-ns trajectories were computed for a 50-50 and a 30-30 stacked G-quadruplex complex. Average relative rotation (θ_{tet}) of the four interface guanine stacks was characterized at regular intervals.

level described in Table 2-3 suggest that the *6-ring* and *5/6-ring* stacking modes are energetically different by roughly 5 kcal/mol. Based on the QM and MD studies described in this work, we provide evidence that accessible base stacking modes are fundamentally different for 3'-3' and 5'-5' stacked G-quadruplexes. Furthermore, we suggest that sizable energetic differences between accessible base stacking modes play a role in the observed preference for G-quadruplexes to stack primarily in a 5'-5' manner.

Understanding the relative energies of accessible interfacial base stacking modes has important implications in high-order stacking of G-quadruplexes. Past studies have suggested that long telomeric repeats form a “bead-on-a-string” model in which long telomeric nucleic acids form a series of intramolecular quadruplex “beads” (48,183). Other studies (21,155,180,184) suggest that further structural organization is possible, in which individual beads stack upon each other on the 3'-end and/or the 5'-end G-tetrad interfaces. Stacking models have been suggested in which telomeric G-quadruplex beads

Figure 2-9: Illustrative interface geometry of MD trajectories: (A) A ‘5/6-ring’ stacking equilibrium is maintained across the 100-ns trajectory for the 5’–5’ stacking model. (B) The interface of the 3’–3’ model is mostly found in a ‘6-ring’ stacking geometry. (C) Occasionally the 3’–3’ model occupies a ‘Partial 6-ring’ stacking mode. Backbone atoms are shown in light gray and the O1’ atoms of sugar are shown in red. Spherical representation of backbone atoms are the approximate Van der Waals surface of atoms as visualized in PyMOL.

stack in a 5’–3’ (21,184), 3’–3’ (155), and 5’–5’ manner (155,180). Crystallographic structural data has shown drug-mediated 5’–3’ stacking interactions in 2-repeat human telomeric DNA (162). Additionally, 5’–5’ stacking has been observed in 2-repeat (150) and 4-repeat (21) human telomeric DNA. NMR solution studies have observed 5’–5’ G-tetrad stacking in 2-repeat human telomeric RNA (155). Additionally, recent studies have suggested that TERRA stacks preferentially in a 2-block manner (180,185). This observation is in line with a 5’–5’ or 3’–3’ model as 5’–3’ stacking is expected to demonstrate continuous stacking beyond two blocks. The preference of 5’–5’ stacking observed in our survey supports 5’–5’ stacking models of higher-ordered telomeric repeat structures and are in agreement with recent studies by Martadinata et al. (180) which report a *5/6-ring* interface stacking geometry of the 5’–5’ stacked TERRA based on experimental and MD studies. While it is probable that multiple types of high-order G-quadruplex assemblies can form in dynamic long telomeric nucleic acids, further work

needs to be done to understand which are most readily adopted in the context of biologically relevant environments.

2.5 Conclusions:

The current study seeks to understand the roles of guanine base stacking in the formation and interaction of G-quadruplex nucleic acids. We undertake an extensive survey of the PDB to characterize guanine base stacking geometries within the core and at the interface of G-quadruplexes. We couple this survey with systematic QM and MM computations of G-tetrad stacking energy landscapes, helping to understand the base stacking energy contributions for different G-tetrad geometries observed experimentally. We also perform MD simulations to investigate the roles of base stacking in high-order G-quadruplex assemblies. We conclude the following from our studies:

- 1) There exist three well-defined base stacking modes within the G-tetrad core of G-quadruplexes as well as numerous commonly occurring stacked G-tetrad geometries at the interface of stacked G-quadruplex structures. Base stacking geometries within the core were unique for the *Anti/Anti*, *Syn/Anti*, and *Anti/Syn* di-nucleotides steps. Additional base stacking geometries were observed at the interface, with the *5-ring* and *5/6-ring* stacking modes being most readily observed.
- 2) In our survey of the PDB, G-tetrad stacking at the interface of G-quadruplexes in both NMR and crystallographic structures always displayed opposite-polarity stacking and were primarily stacked in a 5'–5' manner.
- 3) QM energy landscapes of K⁺-coordinated stacked G-tetrads were calculated for different stacking polarities. Tetrad stacking energy for experimental geometries are ranked as follows, core stacking modes: *Anti/Anti Partial 5/6-ring* < *Syn/Anti 5-ring* < *Anti/Syn Partial 6-ring*, interface stacking modes: *5/6-ring* < *6-ring* < *5-ring* < *Partial 6-ring*. G-tetrad stacking geometries at the interface of G-quadruplex units are found to be energetically different in terms of stacking

energy by up to 12 kcal/mol.

- 4) Aside a few small differences, MM computed G-tetrad stacking energy landscapes are in good agreement with QM landscapes suggesting that relative G-tetrad stacking energies are well represented in MD simulations.
- 5) MD simulations suggest that 5'–5' and 3'–3' stacking of parallel-stranded G-quadruplexes have different accessible base stacking modes, with 5'–5' stacking being more energetically stable in terms of tetrad stacking energy. The results of our survey and computational studies suggest that a 5'–5' arrangement with the *5/6-ring* base stacking geometry of the interfacial G-tetrads is a favorable arrangement of stacked parallel G-quadruplexes.

Chapter 3:

Fundamentals of G-quadruplexes: Charge Transport

This chapter was largely adapted from the authors work: Lech, C.J., Phan, A.T., Michel-Beyerle, M., Voityuk, A.A. (2013) Electron-Hole Transfer in G-quadruplexes with Different Tetrad Stacking Geometries: A Combined QM and MD Study. *Journal of Physical Chemistry B*, 117, 9851-9856

Author Contributions: MD Simulations and analysis of MD data was performed by C.J.L.; Geometrical characterization of G-tetrad stacks was done by A.A.V.; QM computations were performed by A.A.V.; Computational approaches were designed by A.A.V. and C.J.L.; QM data analysis was performed by C.J.L and A.A.V.; All authors contributed to the experimental conceptualization and writing of above the manuscript from which this chapter is based.

3.1 Introduction:

The ability for DNA to host long range charge transport has important implications in both biology and nanotechnology. Charge transport enables the relocation of DNA damage into particular areas of the genome and may play a role in protein signaling (186). From a nanotechnology perspective, the growing need for nanoscale electronics has produced a rising interest in DNA as a potential element for building nanoscale circuitry. Previous studies have demonstrated a wide range of electron hole-transfer (HT) properties of DNA duplexes and related systems (187-190). In particular, 10 years ago, it was shown that the efficiency of HT in DNA strongly depends on conformational fluctuations (191,192). Subsequently, this finding was supported by many other studies (See references (189,190) and references therein). A deeper understanding of the relationship between kinetics and structure is expected to help to improve the design of DNA based

molecular wires (193-196).

As an alternative to double helical structures, four-stranded G-quadruplex nucleic acids represent an attractive biopolymer for the formation of conductive nanoscale wires (G-wires) (91,92,197-199). G-quadruplexes are stabilized by the stacking of Hoogsteen-bound guanine tetrads (G-tetrads) and have a propensity to form in ionic solution, or, perhaps better, in aqueous electrolytes (200). Stacked G-tetrads were reported to be efficient electron traps that may function *in vivo* as electron sinks, diverting oxidative damage away from other genomic regions (201). There are only a few studies focusing on electron-transport properties of G-quadruplexes (202-205). While cation coordinated G-tetrads of G-quadruplexes have more π -contacts along the stack compared to double-stranded DNA, the relative conductive properties of these two classes of structures is still unclear (201,202,205,206).

Computational studies of nucleic acids reveal many important details of the structure and dynamics of systems that are difficult to derive from experimental data (207). Both quantum mechanics (QM) and molecular dynamics (MD) approaches have provided valuable insights into G-tetrad stacking geometries and interaction energies (59,71,143,158,208). Electronic structure calculations have shown that electronic states in G-quadruplexes are more delocalized than in DNA duplexes (209,210). Recently, the electrical conduction properties of a tetrameric parallel-stranded G-quadruplex were thoroughly investigated using a QM/MD approach (211).

Within G-quadruplex nucleic acids, the guanine base stacking geometry of stacked G-tetrads can vary greatly depending on the conformation adopted (67). Several unique G-

tetrad stacking modes can arise from different folding patterns within a single G-quadruplex molecule or at the interface of two stacked G-quadruplex subunits (212). It is currently unclear how the orientation of stacked G-tetrads affects the mobility of electron holes through the G-tetrad stack.

In this study, we address this question by examining how the probability of HT varies with G-tetrad stacking geometry. Through a combined QM and MD study of G-quadruplex systems (Appendix II – Protocols – Chapter 3), we demonstrate that HT rates within the G-tetrad stacks vary greatly for different commonly occurring stacking geometries. By identifying experimentally observable G-tetrad stacking geometries with optimal HT properties, our findings reveal important structural considerations for the design of conductive nanowires built upon the G-quadruplex architecture.

3.2 Theory:

3.2.1 Electronic Coupling

The well established Fragment Charge Difference (FCD) method (213,214) was employed to derive the quantities V from the adiabatic electronic states of the G-tetrad stacks.

In the two-site model of the FCD method, we consider the transfer of an electron hole from a donor “ D ” to an acceptor “ A ” site. The system can be described in terms of the two orthogonal and normalized states, Ψ_1 and Ψ_2 , with corresponding energies E_1 and E_2 :

$$\begin{aligned}\Psi_1 &= c_{D1}\Phi_D^\sim + c_{A1}\Phi_A^\sim \\ \Psi_2 &= c_{D2}\Phi_D^\sim + c_{A2}\Phi_A^\sim\end{aligned}\tag{1}$$

Where the functions $\Phi_D^\sim$ and $\Phi_A^\sim$ correspond to normalized wave functions that are localized on the donor and acceptor sites. The charge on the donor or acceptor fragment, $F=(D,A)$, in the state Ψ_i , $i=(1,2)$, is simply c_{Fi}^2 . By applying the following orthogonal transformation T :

$$T = \begin{pmatrix} \cos(\omega) & -\sin(\omega) \\ \sin(\omega) & \cos(\omega) \end{pmatrix}\tag{2}$$

to the states Ψ_1 and Ψ_2 , we can obtain two new states described by:

$$\begin{aligned}\Phi_D &= \Psi_1 \cos(\omega) + \Psi_2 \sin(\omega) \\ \Phi_A &= -\Psi_1 \sin(\omega) + \Psi_2 \cos(\omega)\end{aligned}\tag{3}$$

The charge localized on the donor, $q(D)$, in the states Φ_D and Φ_A can be expressed as:

$$\begin{aligned} q(D) &= (c_{D1} \cos(\omega) + c_{D2} \sin(\omega))^2 \\ &= q_1(D) \cos^2(\omega) + q_2(D) \sin^2(\omega) + q_{12}(D) \sin(2\omega) \end{aligned} \quad (4)$$

$$\begin{aligned} q(A) &= (c_{A2} \cos(\omega) - c_{A1} \sin(\omega))^2 \\ &= q_2(A) \cos^2(\omega) + q_1(A) \sin^2(\omega) - q_{12}(A) \sin(2\omega) \end{aligned} \quad (5)$$

where $q_{12}(F) = c_{F1}c_{F2}$. By maximizing the value $q(D) + q(A)$ we determine the value of ω that corresponds to maximum charge transfer between the donor and acceptor, leading to:

$$\tan 2\omega = \frac{2\Delta q_{12}}{\Delta q_1 - \Delta q_2} \quad (6)$$

where $\Delta q_{12} = \Delta q_{12}(D) - \Delta q_{12}(A)$. Considering a two-state model, the electronic coupling between donor and acceptor can be written as:

$$V = \frac{1}{2}(E_2 - E_1) \sin 2\omega \quad (7)$$

By combining Eq. 6 and Eq. 7, the electronic coupling matrix element between the donor site D and the acceptor site A can be written as:

$$V = \frac{(E_2 - E_1)\Delta q_{12}}{\sqrt{(\Delta q_1 - \Delta q_2)^2 + 4\Delta q_{12}^2}} \quad (8)$$

Here Δq_1 and Δq_2 are the donor-acceptor charge differences in the adiabatic states with energies E_1 and E_2 , and Δq_{12} is the corresponding off-diagonal term.

These quantities were calculated within the self-consistent field approximation using the semi-empirical INDO/S method (215). The method was shown to be computationally very efficient, offering surprisingly good estimates for electronic couplings in DNA stacks (216). These advantages enable quantum mechanical treatment of several thousand

different conformations of stacked G-tetrads.

3.2.2 Hole Transfer Rates

The non-adiabatic electron transfer rate k_{ET} can be expressed as (217):

$$k_{ET} = \frac{2\pi}{\hbar} V^2 FC \quad (9)$$

where the electronic coupling V is a key parameter that determines the sensitivity of the Electron transfer (ET) rate on the arrangement of the donor and acceptor sites. FC is the nuclear Frank-Condon factor that can be estimated as:

$$FC = \frac{1}{\sqrt{4\pi\lambda k_B T}} e^{\frac{-(\Delta G + \lambda)^2}{4\lambda k_B T}} \quad (10)$$

where ΔG is the free energy of ET and λ is the reorganization energy. In the considered systems, the hole donor and acceptor sites are very similar and we assume that on average $\Delta G \approx 0$. In related DNA systems, the reorganization energy estimated with different approaches for HT between nucleobases ranges from 0.5 to 1.5 eV (218-221). Due to the exponential dependence of FC on λ , the estimated ET rate is extremely sensitive to the reorganization energy value used in Eq. (2) and decreases by a factor of $2 \cdot 10^4$ passing from $\lambda=0.5$ to $\lambda=1.5$ eV. In this study, we are primarily interested in relative rates rather than in the absolute values. Assuming a similar reorganization energy and FC for all stacks considered, we obtain that a relative rate value that depends only on the square of effective electronic coupling,

$$k^{rel}(X) = \frac{V^2(X)}{V^2(0)} \quad (11)$$

where $V^2(0)$ corresponds to the reference system with an ET rate set equal to 1. We

computed the effective coupling for each ensemble of G-tetrad stacking geometries $V^2(X)$ (where X represents a given geometry of Fig 3-2A-E) by averaging of the matrix elements squared over N structures ($N=1000$) extracted from MD trajectories.

$$V^2(X) = \overline{V^2}(X) = \frac{1}{N} \sum_{i=1}^N V_i^2(X) \quad (12)$$

3.3 Results and Discussion:

3.3.1 Geometry of Stacked G-tetrads

G-tetrad stacking geometry can be simply classified by the relative polarity of stacked G-tetrads (Fig. 3-1A). The stacking of G-tetrads in a Same-Polarity or Opposite-Polarity manner defines the relative geometries that may be adopted by stacked guanines within G-tetrad stacks. Stacked G-tetrads are found within the core of a single G-quadruplex unit and at the stacking interface of dimeric G-quadruplexes and higher-order G-quadruplex assemblies. The geometry of stacked G-tetrads within a G-quadruplex or at the interface of stacked G-quadruplexes is highly sequence and structure dependent.

To investigate the electronic properties of stacked G-tetrads, we undertake QM calculations on G-tetrad stacks obtained from a series of MD simulations of G-quadruplex molecules (Fig 3-1B and Appendix II – Protocols – Chapter 3). A total of 1000 G-tetrad stacks are derived from MD data for each of the 5 unique G-tetrad stacking modes (Fig 3-

Figure 3-1. (A) Stacked G-tetrads are in either an Opposite-Polarity or Same-Polarity orientation. (B) Illustrative example of a stacked G-tetrad system with coordinating K⁺ ion (purple) extracted from MD simulations. The C and N atoms of the guanine are colored by tetrad. The H, O, and K atoms are colored by type. (C) Relative position of guanines in the G-tetrad system referenced in Tables 3-2 and 3-4.

Figure 3-2. Illustrative examples of G-tetrad stacking modes studied in this work

2). G-tetrad stacking geometries that commonly occur within the “core” of a single G-quadruplex unit were investigated (Fig 3-2A-C). We also examined “interface” stacking geometries that are observed when two G-quadruplex molecules dimerize at a G-tetrad interface (Fig 3-2D-E).

The *Partial 5/6-ring* G-tetrad stacking mode is a commonly occurring geometry in which the 6-member ring of one guanine base is partially overlapped with the 5-member ring of the other (Fig 3-2A). It is found within the G-tetrad core of a single G-quadruplex unit containing a parallel-stranded configuration. The *5-ring* and *Partial 6-ring* geometries are named after the aromatic overlap of their rings, and are found within a single G-quadruplex unit containing a non parallel-stranded configuration (Fig. 3-2B-C). The *6-ring* and *5/6-ring* geometries are more heavily overlapped geometries which occur at the interface of stacked G-quadruplex units (Fig. 3-2D-E).

The geometrical parameters of stacked G-tetrads were computed to explore the geometries sampled by the various stacking modes described in Figure 3-2. The data presented in Table 3-1 reports the average geometrical parameters and their standard deviations for the simulated G-tetrad systems considered in this chapter.

Table 3-1. Average geometrical parameters of stacked G-tetrads and their standard deviations

Stacking Orientation ^a	Twist ^b (°)	Rise ^c (Å)	Inclination ^d (°)	Planar Shift ^e (Å)
Partial 5/6-ring (A)	32.56 ±1.93	3.43 ±0.06	1.23 ±0.70	0.22 ±0.11
5-ring (B)	11.82 ±1.23	3.49 ±0.07	1.33 ±0.74	0.26 ±0.13
Partial 6-ring (C)	43.42 ±1.20	3.54 ±0.08	1.66 ±0.95	0.24 ±0.12
6-ring (D)	27.10 ±1.99	3.60 ±0.08	2.19 ±1.11	0.39 ±0.21
5/6-ring (E)	-8.73 ±2.45	3.37 ±0.07	1.27 ±0.67	0.31 ±0.16

^a Lettering of stacking orientations refer to the geometries of Figure 3-2.

^b Twist is defined by the dihedral angles between N9 atoms in stacked guanine bases and the center of tetrad planes. Values are averaged over each stacked guanine pair in a single snapshot.

^c Rise is defined as the projection of the vector connecting centers of two tetrads on the normal of the average tetrad plane.

^d Inclination is the angle between the tetrad planes.

^e Planar Shift is the projection of the vector connecting centers of the stacked tetrads on the average tetrad plane.

The “Twist” parameter describes the relative rotation between two stacked tetrads. Variation in this parameter is a main feature of different G-tetrad stacking modes. It is important to note that the *Partial 5/6-ring* geometry arises from stacked G-tetrads of the same polarity. The other four stacking modes studied arise from G-tetrads of the opposite polarity. Relative values of “twist” are only comparable for G-tetrad stacks of similar stacking type (i.e. same polarity or opposite polarity).

The rise parameter represents the distance between G-tetrad planes. Values of this parameter are found to be within the range of 3.4 to 3.6 Å, as expected for π -stacked systems. Additionally, the variation in values of “rise” for different G-tetrad stacking geometries is in good agreement with our experimental survey (Chapter 2) in which we characterize this parameter for individual guanine stacks.

The “inclination” parameter measures the relative angle away from a parallel alignment of G-tetrad planes. A slight average inclination was found for G-tetrads. The “planar shift” parameter is a measure of the displacement of the center of one tetrad plane with respect to the other. No structurally significant variation in “inclination” or “planar shift” is found.

3.3.2 Electronic coupling and hole transfer rates

As described in section 3.2 Theory, relative rates of HT between two stacked G-tetrads are determined by the ratio of electronic coupling squared (Eq. 3-9). In Table 3-2 we list values of the effective coupling squared derived for each type of G-tetrad stacking geometry by averaging over 1000 MD snapshots examined at the QM level.

The left panel of Figure 3-3, shows time evolution of V^2 between stacked guanines G2 and G6 in the G-tetrad stack *E* (Fig. 3-2E). The changes are caused by thermal fluctuations of the structure. As seen, the V^2 values are extremely sensitive to the stack geometry. Quantum mechanical calculations of the coupling for a single or few structures may provide inaccurate estimates of V^2 and the related HT rate. Averaging over many thermally accessible conformations is required to obtain reliable values of the effective coupling. This is evident when examining scatter plots of the V^2 dependence on the twist parameter (Fig. 3-3B). A wide range of V^2 values are observed for similar values of twist with no strong correlation between V^2 and the individual parameters that define the mutual position of G-tetrads in the stack. Nevertheless, one can see that the largest coupling values ($V^2 > 0.2 \text{ eV}^2$) of stacked guanines are found for twist values ranging from

Table 3-2. Electronic coupling squared (in eV^2) between stacked guanine bases in different G-quadruplex structures.

Stacking Orientation ^a	Partial 5/6-ring (A)	5-ring (B)	Partial 6-ring (C)	6-ring (D)	5/6-ring (E)
V_{15}^2	$0.78 \cdot 10^{-3}$	$2.66 \cdot 10^{-3}$	$2.79 \cdot 10^{-3}$	$7.39 \cdot 10^{-3}$	$71.1 \cdot 10^{-3}$
V_{26}^2	$1.38 \cdot 10^{-3}$	$4.75 \cdot 10^{-3}$	$3.20 \cdot 10^{-3}$	$6.27 \cdot 10^{-3}$	$97.6 \cdot 10^{-3}$
V_{37}^2	$1.10 \cdot 10^{-3}$	$2.93 \cdot 10^{-3}$	$2.44 \cdot 10^{-3}$	$5.50 \cdot 10^{-3}$	$93.4 \cdot 10^{-3}$
V_{48}^2	$1.54 \cdot 10^{-3}$	$2.24 \cdot 10^{-3}$	$1.97 \cdot 10^{-3}$	$10.15 \cdot 10^{-3}$	$74.9 \cdot 10^{-3}$
Mean	$1.18 \cdot 10^{-3}$	$3.15 \cdot 10^{-3}$	$2.60 \cdot 10^{-3}$	$7.33 \cdot 10^{-3}$	$84.3 \cdot 10^{-3}$

The calculation were performed within the two state FCD model for all pairs of stacked guanines. The numbering of nucleobases is shown in Figure 3-1C

^a Lettering of stacking orientations refer to the geometries of Figure 3-2.

Figure 3-3. (A) Time evolution of electronic coupling squared V^2 . (B) Scatter plots of the V^2 vs. the twist parameter. In both cases the coupling of G2 and G6 (see Fig. 3-1C) in the π stack E is considered.

-14 to -6 degrees.

The non-adiabatic electron transfer rates calculated using Eq. (3-2) are listed in Table 3-3. Rates are found to vary from 26 ns^{-1} for the *Partial 5/6-ring* geometry (Fig 3-2A) to 1.9 ps^{-1} for the *5/6-ring* geometry (Fig. 3-2E). The absolute values of k_{HT} depend strongly on the value of reorganization energy. For example, k_{HT} decreases by a factor ~ 2.7 when λ increases by 0.1 eV. However, relative HT rates k_{rel} remain unchanged. A 70-fold difference is observed in the value of k_{rel} between the *Partial 5/6-ring* geometry and the *5/6-ring* geometry which is independent of the reorganization energy parameter λ . Thus, we observe that the *5/6-ring* orientation of stacked G-tetrads (Fig 3-2E) facilitates HT significantly as compared with other G-tetrad stacks found experimentally (Fig 3-4). Am-

Table 3-3. Absolute and relative rates of HT between adjacent tetrads in the G-quadruplexes

Stacking Orientation ^a	k_{HT}^{b} (ns^{-1})	k_{rel}
Partial 5/6-ring (A)	26.0	1
5-ring (B)	69.4	2.7
Partial 6-ring (C)	55.3	2.1
6-ring (D)	163.6	6.3
5/6-ring (E)	1912.2	73.5

[a] Codes for stacking orientation refer to the geometries of Figure 3-2.

[b] The rates are computed at $\lambda = 0.7 \text{ eV}$ and $T = 298.15 \text{ K}$.

Figure 3-4. Relative HT rates computed for different stacking orientations.

ong geometries that occur within the core of a G-quadruplex, we note that the G-tetrads stacked in a Same-Polarity manner (Fig 3-2A) are found to be less conductive than those stacked in an Opposite-Polarity orientation (Fig B-D).

This *5/6-ring* orientation (Fig 3-2E) is a unique geometry that occurs at the interface of some stacked parallel G-quadruplex units. Depending on which ends of the G-quadruplex participate in stacking, dimerization can also result in the formation of a *6-ring* geometry (Fig 3-2D) at the stacking interface. By contrast, the *Partial 5/6-ring* geometry found in the core (Fig 3-2A) of these parallel G-quadruplex units show considerably smaller values of k_{HT} . G-tetrad stacking geometries in G-wires formed by long guanine-rich strands of DNA are constrained by the backbone to adopt geometries A-C what are not ideal for CT. Alternatively, it may also be possible to engineer G-tetrads that demonstrate continuous 5/6-ring stacking from the assembly of single guanosine analogs or through modification of the backbone in G-rich DNA.

HT in G-quadruplexes may also occur within a single G-tetrad between hydrogen-bonded guanines. The calculated values of $\overline{V^2}$ for these types of HT are listed in Table 3-4. Co-

Table 3-4. Electronic coupling squared between guanine bases in one G-tetrad

G pair ^a	1-2	2-3	3-4	4-1	1-3	2-4
V^2, eV^2	$7.42 \cdot 10^{-6}$	$5.80 \cdot 10^{-6}$	$5.05 \cdot 10^{-6}$	$4.70 \cdot 10^{-6}$	$5.02 \cdot 10^{-8}$	$1.46 \cdot 10^{-8}$

^aFirst four pairs are formed neighboring guanines. The nucleobases in pairs (1-3) and (2-4) are arranged diagonally.

mparison of these quantities with data collected in Table 3-3 suggests that the probability of the HT within a G-tetrad are roughly 3 orders of magnitude lower than for HT between stacked nucleobases.

3.4 Conclusions

Using a combined quantum mechanics and molecular dynamics approach we have studied hole transport properties in several experimentally observed G-tetrads geometries with different types of π -stacking. The effective electronic coupling values between π -stacked and Hoogsteen bonded guanine nucleobases were derived with the Fragment Charge Difference method applied to adiabatic states obtained using the semi-empirical INDO/S scheme. Our study demonstrates the important role of G-tetrad orientation in electron hole transport within the G-tetrad stacks. The fastest hole hopping between G-tetrad stacks has been for the 5/6-ring geometry commonly found at the interface of stacked G-quadruplex units. The HT rate is at least an order of magnitude slower for other types of G-tetrad structures. Thus, we have identified the stacking configurations of neighboring G-tetrads with enhanced electric conductivity. V^2 values within a single G-tetrad are at least 2 orders of magnitude smaller than between stacked guanines and therefore hole hopping within individual tetrads should not affect the electron hole mobility in G-quadruplexes. The results of this study provide guidance for the design of conductive G-wire systems.

Chapter 4:

Engineering the Sugar Backbone: Locked Nucleic Acid

This chapter was largely adapted from the authors work: Li, Z.*, Lech, C.J.*, Phan, A.T. (2014) Sugar-modified G-quadruplexes: effects of LNA-, 2'F-RNA- and 2'F-ANA-guanosine chemistries on G-quadruplex structure and stability. *Nucleic Acids Research*, [in press] (* Denotes co-first authorship) and unpublished work including contributions from Brahim Heddi and Michael Adrian.

Author Contributions: DNA synthesis, purification and thermal denaturing experiments were carried out by L.Z.; Data analysis of thermal denaturing experiments was performed by C.J.L.; 1D NMR experiments were performed by C.J.L. and L.Z.; 2D NMR Experiments were performed by B.H, M.A. and L.Z.; NMR assignments were performed by M.A. and L.Z.; The NMR solution structure of the L4 sequence was computed by M.A. and L.Z. and B.H.; The above mentioned manuscript was written by C.J.L, L.Z. and A.T.P; All authors contributed to the conceptualization and planning of experiments.

4.1 Introduction:

The introduction of alternative chemistries into G-quadruplexes can be advantageous, making them more suitable for pharmaceutical or engineering applications. Modification to the base (82,107,111) or sugar-phosphate backbone (106,107,130,222-224) can have beneficial effects on the stability, kinetics, enzymatic digestion resistance, and cellular uptake of biologically active G-quadruplexes. One alternative nucleic acid chemistry that has received notable attention is Locked Nucleic Acid (LNA) (225), a ribonucleotide analogue with a 2'-O,4'-C-methylene linkage (Fig. 4-1A). Introduction of LNA can improve oligonucleotide stability towards enzyme digestion as well as the thermal stability of duplexes and triplexes (225,226). Previous studies have shown that LNA-modifications can greatly enhance the RNA cleaving rate of a DNAzyme (227). Additionally, LNA is soluble in water and non-toxic (104,124).

Figure 4-1. Introduction to LNA and G-quadruplexes: (A) Sugar chemistries of DNA and LNA. G-quadruplex scaffolds of the (B) propeller-type (4+0) G-quadruplex formed by the sequence $d[T_2(G_3T)_4]$ and the (C) hybrid (3+1) G-quadruplex formed by the sequence $d[T_2(G_3T_2A)_3G_3A]$. Guanines are colored based on their *syn* (magenta) or *anti* (cyan) conformation.

In the context of G-quadruplexes, it has been reported that the introduction of LNA modified guanosine (^{LNA}G) stabilizes tetramer G-quadruplexes formed by the sequences $d[T^{LNA}G_3T]$, $d[T(G^{LNA}G)_2T]$ and $d[T^{LNA}G_4T]$ (126,127). ^{LNA}G is previously observed to favor an *anti* glycosidic conformation of the base (125) and previous studies have taken advantage of this preference to engineer G-quadruplex folding topology (65,228). Substitutions of ^{LNA}G into positions that adopt a *syn* conformation tend to push structural equilibrium towards a parallel conformation where all guanines adopt an *anti* conformation (125,228,229). Incorporation of ^{LNA}G has also been used to enhance the inhibitory properties of biologically active G-quadruplex molecules that demonstrate activity against HIV-1 (230) or thrombin (231).

Considering the demonstrated potential of LNA for enhancing the drug-like properties of G-quadruplexes, we set out on a systematic study to characterize the effects of LNA

incorporation into G-quadruplex DNA. In this study, two G-quadruplex-forming sequences were site-specifically modified at each guanine residue with ^{LNA}G. Single-position substitutions were made into: 1) The intramolecular parallel-stranded “(4+0)” G-quadruplex formed by the sequence d[T₂(G₃T)₄] (182), termed “PS-series” (Fig. 4-1B). This (4+0) G-quadruplex structure contains three single-nucleotide (1-nt) propeller loops with all guanine bases adopting an *anti* glycosidic conformation. 2) The intramolecular “(3+1)” hybrid G-quadruplex formed by the sequence d[T₂(G₃T₂A)₃G₃A] (28), termed “HT-series” (Fig. 4-1C). The (3+1) G-quadruplex of the HT-series contains 3-nt edge-wise and propeller loops with guanine bases in both *syn* and *anti* conformations. These two sequences were chosen to explore a range of G-quadruplex structural environments.

The folding topology and thermal stability of G-quadruplexes formed by ^{LNA}G-modified sequences (Table 4-1) is determined through a series of nuclear magnetic resonance (NMR) spectroscopic, circular dichroism (CD) spectroscopic, and thermal denaturing studies (Appendix II – Protocols – Chapter 4). The high-resolution NMR solution structure of a LNA-modified (3+1) G-quadruplex forming sequence is determined, providing structural insight into observations from our biophysical studies. This systematic study reveals important and novel limitations of LNA incorporation into G-quadruplex nucleic acid.

Table 4-1. DNA Sequences and Thermal Stability

Name ^a	Sequence (5'→3') ^b	(°C) ^c	
		T _m	ΔT _m
(4+0) native	TTGGGTGGGTGGGTGGGT	79.0 ± 0.5	-
PS-L3	TT L GGTGGGTGGGTGGGT	84.0 ± 0.4	5.0
PS-L4	TT G LGTGGGTGGGTGGGT	87.4 ± 0.3	8.4
PS-L5	TT G GLTGGGTGGGTGGGT	36.5 ± 0.4	-42.5
PS-L7	TTGGGT L GGTGGGTGGGT	86.4 ± 0.1	7.4
PS-L8	TTGGGT G LGTGGGTGGGT	82.2 ± 0.2	3.2
PS-L9	TTGGGTGG L TGGGTGGGT	32.5 ± 0.1	-46.5
PS-L11	TTGGGTGGGT L GGTGGGT	87.0 ± 0.1	8.0
PS-L12	TTGGGTGGGT G LGTGGGT	81.8 ± 0.1	2.8
PS-L13	TTGGGTGGGTGG L TGGGT	32.0 ± 0.5	-47.0
PS-L15	TTGGGTGGGTGGGT L GGT	85.3 ± 0.1	6.3
PS-L16	TTGGGTGGGTGGGT G LGT	83.0 ± 0.1	4.0
PS-L17	TTGGGTGGGTGGGT G LT	82.4 ± 0.1	3.4
(3+1) native	TTGGGTTAGGGTTAGGGTTAGGGA	56.3 ± 0.2	-
HT-L3	TT L GGTTAGGGTTAGGGTTAGGGA	54.3 ± 0.3	-2.0
HT-L4	TT G LGTTAGGGTTAGGGTTAGGGA	60.5 ± 0.0	4.2
HT-L5	TT G GLTTAGGGTTAGGGTTAGGGA	55.8 ± 0.4	-0.6
HT-L9	TTGGGTTA L GGTTAGGGTTAGGGA	-	-
HT-L10	TTGGGTTAG L GTTAGGGTTAGGGA	58.2 ± 0.4	1.9
HT-L11	TTGGGTTAG G LTTAGGGTTAGGGA	57.2 ± 0.3	0.9
HT-L15	TTGGGTTAGGGTTA L GGTTAGGGA	-	-
HT-L16	TTGGGTTAGGGTTAG L GTTAGGGA	-	-
HT-L17	TTGGGTTAGGGTTAG G LTTAGGGA	54.0 ± 0.4	-2.3
HT-L21	TTGGGTTAGGGTTAGGGTTA L GGA	-	-
HT-L22	TTGGGTTAGGGTTAGGGTTAG L GA	-	-
HT-L23 ^d	TTGGGTTAGGGTTAGGGTTAG G LA	59.4 ± 0.1	3.0

[a] "HT-" series denotes a modified (3+1) G-quadruplex forming sequence while "PS-" series denotes a modified (4+0) G-quadruplex forming sequence.

[b] Residues with LNA-modified guanosine are denoted as (**L**)

[c] Thermal stability data was obtained via UV melting (for HT-series) and CD melting (for PS-series) experiments. Salt conditions were (20 mM KPi) for the HT-series and (1.1 mM KPi) for the PS-series. Thermal stability data for the HT-series is presented for sequences which demonstrate a single species in NMR. The "±" values indicate the hysteresis between heating and cooling curves.

[d] Sequence contains a small secondary melting transition at low temperature range

4.2 Results and Discussion:

4.2.1: Substitution of ^{LNA}G into a (4+0) G-quadruplex: LNA modifications are detrimental when substituted prior to short propeller loops.

G-quadruplex formation of ^{LNA}G-modified sequences was evaluated by monitoring the imino proton region (10.5 – 12.5 p.p.m.) of ¹H NMR spectra (232). Chemical shift patterns in this region can be used to determine the number of G-quadruplex conformations present. Similarity between NMR spectra is a strong indicator that sequences adopt a similar folding topology. Such consideration is important as small changes in sequence can have large effects on the conformations adopted by G-quadruplex forming sequences (23,233). CD spectroscopy was also used to probe G-quadruplex folding topology based on well-characterized patterns in CD spectra (234). Additionally, thermal stability of ^{LNA}G-modified sequences was evaluated through a series of UV and CD melting experiments. The melting temperature (T_m) of these sequences is presented as a useful quantitative metric for comparing the thermal stability of modified sequences.

Within the (4+0) G-quadruplex-forming PS-series, nine of the twelve single-position ^{LNA}G-modified sequences were observed to form a single G-quadruplex species in imino proton NMR spectra and demonstrated spectra which were similar to the native sequence (Fig 4-2.A). CD spectra of these sequences were characteristic of a (4+0) G-quadruplex conformation, with a maximum around 260 nm and a minimum around 240 nm (Fig. 4-2.B). Conversely, substitution of ^{LNA}G into positions G5, G9, and G13 lead to multiple conformations in NMR spectra and a reduction in the intensity of CD spectra at 260 nm (Fig. 4-2). Sequences that formed multiple conformations also demonstrated extreme inst-

Figure 4-2. LNA modifications disrupt the (4+0) G-quadruplex conformation of PS-series sequences in a structurally dependent manner: (A) NMR imino proton and (B) CD spectra of select PS-series sequences containing single-position LNA substitutions on the 3'-end of loop, middle tetrad, or 5'-end of loop.

ability with a change in melting temperature (ΔT_m) exceeding -40°C (Fig 4-3). Alternatively, sequences that formed a single conformation displayed ΔT_m values of $+2.8^\circ\text{C}$ and $+8.4^\circ\text{C}$ in CD melting studies.

We note the unfavorable substitutions occur at residues G5, G9 and G13 which are located upstream, at the 5'-end, of 1-nt propeller loops within this intramolecular (4+0) scaffold. These results suggest the incompatibility of LNA incorporation into residues preceding short propeller loops. Considering this observation, we proceeded to explore the role of loop length in the $^{\text{LNA}}\text{G}$ -induced destabilization from modifications upstream

Figure 4-3. ΔT_m of PS-series sequences containing single-position $^{\text{LNA}}\text{G}$ modifications determined by CD melting experiments.

of propeller loops in the (4+0) scaffold. We expanded the 1-nt Thymine (T) loop at position T10 to TT and TTT in both the native and ^{LNA}G9-modified PS-L9 sequences. The effects of loop elongation were monitored using NMR and CD melting experiments (Fig. 4-4). The T_m values of the loop-extended native sequence and PS-L9 sequences converge as the loop length increases. At a loop length of TTT, the difference in T_m between the two sequences converged to a mere 5°C compared to a 46°C difference in sequences containing a 1-nt loop. These changes in T_m values coincide with the emergence of a single conformation in the NMR spectrum of the ^{LNA}G9-modified TTT-loop sequence and a single folding topology maintained for the unmodified TTT-loop sequence.

In this study, we show that ^{LNA}G substitution upstream of short propeller loops is destabilizing to G-quadruplex structure. ^{LNA}G substitution to positions G5, G9 and G13 in the (4+0) G-quadruplex is observed to be disruptive despite the *anti* glycosidic conforma-

Figure 4-4. Effect of loop length on thermal stability: (A) NMR Imino proton spectra. The 1-nt T10 loop of the native (4+0) G-quadruplex forming sequence and PS-L9 containing a LNA substitution at position 9 were elongated. Sequences containing a loop of T, TT, and TTT are shown. (B) T_m of the loop-elongated native (black) and PS-L9 (red) sequences.

-tion of the substituted guanine base. Elongation of these 1-nt propeller loops is shown to reduce the detrimental effects of ^{LNA}G substitutions. It is likely that the conformational constraints on the sugar-backbone geometry imposed by the short propeller loop are incompatible with the conformationally restricted LNA sugar-backbone. The position dependent nature of ^{LNA}G substitutions into *anti*-position guanines is in direct contrast to more versatile nucleoside such as 2'-F-guanoisine and 2'-F-ANA-guanosine (see Chapter 5). The disruptive effect of ^{LNA}G-modification at the 5'-end of short loops constitutes an important consideration for future design of G-quadruplexes containing LNA modifications.

4.2.2 Substitution of ^{LNA}G into a (3+1) G-quadruplex: LNA modifications generally affect structure in a syn/anti dependent manner

Similar to our study of the (4+0) G-quadruplex scaffold, we systematically substituted ^{LNA}G into guanines within the hybrid (3+1) “HT-series” G-quadruplex structure, which contains guanine bases in both *syn* and *anti* conformations (Fig 4-1C). Within the HT-series, seven of the twelve modified sequences demonstrated a single conformation as determined by NMR spectra (Fig. 4-5A). CD spectra of these sequences demonstrated a (3+1) profile similar to the native sequence (Fig. 4-5B). Conversely, the other five ^{LNA}G substitutions induced multiple conformations as observed in NMR spectra (Fig 4-5A). The CD spectra of these five modified sequences exhibit a profile that deviates from the (3+1) native sequence, with an increase in absorbance at 260 nm and a decrease at 295 nm (Fig. 4-5C).

Within the (3+1) G-quadruplex, six of the seven sequences displaying a single G-quadruplex

Figure 4-5. The *syn/anti* preference of LNA substitutions into the HT-series: (A) NMR imino proton spectra and (B-C) CD spectra are shown for select sequences containing single position LNA substitutions to (B) *anti* positions and (C) *syn* positions.

plex conformation contained $^{\text{LNA}}\text{G}$ substitutions to guanosine in an *anti* glycosidic conformation. Substitution with $^{\text{LNA}}\text{G}$ produced a mixture of effects on the thermal stability of modified sequences (Fig. 4-6). Substitution into *anti* guanines displayed ΔT_m values in the range of -2.3°C to $+4.2^\circ\text{C}$. Four of the five $^{\text{LNA}}\text{G}$ substitutions that induced multiple conformations with made to *syn* position guanines. Substitution to *syn*-position G3 led to a destabilization, with a ΔT_m of -1.4°C despite the observation of a single (3+1) conformation. Comparison of T_m values for sequences that demonstrate multiple species is complicated by complex melting curves and an uncertainty about which structural transition is being analyzed. For this reason we don't discuss these sequences in detail.

It is generally observed that substitution of $^{\text{LNA}}\text{G}$ into *syn*-position guanines disrupts the folding topology of the G-quadruplex and lead to co-existence of multiple species while *anti* positions substitutions are generally tolerated. This is not surprising as $^{\text{LNA}}\text{G}$ has been reported to favor an *anti* conformation (65). Previous studies have observed that $^{\text{LNA}}\text{G}$ su-

Figure 4-6. ΔT_m upon LNA modification to HT-series G-quadruplexes: Values are plotted for sequences containing single-position LNA substitutions that demonstrated a single conformation. Cyan and magenta indicate the *anti* or *syn* conformation respectively of the substituted guanine in the unmodified sequence.

substitution into *anti* guanines stabilize G-quadruplexes (126,127) while substitution to *syn* positions has a destructive effect on the native G-quadruplexes conformation (65,125,229).

Despite the general *syn/anti* dependence the ^{LNA}G modifications, we note some important exceptions to this trend. Within the (4+0) G-quadruplex-forming PS-series discussed above, substitutions were generally stabilizing, with increases in T_m observed between +2.8°C and +8.4°C. However, ^{LNA}G modifications directly upstream of 1-nt propeller loops were highly disruptive (Fig 4-2 and 4-3). Interestingly, ^{LNA}G modification to G5, G11, and G17 within the HT-series, located upstream of a 3-nt propeller or edge-wise loop, were the least favorable *anti*-position substitutions tested in the (3+1) G-quadruplex with ΔT_m values of -2.1°C, +0.4°C and -3.2°C respectively (Fig 4-6). These results, in conjunction with results of our loop-elongation experiments (Fig 4-4), suggest that the negative effects of ^{LNA}G modification upstream of loops may not be limited to short propeller loops.

Figure 4-7. The (A) NMR imino proton spectra and (B) CD spectra are presented for the *syn*-position modified HT-L3 sequence and the *anti*-position modified HT-L22 sequence

Other exceptions to the *syn/anti* dependence of $^{\text{LNA}}\text{G}$ modifications are also observed. Within the (3+1) G-quadruplex, $^{\text{LNA}}\text{G}$ substitution to an *anti*-position G22 is observed to disrupt the native conformation (Fig 4-7). It is not clear why G22 does not tolerate $^{\text{LNA}}\text{G}$ modification, as modification to other central-tetrad *anti*-position guanines G4 and G10 does not disturb the (3+1) conformation. Additionally, a single instance of *syn*-position substitution to residue G3 unexpectedly resulted in an undisrupted (3+1) conformation (Fig 4-7). The NMR spectra of this sequence contains sharp peaks at 9.7 ppm and 13.4 ppm not visible in the (3+1) native sequence suggesting enhanced external base pairing (Fig 4-7A). However, this *syn*-substitution comes at a cost of reduced thermal stability (Fig. 4-6). The exceptions to the generalized glycosidic-dependence of $^{\text{LNA}}\text{G}$ substitutions observed in this work are important considerations when incorporating these modifications into G-quadruplex nucleic acids.

4.2.3 LNA modification induces local backbone rearrangement: NMR solution structure of a (3+1) G-quadruplex containing a single ^{LNA}G modification

The experiments and results in this section are largely the work of collaborators Li, Z., Adrian, M., and Heddi, B. A summary of these results in relation to the over-all goal of our LNA studies is presented.

The NMR solution structure of one of the (3+1) G-quadruplex-forming HT-series sequences was solved in order to investigate the effects of a single ^{LNA}G modification on G-quadruplex structure in atomic detail. We choose to study the sequence containing an ^{LNA}G modification to G4, termed “HT-L4”, as this sequence was particularly stabilized upon ^{LNA}G-modification and demonstrated a well resolved ¹H NMR spectra.

We performed unambiguous assignments of aromatic H8 protons for seven of the guanines in HT-L4 by site-specific deuterium labeling. The remaining H8 and H1 protons were assigned by sequential connectivity of the H8-H1' region in NOESY experiments and through-bond correlation of H1-H8 protons in ¹H-¹³C HMBC experiments. NOEs between H1 imino and H8 protons in H₂O NOESY experiments are used to determine the alignment of guanines within G-tetrads. These series of NMR experiments indicate that HT-L4 adopts the same (3+1) G-quadruplex conformation as the unmodified sequence (Fig 4-1B). This folding topology is in agreement with a CD spectrum that is characteristic of a (3+1) conformation.

A collection of NMR restraints including NOE-derived distance restraints were used to compute the NMR solution structure of the (3+1) G-quadruplex formed by the HT-L4 sequence (Fig 4-8A). Similar to the native sequence, base pairing is observed between T1•A20 and T13•A24, with these bases stacking on external tetrads (Fig. 4-8B). The most pronounced difference between the (3+1) native and HT-L4 structures is a rearrangement

of the sugar and phosphate-backbone geometry at the ^{LNA}G4-to-G5 di-nucleotide step (Fig 4-8C and Fig 4-9). The sugar pucker of ^{LNA}G4 adopts a C3'-endo conformation as expected for a LNA sugar moiety. The χ and δ angles of ^{LNA}G4 vary notably from G4 of the native structure. The χ angle of ^{LNA}G4 adopts a value of $\sim -159^\circ$ compared to $\sim -124^\circ$ in the native structure, while a value of $\sim 67^\circ$ is observed for δ compared to $\sim 123^\circ$ in the native G-quadruplex. Both the χ and δ dihedral angles of G4 are well converged in our structure (Fig. 4-9). Additionally, the relative χ values for ^{LNA}G and ^{DNA}G are in good agreement with values predicted by quantum chemical computations (125). The LNA-induced changes to sugar pucker as well as χ and δ dihedral angles cause a projection of the backbone that is notably different than the native structure (Fig 4-8C). Despite this different orientation, the α , β , γ and δ dihedral angles in ^{LNA}G4-to-G5 step were largely unaffected, with the α/γ dihedrals adopting a similar g^-/g^+ orientation as observed in the native structure (Fig 4-9).

As sugar pucker is related to backbone conformation, it is not surprising that reorientation

Figure 4-8. NMR Solution structure of ^{LNA}G4-modified HT-series sequence: (A) Alignment of the 10 lowest energy structures. (B) Representative ribbon structure highlighting the (3+1) conformation and A•T base pairing on the 3' and 5'-ends. The sugar bridge of ^{LNA}G4 is shown in black. Guanine bases are colored by *anti* (cyan) and *syn* (magenta) glycosidic conformation. Adenines (green) and thymines (orange) are colored by type. Atoms are colored as such: Backbone and sugar (grey); O4' (yellow); and P (red). (C) Local structural deviation in the sugar-backbone of the ^{LNA}G4 modified sequence (grey) compared to the unmodified HT-series structure (red).

Figure 4-9. Dihedral angle comparison of the (3+1) native structure and the ^{LNA}G4-modified (3+1) G-quadruplex forming HT-L4 sequence: Plots of standard backbone angles (α , β , γ , δ , ϵ , ζ), glycosidic angle (χ) and Pseudo-rotation angle of the sugar (P) for the G3→G5 residues in the (3+1) native (red x) and HT-L4 (black o) sequences.

occurs around the modified residue as previously reported (126,127). Similar values of χ and δ dihedral angles have been observed upon ^{LNA}G modification into a tetramer G-quadruplex (126). Our NMR studies of the HT-L4 sequence reveal that LNA incorporation can induce local re-arrangement of the sugar-phosphate backbone, especially downstream in the linkage to the 3'-neighboring residue. These observations provide a structural explanation for the observed intolerance of ^{LNA}G modification upstream to short-propeller loops in G-quadruplex DNA as such rearrangement may be incompatible with the fold-back geometry required of a short-propeller loop.

4.3 Conclusions:

Through systematic single-position LNA modifications on well-defined intramolecular (4+0) and (3+1) G-quadruplex scaffolds, we discover that modification of ^{LNA}G directly upstream of short propeller loops is highly disruptive to G-quadruplex formation. The NMR solution structure of a (3+1) G-quadruplex containing a single LNA modification is solved, revealing local backbone rearrangement downstream of the ^{LNA}G modification and providing a structural explanation for the severe destabilization observed when ^{LNA}G substitution precedes short-propeller loops in our studies. ^{LNA}G substitutions are generally tolerated in a manner dependent on the *syn* or *anti* glycosidic conformation of the guanine base. Substitution of ^{LNA}G into most *anti* positions leads to more stable G-quadruplex, while substitution into most *syn* positions generally disrupts the native (3+1) G-quadruplex conformation. However, we indentify some noteworthy exceptions to these generalizations in the course of our study. The insight gained from this work will be valuable to the future design of LNA-modified G-quadruplexes for pharmaceutical and engineering applications.

Chapter 5:

Engineering the Sugar Backbone: 2'-F-Guanosine and 2'-F-ANA-Guanosine Modifications

This chapter was largely adapted from the authors works: Li, Z.* , Lech, C.J.*, Phan, A.T. (2014) Sugar-modified G-quadruplexes: effects of LNA-, 2'-F-RNA and 2'-F-ANA-guanosine chemistries on G-quadruplex structure and stability. *Nucleic Acids Research*, [in press] (* Denotes co-first authorship) and Lech, C.J.*, Zhe, L.,* Heddi, B., Phan, A.T. (2012) 2'-F-ANA-guanosine and 2'-F-guanosine as powerful tools for structural manipulation of G-quadruplexes. *Chemical Communications*. 48. 11425-11427. (* Denotes co-first authorship)

Author Contributions: DNA synthesis, purification and thermal denaturing experiments were carried out by L.Z.; Data analysis of thermal denaturing experiments was performed by C.J.L.; 1D NMR experiments were performed by C.J.L. and L.Z.; All authors contributed to the conceptualization and planning of experiments as well as preparation of manuscripts. We would like to thank Mee Chea Chin for her participation in the early stages of this work

5.1 Introduction:

The incorporation of alternative nucleic acid chemistries can enhance the pharmacological properties of G-quadruplex-based drugs. For example, past studies have investigated the effects of introducing chemically modified base and sugar-backbone chemistries into the thrombin binding aptamer (82), known for its anti-coagulant properties. The use of modified chemistries in the thrombin binding aptamer have lead to higher stability (106,128-131,231,235), increased binding affinity (106,128), and enhanced biological activity (122,128,231,236,237), including studies *in vivo*. In a similar manner, studies involving modified nucleic acid chemistries have enhanced the properties of anti-HIV aptamers (106,123,230,238-241).

Figure 5-1. Modified nucleosides and G-quadruplex scaffolds: **(A)** Sugar chemistry of DNA, 2'-F-ribose, and 2'-F-ANA-ribose. **(B)** Hybrid (3+1) G-quadruplex, formed by the “HT-series” sequence d[T₂(G₃T₂A)₃G₃A]. **(C)** Parallel (4+0) G-quadruplex, formed by the “PS-series” sequence d[T₂(G₃T)₄]. Guanine bases are colored according to their *syn* (magenta) or *anti* (cyan) glycosidic conformations.

Two promising alternative nucleosides are 2'-deoxy-2'-fluoro-riboguanosine (^FG) and 2'-deoxy-2'-fluoro-arabinoguanosine (^{FANA}G) which contain a fluorine atom substitution at the C2' position of the sugar (Fig. 5-1). These chemistries have shown promise for increasing the stability and anti-sense potency of duplexes formed by short interference RNAs (242,243). Past studies have observed that 2'-deoxy-2'-fluoro-ribo- (2'-F) and 2'-deoxy-2'-fluoro-arabino- (2'-F-ANA) nucleosides can increase the resistance of modified oligonucleotides to degradation by nuclease (103,105,106). In the context of G-quadruplex, modification with 2'-F-ANA nucleosides has also allowed for enhanced G-quadruplex aptamer stability and nuclease resistance (106). Considering these beneficial properties, 2'-F and 2'-F-ANA modified nucleosides are attractive tools for use in the design and optimization of G-quadruplex based aptamers.

The goal of this study is to understand how to effectively incorporate ^FG and ^{FANA}G into G-quadruplex DNA by examining the effects of modifications on G-quadruplex

conformation and thermal stability. We characterized the effects of single-position substitutions of ^FG and ^{FANA}G into two G-quadruplex scaffolds. 1) The first is the intramolecular (3+1) hybrid G-quadruplex formed by the *human telomeric* sequence d[T₂(G₃T₂A)₃G₃A] of the “HT-series” (28). This structure contains three strands oriented in one direction with one strand running in the opposite direction (Fig 5-1B). This strand arrangement is connected by edgewise and propeller loops three nucleotides (3-nt) in length. Furthermore, guanine bases within the G-tetrad core adopt a mixture of *syn* and *anti* glycosidic conformations. 2) The second scaffold is an intramolecular parallel-stranded (4+0) G-quadruplex formed by the “PS-series” sequence d[T₂(G₃T)₄] (182). This structure contains all strands pointed in the same direction connected by short 1-nt loops, with all guanines adopting an *anti* conformation. These two G-quadruplex scaffolds were chosen for study as they are structurally distinct, well characterized, and demonstrate a well-defined major conformation. We evaluate the G-quadruplex conformation formed by modified sequences along with their thermal stability using a combination of nuclear magnetic resonance (NMR), ultraviolet (UV) absorption and circular dichroism (CD) spectroscopic methods (Appendix II – Protocols – Chapter 5). By testing the effects of ^FG and ^{FANA}G substitutions into a variety of G-quadruplex structural environments, we aim to broadly investigate how to effectively incorporate these modified nucleosides into G-quadruplex structure. Such knowledge will enable the rational design of engineered G-quadruplexes containing ^FG and ^{FANA}G chemistries.

5.2 Results and Discussion:

5.2.1: 2'-F-guanosine and 2'-F-ANA-guanosine substitutions destabilize *syn* positions and are universally tolerated in *anti* positions

Substitutions of ^FG and $^{\text{FANA}}\text{G}$ were made in a systematic manner into the (3+1) G-quadruplex scaffold of the HT-series (Table 5-1). The modifications of guanines that adopt a *syn* conformation in the native (3+1) G-quadruplex yielded sequences that demonstrated multiple sets of imino proton peaks in NMR spectra (Fig 5-2). Additionally, these sequences generally demonstrated notable changes in CD spectra when compared with the unmodified sequence, with a decrease in signal at 295 nm and an increase at 260 nm being observed for most sequences (Fig 5-2). Collectively, this data suggests that ^FG or $^{\text{FANA}}\text{G}$ modifications perturb the particular folding topology of the G-quadruplex when substituted into *syn*-position guanines.

Alternatively, ^FG and $^{\text{FANA}}\text{G}$ substitutions were well tolerated when substituted into *anti*

Figure 5-2. Effect of glycosidic conformation on ^FG and $^{\text{FANA}}\text{G}$ substitutions into the (3+1) G-quadruplex: (A) Illustrative NMR spectra of the “(3+1) native” sequence and sequences containing $^F\text{G}/^{\text{FANA}}\text{G}$ substitutions to *syn* and *anti* positions. CD spectra are shown for $^F\text{G}/^{\text{FANA}}\text{G}$ substitutions to (B) *syn* guanine 9 and (C) *anti* guanine 11.

Table 5-1. DNA Sequences and Melting Temperature Data

Name ^a	Sequence (5'→3') ^b	(°C)	
		T _m ^c	ΔT _m
(3+1) native	TT GGG TTA GGG TTA GGG TTA GGG A	50.4 ± 0.2	-
HT-F-3	TT F GG TTA GGG TTA GGG TTA GGG A	-	-
HT-F-4	TT G FG TTA GGG TTA GGG TTA GGG A	50.6 ± 0.4	0.2
HT-F-5	TT G G F TTA GGG TTA GGG TTA GGG A	50.2 ± 0.3	-0.2
HT-F-9	TT GGG TTA F GG TTA GGG TTA GGG A	-	-
HT-F-10	TT GGG TTA G FG TTA GGG TTA GGG A	47.9 ± 1.3	-2.5
HT-F-11	TT GGG TTA G G F TTA GGG TTA GGG A	48.8 ± 0.8	-1.5
HT-F-15	TT GGG TTA GGG TTA F GG TTA GGG A	-	-
HT-F-16	TT GGG TTA GGG TTA G FG TTA GGG A	-	-
HT-F-17	TT GGG TTA GGG TTA G G F TTA GGG A	47.7 ± 0.5	-2.6
HT-F-21	TT GGG TTA GGG TTA GGG TTA F GG A	-	-
HT-F-22	TT GGG TTA GGG TTA GGG TTA G FG A	48.8 ± 0.8	-1.6
HT-F-23	TT GGG TTA GGG TTA GGG TTA G G F A	48.5 ± 0.1	-1.8
HT-FANA-3	TT F GG TTA GGG TTA GGG TTA GGG A	-	-
HT-FANA-4	TT G F G TTA GGG TTA GGG TTA GGG A	53.4 ± 0.3	3.1
HT-FANA-5	TT G G F TTA GGG TTA GGG TTA GGG A	50.8 ± 0.6	0.5
HT-FANA-9	TT GGG TTA F GG TTA GGG TTA GGG A	-	-
HT-FANA-10	TT GGG TTA G F G TTA GGG TTA GGG A	53.5 ± 0.7	3.1
HT-FANA-11	TT GGG TTA G G F TTA GGG TTA GGG A	51.8 ± 0.5	1.5
HT-FANA-15	TT GGG TTA GGG TTA F GG TTA GGG A	-	-
HT-FANA-16	TT GGG TTA GGG TTA G F G TTA GGG A	-	-
HT-FANA-17	TT GGG TTA GGG TTA G G F TTA GGG A	52.8 ± 0.1	2.4
HT-FANA-21	TT GGG TTA GGG TTA GGG TTA F GG A	-	-
HT-FANA-22	TT GGG TTA GGG TTA GGG TTA G F G A	52.5 ± 0.7	2.2
HT-FANA-23	TT GGG TTA GGG TTA GGG TTA G G F A	50.9 ± 0.5	0.5
(4+0) native	TT GGG T GGG T GGG T GGG T	74.9 ± 0.8	-
PS-F-3	TT F GG T GGG T GGG T GGG T	75.6 ± 0.1	0.7
PS-F-4	TT G FG T GGG T GGG T GGG T	75.8 ± 0.0	0.8
PS-F-5	TT G G F T GGG T GGG T GGG T	74.2 ± 0.1	-0.7
PS-F-8	TT GGG T G FG T GGG T GGG T	75.7 ± 0.1	0.7
PS-F-11	TT GGG T GGG T F GG T GGG T	76.0 ± 0.2	1.1
PS-F-12	TT GGG T GGG T G F G T GGG T	74.3 ± 0.1	-0.6
PS-F-13	TT GGG T GGG T G G F T GGG T	75.4 ± 0.3	0.5
PS-F-17	TT GGG T GGG T GGG T G G F T	74.0 ± 0.0	-1.0
PS-FANA-3	TT F GG T GGG T GGG T GGG T	75.9 ± 0.2	1.0
PS-FANA-4	TT G F G T GGG T GGG T GGG T	78.5 ± 0.0	3.6
PS-FANA-5	TT G G F T GGG T GGG T GGG T	75.9 ± 0.3	1.0
PS-FANA-8	TT GGG T G F G T GGG T GGG T	78.5 ± 0.0	3.6
PS-FANA-11	TT GGG T GGG T F GG T GGG T	75.9 ± 0.2	1.0
PS-FANA-12	TT GGG T GGG T G F G T GGG T	78.0 ± 0.0	3.1
PS-FANA-13	TT GGG T GGG T G G F T GGG T	75.4 ± 0.3	0.5
PS-FANA-17	TT GGG T GGG T GGG T G G F T	75.1 ± 0.2	0.2

[a] "HT-" series denotes a modified (3+1) G-quadruplex forming sequence while the "PS-" series denotes a modified (4+0) G-quadruplex forming sequence.

[b] Residues with modified nucleosides are denoted as such: 2'-F-guanosine (**F**) and 2'-F-ANA-guanosine (**F**).

[c] Salt conditions were (5 mM KCl and 5 mM KPi) for the HT-series and (1 mM KPi) for the PS-series.

position guanines within the (3+1) G-quadruplex. Sequences containing modifications to *anti*-position guanines were observed to form a single conformation in NMR spectra with chemical shift patterns that were highly similar to that of the unmodified sequence. CD

spectra of these sequences were also similar to that of the unmodified sequence, suggesting that modified sequences maintain the same (3+1) G-quadruplex conformation upon substitution of ^FG or $^{\text{FANA}}\text{G}$ (Fig 5-2). Additionally, substitutions were made into the (4+0) G-quadruplex which contains all guanines in an *anti* conformation. Within the (4+0) G-quadruplex, substitutions were made into 8 select positions to include a variety of structural environments including G3-G5, G8, G11-G13, and G17. All of the ^FG and $^{\text{FANA}}\text{G}$ modifications into the (4+0) G-quadruplex demonstrated highly similar NMR and CD spectra compared to the unmodified sequence with no sign of disruption to the major folding topology adopted (Fig 5-3). Collectively, our results from ^FG and $^{\text{FANA}}\text{G}$ substitutions into the (3+1) and (4+0) G-quadruplex scaffolds suggest that these chemistries are well-suited for substitution into *anti*-conformation guanines in variety of structural environment.

This study reports that modification of ^FG or $^{\text{FANA}}\text{G}$ to *syn*-position guanines broadly pert-

Figure 5-3. Incorporation of ^FG and $^{\text{FANA}}\text{G}$ substitutions into the (4+0) G-quadruplex scaffold: (A) NMR and (B) CD spectra of select PS-series samples containing single ^FG and $^{\text{FANA}}\text{G}$ substitutions to guanines located in a variety of structural environments. ^FG and $^{\text{FANA}}\text{G}$ are well tolerated in all positions of the PS-series.

urbs the folding topology of the (3+1) G-quadruplex. Previous studies have shown that modifications of ^{FANA}G to multiple *syn*-positions within a G-quadruplex scaffold can drive conformational changes (106). Others have expanded upon this to shown that rationally-placed ^{FANA}G modifications can alter structural equilibrium (244). In the current work, we demonstrate that a single substitution of either ^FG or ^{FANA}G into any of the *syn*-positions tested is powerful enough to induce a mixture of conformers and perturb the native (3+1) G-quadruplex folding topology. These findings suggest the general *syn*-destabilization properties of ^FG or ^{FANA}G modified nucleosides.

In contrast, *anti*-position substitutions of ^FG and ^{FANA}G chemistries are tolerated in both the (3+1) and (4+0) G-quadruplex scaffold without disrupting the folding topology. The tolerance of these chemistries in all *anti* positions is interesting considering the wide range of structural environments tested. ^FG and ^{FANA}G chemistries were successfully substituted with guanines within the central tetrad layer, adjacent to short 1-nt propeller loops, and adjacent to 3-nt edgewise and propeller loops without disrupting the native G-quadruplex conformation. Our work here suggests that, as a general rule, the ^FG and ^{FANA}G chemistries can be universally incorporated into *anti*-position guanines in G-quadruplex DNA.

5.2.2: 2'-F-ANA-guanosine substitutions stabilize G-quadruplex DNA while 2'-F-guanosine substitutions induce mixed effects on stability

The effect of single-position ^FG and ^{FANA}G substitutions on the melting temperature (T_m) of G-quadruplexes was determined through thermal denaturing experiments employing UV absorbance spectroscopy (Fig 5-4 and Table 5-1). The T_m values of select sequences

were also confirmed using CD spectroscopy. Samples from the HT-series were analyzed in 5 mM KCl and 5 mM KPi salt conditions while samples from the PS-series were analyzed in 1 mM KPi. Sequences containing single-position substitutions into *anti*-position guanines were analyzed. These sequences were chosen for analysis as they adopt the same conformation as their parent sequences, a necessary prerequisite to a meaningful quantitative comparison of thermal stability.

^{FANA}G substitutions were observed to moderately stabilize the (3+1) G-quadruplex scaffold with ΔT_m values in the range of +0.5°C to +3.1°C (Fig. 5-4A). Within the (4+0) G-quadruplex scaffold, ^{FANA}G substitutions were also stabilizing with ΔT_m values in the range of +0.2°C to +3.6°C (Fig. 5-4B). Substitutions of ^{FANA}G into central tetrad layers in both scaffolds were observed to be particularly stabilizing, with ΔT_m values of +2.2°C to +3.1°C observed in the HT-series and values of +3.1°C to +3.6°C observed for the PS-series. On the contrary, sequences containing ^FG substitutions were generally mildly dest-

Figure 5-4. Change in the melting temperature for single position ^FG (blue) and ^{FANA}G (red) substitutions to (A) *anti* positions in the (3+1) G-quadruplex of the HT-series and (B) the (4+0) G-quadruplex of the PS-series. Error bars indicate the hysteresis between heating and cooling melting curves.

abilized in the HT-series with ΔT_m values between -2.6°C and $+0.2^\circ\text{C}$. ^FG substitutions were slightly more favorable in the PS-series with ΔT_m values between -0.7°C and $+1.1^\circ\text{C}$. No clear tetrad-layer preference is observed for ^FG substitutions.

The results of our thermal stability studies indicate that $^{\text{FANA}}\text{G}$ modifications are moderately stabilizing. ^FG substitutions are observed to be tolerated within the two scaffolds, generally destabilizing the (3+1) G-quadruplex by $1\text{-}2^\circ\text{C}$ while having minor and mixed effects on the stability of the (4+0) G-quadruplex. In all *anti* positions tested over both scaffolds, $^{\text{FANA}}\text{G}$ is observed to be of equal or greater effectiveness in stabilizing G-quadruplex structure compared to its ^FG counterpart. This observation is in agreement with recent work describing $^{\text{FANA}}\text{G}$ to be a more powerful substituent than ^FG for driving structural equilibrium (244). Interestingly, substitutions of $^{\text{FANA}}\text{G}$ to guanosines in the central G-tetrad of the 3-layered (3+1) and (4+0) G-quadruplexes were found to be most stabilizing compared to other positions (Fig 5-4).

5.2.3: ^FG and $^{\text{FANA}}\text{G}$ are powerful tools for structural manipulation of G-quadruplex

Alternative nucleic acids can be used to manipulate G-quadruplex structure. As the conformation of the guanine base about the glycosidic bond is intrinsically related to the folding topology of G-quadruplexes (67), one approach is to use modified nucleotides that favour a *syn* or *anti* guanine base conformation (25,125,129,229). For example, substitutions of a guanine with C8-modified guanine bases, such as 8-bromoguanine (25,32), is one technique for manipulating structure, as these modifications favor substitution into *syn* positions (113). Considering this, we sought to explore the ability of ^FG and $^{\text{FANA}}\text{G}$ to manipulate folds adopted by G-quadruplex forming sequences.

Figure 5-5: (A) Fig. 1 (A) Sugar schematic of DNA, 2'-F-ANA, and 2'-F chemistries. (B) Sequences explored in this work. "F" denotes a ^{FANA}G or ^FG modification. The HT1-syn and HT2-syn nomenclature signifies substitution to all guanines that adopt syn conformations in the HT1 or HT2 sequences. (C,D) The major form (3+1) hybrid G-quadruplex folding topologies of the (C) HT1 and (D) HT2 sequences. Syn and anti guanine glycosidic conformations are shown in magenta and cyan respectively.

First, We examine the effectiveness of ^{FANA}G and ^FG in decreasing structural polymorphism when substituted within sequences that adopt multiple G-quadruplex forms in solution. Two G-quadruplex forming human telomeric sequences are examined, both of which demonstrate a (3+1) hybrid folding topology along with minor conformations (27,28). The sequence d[TA(G₃T₂A)G₃], termed HT1, exists predominantly in Form 1, (28) (Fig 5-5C) while d[TA(G₃T₂A)G₃T₂], termed HT2, has a major fold of Form 2 (27) (Fig 5-5D). The folding topology of G-quadruplexes is very sensitive to sequence changes, even amongst similar sequences (67,232). Although the HT1 and HT2 sequences differ only by the presence of two extra thymine bases at the 3' end of HT2, they adopt different major folding topologies. While both sequences form a (3+1) hybrid folding topology, the two structures are uniquely different. The loop arrangement of Form 1 (Fig 5-5C) (propeller → edge-wise → edge-wise) is quite

different from that of Form 2 (Fig 5-5D) (edge-wise → edge-wise → propeller), resulting in different glycosidic conformations of guanine bases G10 and G16 (Fig 5-5B).

We use rational substitutions of a single $^{\text{FANA}}\text{G}$ or $^{\text{F}}\text{G}$ chemistry in order to further favor the major conformation in HT1 and HT2 as our studies above indicate their *anti*-favoring capabilities. To accomplish this, we substitute the *anti*-favoring $^{\text{FANA}}\text{G}$ and $^{\text{F}}\text{G}$ nucleotides into *anti*-position guanines in one form that adopt a *syn* conformation in the other form. As positions 10 and 16 adopt different glycosidic conformations in the hybrid (3+1) Form 1 and Form 2, substitutions were made to HT1 in the position that adopts *anti* in Form 1 and *syn* in Form 2, and vice versa (Fig 5-5B). It was hypothesized that a substitution at these positions should stabilize the desired form while potentially destabilizing undesired forms.

The effects of $^{\text{FANA}}\text{G}$ substitutions on G-quadruplex folding topology and polymorphism were examined by CD and ^1H NMR spectroscopy (Fig 5-6). As predicted, imino proton spectra reveal the reduction of minor conformations, favoring a single conformer. The high similarity between the major form in unmodified and modified NMR spectra strong-

Figure 5-6: Decreasing polymorphism: (A,B) NMR imino proton and (C,D) CD spectra of (A,C) Form 1 and (B,D) Form 2 unmodified and $^{\text{FANA}}\text{G}$ modified sequences. Proton peaks of the major form are indicated by asterisks (*). Arrows highlight the reduction of minor conformations in modified sequences.

Table 5-2. Thermal stability analysis

Sequence name	T _m (°C)	ΔT _m (°C)
HT1	61.1	-
HT1-10 FANA	64.6	+ 3.4
HT1- <i>syn</i> FANA	71.4	-
HT2	58.7	-
HT2-16 FANA	62.0	+3.3
HT2- <i>syn</i> FANA	69.9	-

ly suggests the same major fold is adopted (Fig 5-6). CD spectra also support that (3+1) hybrid folding topologies are adopted. UV melting experiments were performed to analyze the thermal stability of modified sequences (Table 5-2). Data indicate that modified sequences containing a single ^{FANA}G substitution maintain stable G-quadruplex structures upon modification. The effects of modifications by ^FG on NMR imino proton spectra were also examined (Fig 5-7). ^FG is observed to be less effective at decreasing the minor conformations present in HT1 and HT2 compared to ^{FANA}G. Collectively, these results indicate that a single ^{FANA}G substitution is powerful enough to favor a single conformation from polymorphic sequences. The approach presented here has general application towards facilitating the structural studies of polymorphic G-quadruplexes sequences (67,121).

In addition to ^{FANA}G being a powerful tool in decreasing polymorphism, 2'-F-modified nucleotides such as ^{FANA}G and ^FG have the added potential to be used as ¹⁹F NMR struct-

Figure 5-7: Decreasing polymorphism: NMR imino proton (A) Form 1 and (B) Form 2 unmodified and ^FG modified sequences. Proton peaks of the major form are indicated by asterisks (*). Arrows highlight the presence of minor conformations in unmodified sequences.

Figure 5-8: Conformational switching: (A,B) NMR imino proton and (C,D) CD spectra of (A,C) Form 1 and (B,D) Form 2 unmodified and ^{FANA}G *syn*-modified sequences. Proton peaks of the major form are indicated by asterisks (*) in the unmodified sequences and by (°) in the *syn*-modified sequences.

ural probes. Rational substitution allows for ¹⁹F NMR capabilities without causing large changes in G-quadruplex structure. Such ¹⁹F NMR probes can be used to understand the number of conformations present in solution due to the simplicity of ¹⁹F spectra. Similarly, ¹⁹F NMR could be used as a probe to study the interaction of G-quadruplexes with protein or small molecules.

Secondly, we examine the ability of ^{FANA}G and ^FG substitutions to drive conformational switching of G-quadruplex folding topology. We attempt to push structural equilibrium from the native (3+1) hybrid folding of HT1 and HT2 towards a parallel-stranded conformation by rational ^{FANA}G substitutions to *syn*-position guanines in the major folds of these sequences. NMR imino proton spectra show 12 imino proton peaks indicating that a single conformation is formed in modified sequences (Fig. 5-8). CD spectra with a positive peak at 260 nm and not at 290 nm suggest a parallel folding topology as previously reported (121). Thermal stability analysis reveals that the structures adopted by modified sequences are highly stable (Table 5-2). Modifications with ^FG to *syn* positions in a similar manner also demonstrate the capacity to drive structural switching (Fig. 5-9). However, NMR imino proton spectra of ^{FANA}G *syn*-position modified sequenc-

Figure 5-9: Conformational switching: NMR imino proton spectra of (A) Form 1 and (B) Form 2 unmodified and $^{\text{F}}\text{G}$ *syn*-modified sequences. Proton peaks of the major form are indicated by asterisks (*) in the unmodified sequences.

es are observed to be marginally cleaner than their $^{\text{F}}\text{G}$ counterpart. The different orientation of the 2'-fluorine atom in $^{\text{FANA}}\text{G}$ and $^{\text{F}}\text{G}$ appears to influence the effectiveness of these tools. This may be due to a difference in the preferred sugar pucker of these two nucleotides or differences in the favorable intra- and inter-nucleotide fluorine-hydrogen interactions such as those previously observed in duplex DNA (245). Collectively, our data show that modifications by $^{\text{FANA}}\text{G}$ or $^{\text{F}}\text{G}$ can drive a single parallel conformation from the polymorphic HT1 and HT2 human telomeric sequences.

While the first crystallographic structure of a human telomeric G-quadruplex revealed a parallel folding topology (21), obtaining a NMR solution structure has been a difficult undertaking as such a form has only been observed in crowded solution (246). The ability of $^{\text{FANA}}\text{G}$ and $^{\text{F}}\text{G}$ modifications to drive a single parallel conformation of human telomeric DNA in solution speaks of their effectiveness as tools for controlling the fold of G-quadruplexes. Conformational switching using modified nucleic acid chemistries has been explored in the past. Studies have driven G-quadruplex conformational changes using *syn*-favoring 8-bromoguanine substitutions (25,32). C8-modified nucleotides are powerful, but cannot be used to favor parallel structures or other folding topologies that lack *syn* positions. To complement this tool, the development of *anti*-favoring tools is crucial. A study by Tang and Shafer used substitutions of ribonucleotides to drive

conformational switching in G-quadruplexes (129). The anti-favoring locked nucleic acid, or LNA, has also been used to drive conformational changes in G-quadruplex DNA (125,229). Being easier to handle than ribonucleotides, less structurally rigid than LNA, and a useful ^{19}F NMR probe, $^{\text{FANA}}\text{G}$ and $^{\text{F}}\text{G}$ represent powerful new tools in the toolbox of *anti-favoring* nucleotides.

5.3 Conclusions:

In summary, this work examines the effects of systematic single-substitutions of ^FG and ^{FANA}G nucleosides into a (3+1) hybrid and a (4+0) parallel G-quadruplex scaffold. We demonstrate the versatility of 2'-F-ANA-guanosine and 2'-F-guanosine incorporation into G-quadruplex DNA. A single modification of ^FG or ^{FANA}G to a *syn* guanine in the (3+1) hybrid G-quadruplex perturbs this conformation. Alternatively, substitutions into all *anti*-positions are well tolerated and do not disrupt the conformation of the (3+1) or the (4+0) G-quadruplexes, suggesting these nucleosides are universally well-suited for substitution into *anti*-conformation guanine within the G-tetrad core of G-quadruplexes. ^{FANA}G is observed to be better tolerated than ^FG in both scaffolds, with ^{FANA}G substitutions into central G-tetrad guanines being particularly stabilizing. Modification by 2'-F-ANA-guanosine is demonstrated to be a powerful tool in driving a single conformation of G-quadruplex DNA from a mixture of conformers. Such modifications along with 2'-F-guanosine can be used to convert the folding topology of G-quadruplexes. These 2'-F-modified nucleotides represent new tools in the study, design, and manipulation of G-quadruplex structure.

Chapter 6:

Engineering the Base: C8-modified Guanines

This chapter was largely adapted from the authors work: Lech, C.J., Lim, J.K.C., Lim, J.M.W., Amrane, S., Heddi, B., Phan, A.T. (2011) Effects of Site-Specific Guanine C8-Modifications on an intramolecular DNA G-quadruplex. *Biophysical Journal*. 101. 1987-1996.

Author Contributions: DNA synthesis, purification and thermal denaturing experiments were carried out by S.A., J.K.C.L., J.M.W.L. and C.J.L.; Data analysis of thermal denaturing experiments was performed by C.J.L., S.A., J.K.C.L. and J.M.W.L.; Circular Dichroism studies were performed by C.J.L., S.A., J.K.C.L. and J.M.W.L.; 1D NMR experiments including N15 filtered experiments were performed by S.A., J.K.C.L. and J.M.W.L. and C.J.L.; QM Computations and data analysis were performed by C.J.L., B.H. and A.T.P.; The above mentioned manuscript was written by C.J.L., B.H. and A.T.P; All authors contributed to the conceptualization and planning of experiments.

6.1 Introduction:

The preferred folding topology of a G-quadruplex forming nucleic acid sequence can be altered by introducing a guanine-like aromatic base within a G-tetrad. For example, guanine derivatives such as inosine (121,247,248) and 8-bromo-guanine (113,249) have been used as tools in structural studies to favor a single G-quadruplex conformation from a sequence that forms multiple conformations. The guanine derivative 8-bromo-guanine belongs to a specific class of modified bases involving substitutions of the C8-hydrogen with other modification groups (Fig. 6-1). C8-modifications can be made without disrupting hydrogen bonding within the G-tetrad. This strategy was successfully used to incorporate a fluorescent probe within a G-quadruplex structure (250). In contrast to base modifications that disrupt hydrogen bonding and destabilize G-quadruplexes (111,112), C8-modified derivatives 8-bromo-guanine (113,249) and 8-methyl-guanine (118) have shown a tendency to increase the thermal stability of a G-quadruplex when substituted

with guanines that originally adopt *syn* glycosidic conformations in the G-tetrad core. Additionally, C8-modified derivatives have been shown to influence the folding kinetics of G-quadruplex structures (111,116,117).

Past research investigating the effects of C8-modifications on G-quadruplexes has been carried out on both intramolecular (112,113,118,249,250) and tetramer structures (111,114-117). Intramolecular G-quadruplex scaffolds allow for single-position modifications to be implemented and to probe the effects of modifications on *syn/anti* glycosidic conformations. In parallel-stranded tetrameric G-quadruplexes all core guanines originally adopt *anti* conformations. In a tetrameric system, the substitution of a guanine by a C8-modified derivative is reflected about the entire tetrad due to the symmetry of the structure. C8-modified guanine substitutions in a tetrameric system have been reported to cause a change in the polarity of the modified tetrad (114,115,117). Some studies have noted position-dependent effects for the derivatives 8-bromo-guanine (111,114), 8-methyl-guanine (115,117), and 8-amino-guanine (116), including the ability of substitutions at the 5' end to stabilize G-quadruplexes. While studies involving tetrameric G-quadruplexes are valuable, conclusions arising from these studies could be fundamentally different in comparison to those arising from intramolecular scaffolds where only one guanine within a G-tetrad is substituted at a time. With mounting evidence for the *in vivo* roles of G-quadruplexes, the effects of C8-modified derivatives on these structures are of physiological importance considering the numerous naturally occurring C8-modifications with suggested roles in mutagenesis including 8-oxo-guanine (251,252), 8-amino-guanine (253,254), and 8-methyl-guanine (255). From an engineering perspective, investigating the effects of various C8-modified derivatives on G-quadruplexes allows for their effective application in engineered systems to achieved desired properties.

Figure 6-1: (A) Schematic structure of the (3 + 1) G-quadruplex platform used in this study. Anti and syn guanines are shown in light gray and dark gray, respectively. (B) Chemical schematic of a modified guanine tetrad with a centrally located K⁺ ion. The C8-modified guanine derivative is depicted by a modification group R. (C–G) The chemical schematics of guanine and the guanine derivatives explored in this work: (C) guanine, (D) 8-bromo-guanine, (E) 8-O-methyl-guanine, (F) 8-amino-guanine, and (G) 8-oxo-guanine.

In order to understand how site-specific C8-modifications of guanines affect G-quadruplex stability and structure, this work explores the effects of substituting guanine derivatives 8-bromo-guanine (^{Br}G), 8-O-methyl-guanine (^{Omet}G), 8-amino-guanine (^{Am}G), and 8-oxo-guanine (^{Oxo}G) within a well-defined intramolecular (3+1) telomeric G-quadruplex structure (256) (Fig. 6-1) using a combination of UV, CD, NMR spectroscopy and *ab initio* quantum mechanical calculations (Appendix II – Protocols – Chapter 6). The site-specific effects of substitutions to both syn and anti guanines were examined. Folding topology was monitored, allowing for a meaningful thermal denaturing analysis within the context of a single conformer. The observation of an interesting pattern in NMR chemical shift change was reproduced using *ab initio* computational methods, providing insight into the structural effects of these modifications on G-tetrad geometry.

6.2 Results:

6.2.1: Site-specific substitutions of a guanine with an 8-bromo-guanine, 8-O-methyl-guanine, 8-amino-guanine, or 8-oxo-guanine:

In this study, we explored the effects of base modifications on an intramolecular (3+1) G-quadruplex structure (Fig. 6-1A) formed by the human telomeric d[T₂(G₃T₂A)₃G₃A] sequence (256), (henceforth referred to as the unmodified sequence). Individual guanines (G) were site-specifically substituted with four different guanine derivatives (^{Br}G, ^{Omet}G, ^{Am}G, ^{Oxo}G) (Fig. 6-1). Substitutions were made to guanines which adopt a *syn* (residues 3, 9, 15, 16 and 21) or *anti* (residue 22) glycosidic conformations in the original (3+1) G-quadruplex (Fig. 6-1A). Each modified sequence used in this work contained only one or two substitutions (Table 6-1).

Table 6-1: List of DNA Sequences Examined in this work

Sequence Name	Sequence (5'→3')
Unmodified	TT GGG TTA GGG TTA GGG TTA GGG A
Br-3	TT B GG TTA GGG TTA GGG TTA GGG A
Br-9	TT GGG TTA B GG TTA GGG TTA GGG A
Br-15	TT GGG TTA GGG TTA B GG TTA GGG A
Br-16	TT GGG TTA GGG TTA G B G TTA GGG A
Br-21	TT GGG TTA GGG TTA GGG TTA B GG A
Br-22	TT GGG TTA GGG TTA GGG TTA G B G A
Br-9-22	TT GGG TTA B GG TTA GGG TTA G B G A
Omet-3	TT O GG TTA GGG TTA GGG TTA GGG A
Omet-9	TT GGG TTA O GG TTA GGG TTA GGG A
Omet-15	TT GGG TTA GGG TTA O GG TTA GGG A
Omet-16	TT GGG TTA GGG TTA G O G TTA GGG A
Omet-21	TT GGG TTA GGG TTA GGG TTA O GG A
Omet-22	TT GGG TTA GGG TTA GGG TTA G O G A
Br-9-Omet-22	TT GGG TTA B GG TTA GGG TTA G O G A
Am-3	TT M GG TTA GGG TTA GGG TTA GGG A
Am-9	TT GGG TTA M GG TTA GGG TTA GGG A
Am-15	TT GGG TTA GGG TTA M GG TTA GGG A
Am-16	TT GGG TTA GGG TTA G M G TTA GGG A
Am-21	TT GGG TTA GGG TTA GGG TTA M GG A
Am-22	TT GGG TTA GGG TTA GGG TTA G M G A
Br-9-Am-22	TT GGG TTA B GG TTA GGG TTA G M G A
Oxo-3	TT X GG TTA GGG TTA GGG TTA GGG A
Oxo-9	TT GGG TTA X GG TTA GGG TTA GGG A
Oxo-15	TT GGG TTA GGG TTA X GG TTA GGG A

Modified residues are depicted as “**B**” for 8-bromo-guanine, “**O**” for 8-O-methyl-guanine, “**M**” for 8-amino-guanine, and “**X**” for 8-oxo-guanine. Columns of modified positions are shaded.

Circular dichroism (CD) profiles of modified sequences containing ^{Br}G, ^{Omet}G, and ^{Am}G modifications displayed a positive peak at 290 nm and a shoulder at 260 nm, similar to the CD profile of the unmodified sequence (Fig. 6-2), consistent with the formation of a (3+1) G-quadruplex as the major form in these sequences (257).

Imino proton NMR spectra were used to further assess the formation of G-quadruplexes. In all sequences containing ^{Br}G, ^{Omet}G, and ^{Am}G, the observation of roughly twelve major imino protons at 10.5-12.5 ppm indicated the formation of a predominant G-quadruplex conformation (Fig. 6-3A-D). The similar spectral pattern between these peaks and those of the unmodified sequence strongly suggested the formation of the same (3+1) G-quadruplex fold. Note that most changes in chemical shifts were observed only for protons from the G-tetrad containing the modified base.

Figure 6-2: A comparison of CD spectra for sequences containing (A) single substitutions to syn residue 9, (B) single substitutions to anti residue 22, and (C) double substitutions to both syn residue 9 and anti residue 22. The CD spectrum of the unmodified sequence (dotted line) is shown as a reference

Figure 6-3: Imino proton NMR spectra for (A) the unmodified sequence, as well as modified sequences (B) Br-3, (C) Omet-3, (D) Am-3, and (E) Oxo-3 containing a single substitution to syn residue 3. The peaks in the unmodified spectrum are labeled with residue numbers, circled numbers representing guanines within the modified G3•G21•G17•G9 tetrad and the modified residue 3 shaded red. Dotted lines identify the chemical shift of imino protons from the unmodified spectra for guanines located outside of the modified tetrad. Black dots indicate the presence of individual imino proton resonances.

For example, in the spectra of sequences with a single $^{\text{Br}}\text{G}$, $^{\text{Omet}}\text{G}$, or $^{\text{Am}}\text{G}$ substitution at position 3 (Fig. 6-3), most changes were observed only for peaks from the modified G-tetrad G3•G21•G17•G9. This specific pattern in NMR chemical shift change is further investigated below. The NMR spectra of sequences containing $^{\text{Oxo}}\text{G}$ also gave imino proton peaks at 10.5-12.5 ppm (Fig. 6-3E), indicating the formation of G-quadruplexes. However, these spectra were markedly different from that of the unmodified sequence, suggesting $^{\text{Oxo}}\text{G}$ -containing sequences adopt different topologies.

6.2.2: Thermal stability of modified sequences

To characterize and compare the stability of G-quadruplexes formed by different modified sequences, UV melting experiments were performed by monitoring the absorption at 295 nm (258) (Fig. 6-4). Reversible denaturing profiles were recorded for all sequences and

Table 6-2: Melting temperatures (°C) determined by UV absorption at 295 nm

Sequence Name	T _m	ΔT _m	Average ΔT _m ^a
Unmodified	57.0	---	---
Br-3	59.5	2.5	5.2 ± 2.7
Br-9	63.9	6.9	
Br-15 ^b	62.3	5.3	
Br-16	62.2	5.2	
Br-21 ^b	63.2	6.2	
Br-22	55.3	-1.7	---
Br-9-22	60.7	3.7	---
Omet-3	59.6	2.6	3.1 ± 1.4
Omet-9	60.5	3.5	
Omet-15	58.9	1.9	
Omet-16	60.1	3.1	
Omet-21 ^b	61.5	4.5	
Omet-22	59.0	2.0	---
Br-9-Omet-22	64.6	7.6	---
Am-3	57.5	0.5	1.1 ± 1.1
Am-9	58.7	1.7	
Am-15	57.0	0.0	
Am-16	58.4	1.4	
Am-21	59.1	2.1	
Am-22	54.0	-3.0	---
Br-9-Am-22	59.3	2.3	---
Oxo-3	47.2	-9.8	---
Oxo-9	38.7	-18.3	---
Oxo-15	38.5	-18.5	---

[a] Average T_m was calculated considering only *syn* position substitutions for each modification type as indicated in grey shaded regions. The “±” values indicate the maximum deviations about the average.

[b] Samples with a small structural transition at low temperature in their denaturing profile.

their melting temperature (T_m) values are summarized in Table 6-2.

We observed that single substitutions (^{Br}G, ^{Omet}G and ^{Am}G) at *syn* residues resulted in an increase in T_m (except for the ^{Am}G substitution at position 15 which showed no change), while single substitutions at *anti* position 22 showed T_m changes that were small and varying (T_m decreased for ^{Br}G and ^{Am}G and increased for ^{Omet}G) (Table 4-2). Among different *syn* guanines, the stabilization effect of base substitutions was highly position-dependent, with similar trends observed for the three types of substitutions (Fig. 6-4C). Some residues showed low stability enhancement when substituted with C8-modified guanine derivatives such as residues 3 and 15, while others showed relatively high stability enhancement such as residues 9 and 21. When comparing the stability of sequen-

Figure 6-4: (A) The additive effect of multiple substitutions on the T_m can be seen. Melting curves for sequences Br-9 (magenta upward triangle), containing a single *syn* substitution; Br-22 (cyan downward triangle), containing a single *anti* substitution; and Br-9-22 (orange diamonds), containing both *syn* and *anti* substitutions. The curve for the unmodified sequence (grey solid line) serves as a reference. (B) Modification dependence stability ($^{Br}G > ^{Omet}G > ^{Am}G$) was observed for substitutions to *syn* guanines, as illustrated by the melting curves of Br-9 (circles), Omet-9 (squares), and Am-9 (diamonds). The melting curve of the unmodified sequence is shown for reference. (C) Comparison of the change in T_m values for each guanine derivative over substitutions to various *syn* residues. Modifications shown as ^{Br}G (blue), ^{Omet}G (red) and ^{Am}G (green).

nces containing single-position *syn* substitutions, ^{Br}G generally had the highest stabilizing effect, followed by ^{Omet}G and ^{Am}G , respectively (Fig. 6-4B - C). Alternatively, when substituting ^{Oxo}G to *syn* positions 3, 9, and 15 a large destabilizing effect was observed. Due to the large differences in NMR spectra of ^{Oxo}G -containing sequences compared to that of the unmodified sequence, we do not discuss the destabilizing effects of ^{Oxo}G

modifications in terms of the (3+1) G-quadruplex scaffold.

Sequences containing double substitutions showed a melting temperature reflective of the additive contributions of individual single substitution (Table 6-2). For example, the changes in T_m for sequences harboring ^{Br}G substitutions to only *syn* residue 9, to only *anti* residue 22 and to both residues 9 and 22, were +6.9, -1.7 and +3.7°C, respectively (Fig. 6-4A and Table 6-2).

6.2.3: A single guanine substitution in a G-tetrad results in specific patterns of NMR chemical shift changes: Experimental observations

Analysis of the imino proton spectra of various DNA sequences containing a single ^{Br}G , ^{Omet}G , or ^{Am}G substitution at a *syn* guanine revealed specific patterns of chemical shift changes: (i) the most significant chemical shift changes were observed for the modified G-tetrad; (ii) the largest change was observed for the guanine that is hydrogen-bonded to the Hoogsteen edge of the modified base (note that these residues, although being neighbors in the structure, are usually remote from each other in the sequence); (iii) the magnitude and direction of chemical shift changes depended on the type of modification.

Consider a $G_\alpha \cdot G_\beta \cdot G_\gamma \cdot G_\delta$ tetrad, in which G_α is the modified base (Fig. 6-5A), the largest change in imino proton chemical shift would be observed for G_δ , which is hydrogen-bonded to the Hoogsteen edge of G_α . For each sequence with a single ^{Br}G , ^{Omet}G or ^{Am}G substitution at a *syn* guanine, the imino proton of G_δ was unambiguously assigned using site-specific ^{15}N -labeling approach (259) (e.g. Fig. 6-5F). Table 6-3 lists the changes in chemical shifts for sequences containing single substitutions at *syn* guanines. For ^{Br}G substitutions, the G_δ imino proton chemical shift decreased by 0.26-0.39 ppm (Fig. 6-5C

Figure 6-5: (A) Schematic of the modified G16•G22•G4•G10 tetrad is presented with the $G_\alpha \cdot G_\beta \cdot G_\gamma \cdot G_\delta$ naming convention. Special attention is brought to the hydrogen bonds of the $G_\delta \cdot G_\alpha$ interface. (B–E) The NMR imino proton spectrum of (B) the unmodified sequence is presented along with the spectra of (C) Br-16, (D) Omet-16, and (E) Am-16. The chemical shift of the imino proton peak from the G_δ residue, G10, in the unmodified sequence is indicated by the dotted black line. Arrows indicate the positions of the G_δ imino proton peaks in the modified spectra. (F) Example of the unambiguous G10 imino proton assignment for Omet-16 using ^{15}N -labeling.

Table 6-3. Experimentally observed changes in NMR H1 chemical shift of the G_δ imino proton

Modification Type	Change in G _δ H1 Chemical Shift (ppm)				
		Neighboring residue (G _δ) • Modified Residue (G _α)			
	9 • 3	17 • 9	11 • 15	10 • 16	3 • 21
8-BromoG	-0.26	-0.32	-0.28	-0.39	-0.28
8-O-methylG	0.22	0.24	0.40	0.39	0.30
8-AminoG	0.21	0.26	0.26	0.27	0.19

Values indicate the observed change in H1 imino proton chemical shift of the G_δ residue as measured for single-position substitutions to *syn* residues and verified by unambiguous ¹⁵N-labeling experiments. Change in chemical shift is measured with respect to the spectra of the unmodified sequence.

and Table 6-3). Conversely, for ^{Omet}G and ^{Am}G substitutions, the G_δ imino proton peak was downfield-shifted; the chemical shift increased by 0.22-0.40 and 0.19-0.27 ppm, respectively (Fig. 6-5D-E and Table 6-3).

Tentative spectral assignments also suggested a specific pattern of chemical shift changes for the imino proton of the modified guanine G_α, which was in the opposite direction of the G_δ imino proton and in agreement with computational studies. However, as these assignments were not unambiguously confirmed, we will not discuss their patterns here.

6.2.4: A single guanine substitution in a G-tetrad results in specific patterns of NMR chemical shift changes: Computational studies

In order to better understand the chemical shift patterns described above, we attempted to reproduce the experimentally observed patterns of chemical shift change due to base modification using a series of *ab initio* computational studies. Computations were performed at the Hartree-Fock level (260) using a variety of basis sets as described in Appendix II – Protocols – Chapter 6. NMR chemical shift calculations were performed on optimized molecular geometries for modified and unmodified tetrads. The changes in NMR chemical shifts between the unmodified and modified systems are shown in Table 6-4. All calculation protocols employed were able to reproduce the experimentally observed trends in G_δ imino proton chemical shift changes, with a decrease of 0.21-0.31

Table 6-4: Computed change in imino proton (H1) NMR chemical shift of the G₈ residue

Modification Type	Optimization Basis Set	Computed Change in G ₈ H1 Chemical Shift (ppm)				
		NMR Basis Set				
		6-31G(d)	6-31G(d,p)	6-31++G(d,p)	6-311+G(d)	6-311++G(d,p)
8-BromoG	6-31G(d)	-0.289	-0.311	-0.304	-0.300	-0.307
	6-31G(d,p)	-0.289	-0.311	-0.305	-0.300	-0.307
	6-31++G(d,p)	-0.235	-0.249	-0.243	-0.245	-0.242
	6-311++G(d,p)	-0.208	-0.216	-0.208	-0.214	-0.205
8-O-methylG	6-31G(d)	0.398	0.461	0.442	0.347	0.453
	6-31G(d,p)	0.402	0.465	0.447	0.351	0.458
	6-31++G(d,p)	0.398	0.461	0.443	0.350	0.455
	6-311++G(d,p)	0.424	0.489	0.472	0.376	0.484
8-AminoG	6-31G(d)	0.235	0.272	0.261	0.234	0.279
	6-31G(d,p)	0.241	0.279	0.267	0.239	0.285
	6-31++G(d,p)	0.242	0.280	0.269	0.242	0.288
	6-311++G(d,p)	0.248	0.286	0.275	0.247	0.294

Values indicate the computed change in NMR H1 imino proton chemical shift of the G₈ residue. Change in chemical shift is measured with respect to the computed spectra of the unmodified tetrad. NMR chemical shift calculations were performed using multiple basis sets for each method of geometry optimization.

ppm calculated for the ^{Br}G-containing model and an increase of 0.35-0.49 ppm and 0.23-0.29 ppm calculated for the ^{Omet}G- and ^{Am}G-containing models, respectively (Fig. 6-6).

Figure 6-6: Comparison of experimental and computed NMR chemical shift changes. Experimental data are observed chemical-shift changes of the G₈ imino protons upon substitutions to syn residues 3, 9, 15, 16, and 21 by guanine derivatives ^{Br}G, ^{Omet}G, and ^{Am}G. Computational data are the results of different NMR chemical-shift calculations, as shown in Table 6-4. The solid black line represents exact agreement between computational and experimental results. Data points are shown for every combination of experimentally observed and computed changes in chemical shift for each type of guanine derivative.

6.2.5: Structural effects of C8-modifications on G-tetrad geometry: An *ab initio* computational study

As our computational studies correctly reproduced the experimentally observed chemical shift patterns, we used these computational methods to analyze the structural effects of C8-base modifications on G-tetrad geometry. It is generally revealed that the most significant changes in geometry occurred at the $G_\delta \bullet G_\alpha$ and $G_\alpha \bullet G_\beta$ interfaces (refer to Fig. 6-5A). Calculated changes to imino and amino hydrogen-bond geometries at the $G_\delta \bullet G_\alpha$ interface are presented in Table 6-5 and Table 6-6, respectively. Comparison between the changes in G_δ imino proton chemical shifts and the changes in $H_{1\delta} \bullet \bullet \bullet O_{6\alpha}$ bond length relates an increase in imino proton chemical shifts with a decrease in hydrogen-bond lengths, and vice versa. In addition, partial atomic charges were derived from the electrostatic potentials of tetrads with optimized geometries. We were unable to observe a relationship between partial atomic charges and NMR chemical shift changes, as the calculated partial charge highly depended on the basis set used for geometry optimization.

Table 6-5. Computed effects of guanine substitutions on $N1-H \bullet \bullet \bullet O6$ geometry of the tetrad

Modification Type	Optimization Basis Set	$\Delta H1 \bullet \bullet \bullet O6$ (pm)				$\Delta N1 - H1 \bullet \bullet \bullet O6$ Angle (deg)			
		$G_\alpha \bullet G_\beta$	$G_\beta \bullet G_\gamma$	$G_\gamma \bullet G_\delta$	$G_\delta \bullet G_\alpha$	$G_\alpha \bullet G_\beta$	$G_\beta \bullet G_\gamma$	$G_\gamma \bullet G_\delta$	$G_\delta \bullet G_\alpha$
8-BromoG	6-31G(d)	-0.97	0.99	1.57	5.02	-1.12	-0.12	0.00	-1.51
	6-31G(d,p)	-0.96	0.97	1.52	4.92	-1.07	-0.11	0.00	-1.45
	6-31++G(d,p)	-1.05	0.44	0.66	2.96	-0.60	0.00	-0.01	-0.69
	6-311++G(d,p)	-0.50	0.18	0.63	1.80	-0.11	0.08	-0.08	-0.18
8-O-methylG	6-31G(d)	1.08	-1.97	-2.11	-10.14	3.80	0.46	-0.22	3.35
	6-31G(d,p)	1.00	-1.98	-2.11	-10.10	3.77	0.45	-0.22	3.31
	6-31++G(d,p)	1.13	-2.10	-2.11	-10.26	3.89	0.47	-0.21	3.41
	6-311++G(d,p)	0.92	-2.44	-2.23	-11.02	4.07	0.56	-0.18	3.60
8-AminoG	6-31G(d)	-0.15	-1.06	-2.49	-6.25	2.15	0.21	0.19	1.60
	6-31G(d,p)	-0.14	-1.08	-2.50	-6.30	2.12	0.21	0.18	1.60
	6-31++G(d,p)	-0.08	-1.18	-2.51	-6.51	2.21	0.23	0.18	1.71
	6-311++G(d,p)	-0.35	-1.36	-2.70	-6.83	2.30	0.29	0.24	1.79

Geometry changes listed reflect variation from the original values of bond length and angle as found in the unmodified optimized structures. Unmodified tetrad hydrogen bond geometries obtained are as follows: 6-31G(d): 214.33 pm and 157.04°, 6-31G(d,p): 214.07 pm and 157.18°, 6-31++G(d,p): 215.08 pm and 157.76°, 6-311++G(d,p): 216.27 pm and 157.14°. Listed angles were measured toward the center of the tetrad.

Table 6-6. Computed effects of guanine substitutions on N2-H...N7 geometry of the tetrad

Modification Type	Optimization Basis Set	Δ H2 ... N7 (pm)				Δ N2 – H2 ... N7 Angle (deg)			
		$G_\alpha \bullet G_\beta$	$G_\beta \bullet G_\gamma$	$G_\gamma \bullet G_\delta$	$G_\delta \bullet G_\alpha$	$G_\alpha \bullet G_\beta$	$G_\beta \bullet G_\gamma$	$G_\gamma \bullet G_\delta$	$G_\delta \bullet G_\alpha$
8-BromoG	6-31G(d)	0.46	0.41	0.02	1.95	-0.90	-0.13	-0.06	-2.58
	6-31G(d,p)	0.41	0.38	-0.01	2.03	-0.86	-0.12	-0.05	-2.48
	6-31++G(d,p)	-0.44	0.09	-0.07	1.92	-0.54	-0.02	-0.03	-1.23
	6-311++G(d,p)	-0.58	-0.17	-0.13	2.76	-0.17	0.05	-0.08	-0.49
8-O-methylG	6-31G(d)	-2.77	-1.64	-0.37	2.50	2.31	0.42	-0.11	4.17
	6-31G(d,p)	-2.88	-1.67	-0.36	2.50	2.30	0.42	-0.11	4.12
	6-31++G(d,p)	-3.01	-1.75	-0.45	3.01	2.40	0.44	-0.11	4.24
	6-311++G(d,p)	-3.25	-1.89	-0.52	2.97	2.52	0.52	-0.08	4.33
8-AminoG	6-31G(d)	-0.84	-0.76	-0.28	-2.78	1.02	0.20	0.22	2.53
	6-31G(d,p)	-0.86	-0.77	-0.27	-2.91	1.00	0.19	0.21	2.53
	6-31++G(d,p)	-0.98	-0.82	-0.30	-2.56	1.07	0.21	0.21	2.65
	6-311++G(d,p)	-1.11	-0.91	-0.36	-2.72	1.12	0.27	0.26	2.70

Geometry changes listed reflect variation from the original values of bond length and angle as found in the unmodified optimized structures. Unmodified tetrad hydrogen bond geometries obtained are as follows: 6-31G(d): 214.33 pm and 157.04°, 6-31G(d,p): 214.07 pm and 157.18°, 6-31++G(d,p): 215.08 pm and 157.76°, 6-311++G(d,p): 216.27 pm and 157.14°. Listed angles were measured toward the center of the tetrad.

6.3 Discussion:

This study examines the effects of site-specific base modifications of four C8-substituted guanine derivatives (^{Br}G, ^{Omet}G, ^{Am}G, and ^{Oxo}G) into various positions of a G-quadruplex structure. The chosen DNA sequence forms an intramolecular (3+1) G-quadruplex with well-defined spectroscopic characteristics, and the folding topology of the G-quadruplex was controlled and monitored by CD and NMR spectra, allowing a quantitative comparison of the effects of base modifications in different positions of the same G-quadruplex fold for ^{Br}G, ^{Omet}G and ^{Am}G modifications. Although minor conformations were present for some modified sequences, their relatively small populations should not affect the interpretations regarding the stability of the major fold.

This work reports that single-position ^{Br}G, ^{Omet}G, and ^{Am}G substitutions to the five *syn* guanines of a (3+1) G-quadruplex scaffold generally lead to an increase in thermal stability. These results agree with past findings that substitutions of *syn* guanines by C8-modified guanine derivatives 8-bromo-guanines (25,113) and 8-methyl-guanines (118) stabilize G-quadruplexes and extend this conclusion for 8-O-methyl-guanine and 8-amino-guanine substitutions. Among the C8-modifications explored, ^{Br}G was found to be generally most stabilizing followed by ^{Omet}G and ^{Am}G respectively (Fig. 6-4B and Table 6-2). Thermal denaturing results indicate a position-dependent stabilization effect of single C8-modified derivatives among the various *syn* residues (Fig. 6-4C). This position-dependency may be explained by the different local structural environments among the various *syn* positions, such as neighboring loops, grooves, and tetrad layers. A position-dependence effect has also been demonstrated in other scaffolds containing C8-modifications (113,118).

While the expected stabilization of G-quadruplexes by C8-modified guanine substitutions ^{Br}G , ^{Omet}G and ^{Am}G to *syn* positions was confirmed, it was unanticipated to observe the stabilization effect of an ^{Omet}G substitution to *anti* guanine G22 as previous studies exploring C8-modified guanine substitutions to *anti* positions in intramolecular scaffolds have reported destabilizing effects (25,113,261). To our knowledge, this is the first reported observation of a site-specific C8-modified substitution (8-O-methyl-guanine) stabilizing an intramolecular G-quadruplex system when substituted into an *anti* position. Similarities between the CD and imino proton NMR spectra of the sequence containing an ^{Omet}G substitution at position 22 and those of the unmodified sequence suggests that the same folding topology is maintained and the 8-O-methyl-guanine adopts an *anti* conformation in this modified sequence. It is speculated that ^{Omet}G may constitute a unique type of modification due to its ability to rotate about the $N_7-C_8-O_R-C_R$ bond. The ability for *anti* positions to tolerate the inclusion of C8-modified guanine derivatives may be dependent on specific structural features. However, the generalization of such dependence would require more systematic study and is outside the scope of this work. Substitutions were also made to *anti* residue 23 with destabilizing effects observed for ^{Br}G , ^{Am}G , and ^{Omet}G . The effects of modifications to residue 23 are not discussed here as the folding topology could not be confirmed due to significant changes in CD and NMR spectra.

The observation of specific NMR chemical shift patterns due to base modifications could potentially be a tool in structural elucidation or verification, as the observed cross-hydrogen-bond effect occurs at a remote site in the sequence. For example, in the (3+1) human telomeric G-quadruplex structure considered above, a modification at position 3 would cause a predictable change in imino proton chemical shift of residue 9, while a modification at position 9 would cause a change in position 17, and so forth. In this

manner, site-specific modifications could be used in NMR studies to identify Hoogsteen hydrogen-bound guanines within a G-quadruplex structure.

Quantum mechanical calculations of NMR chemical shifts provide a valuable link between experimentally observable chemical shifts and calculated molecular geometries (262,263). Previously, NMR was used to probe the effects of temperature and ion type on hydrogen-bond lengths within G-tetrads (264). The changes in NMR observables, namely N7 chemical shifts and J-couplings, were interpreted in terms of hydrogen-bond length and substantiated by quantum mechanical calculations. A similar approach was also used to study hydrogen-bond length and strength in a triple helix (263).

In this manner, observed chemical shift patterns were used to investigate the effects of modifications on hydrogen-bond geometry. In this study, *ab initio* NMR chemical shift calculations were found to reproduce the observed changes in G₈ imino proton chemical shifts to a high degree (Fig. 6-6, Table 6-3 and Table 6-4). The difference between modification-driven chemical shift changes calculated by different methods was within a range of 0.11 ppm, 0.14 ppm and 0.06 ppm for the ^{Br}G-, ^{Omet}G- and ^{Am}G-containing models, respectively. These data are in agreement with studies by Kumar and McAllister (262), indicating that relative NMR chemical shift calculations are quite accurate.

The computed effects of modifications on tetrad geometries revealed the largest change in hydrogen-bond geometry at the G₈•G_α interface (Table 6-5 and Table 6-6). A relationship between decreasing hydrogen-bond length and downfield-shifting (increased ppm) of imino proton chemical shifts, and vice-versa, was observed. This trend was obtained across all choices of geometry optimization basis sets explored in this study. These results are in agreement with previous studies (262,263) which reported a similar relationship

between hydrogen-bond length and proton chemical shift. It is important to note that NMR chemical shift calculations performed on modified tetrads prior to geometry re-optimization (data not shown) did not fully reproduce experimental chemical shifts, especially for the $^{\text{Omet}}\text{G-}$ and $^{\text{Am}}\text{G-}$ containing models. This indicates that a structural change in G-tetrad geometry is an important feature of modification-driven changes in NMR chemical shift.

Modification-driven change in hydrogen-bond geometry may not be the only factor contributing to the observed changes in imino proton chemical shift. Changes in base stacking geometry caused by C8-substitutions may lead to changes in the ring-current driven magnetic shielding experienced by imino protons. However, a similar pattern in NMR chemical shift change was experimentally observed for guanines from all three G-tetrad layers in the structure. This indicates that for the modifications explored here, the effect of base stacking changes may be small.

Understanding the effects of C8-modifications on stability, as well as the hydrogen-bond geometry of a G-tetrads, should be valuable in the engineering of new G-quadruplexes, as well as the design of molecules targeting these structures. Computational models explored here, as supported by the agreement between computational and experimental NMR data, show great potential in investigation of the effects of different guanine derivatives on tetrad structure.

6.4 Conclusion:

We examined the effects of site-specific substitution of guanines with the guanine derivatives 8-bromo-guanine, 8-O-methyl-guanine, 8-amino-guanine, and 8-oxo-guanine, using a well-defined (3+1) G-quadruplex as the platform. It was observed that 8-bromo-guanine has the highest stabilizing effect when substituted with a single *syn* guanine, followed by 8-O-methyl-guanine and 8-amino-guanine. Substitutions of 8-oxo-guanine lead to drastic changes in NMR imino proton spectra and a large decrease in stability. The effect of substitution on the stability of the G-quadruplex is highly position-dependant among different guanines in the structure. The C8-modification of a guanine results in a specific pattern of chemical shift changes: the imino proton of the guanine that is hydrogen-bonded to the modified base experiences the largest chemical shift change, which depends on the type of modification. This specific chemical shift pattern was successfully reproduced by *ab initio* quantum mechanical computations. The computational study performed here suggests the effects of various C8-modified guanine substitutions on the Hoogsteen hydrogen-bond geometry within a G-tetrad.

Chapter 7:

Engineering the Base: N2-modified Guanines

This chapter was largely adapted from the authors work: Lech, C.J. and Phan, A.T. (2013) Ball with Hair: functionalization of extremely stable G-quadruplex nanoparticles through N2-guanine modifications. (*Submitted*)

Author Contributions: DNA synthesis, purification and biophysical experiments were carried out by C.J.L.; Data analysis of thermal denaturing experiments was performed by C.J.L.; Both Authors wrote the above mentioned manuscript and contributed to the conceptualization and planning of experiments.

7.1 Introduction:

G-quadruplexes are useful molecular scaffolds, self-assembling and self-folding in an ion-dependent manner into thermally stable nanoparticles (57). A major advantage of nucleic acid-based nanoparticles is their ability to be synthesized and functionalized in a controlled and spatial-specific manner. For example, functionalization of strand termini can enhance the activity of G-quadruplex based aptamers (265) and enables their use as FRET based sensors (266). While modification of strand termini is useful, such modifications are restricted by the location and number of strand termini in the G-quadruplex molecule. Modifications can also be made to the loops of G-quadruplexes, but even slight changes in loop base chemistry can often alter the G-quadruplex conformations adopted (23,233). Another strategy for modification of G-quadruplex is to substitute chemically-altered guanines bases into the G-tetrad core of the G-quadruplex (107). In principle, modification of the guanine base allows for functionalization at a

desired location on the G-quadruplex nanoparticle, and can accommodate multiple modified guanines per tetrad layer. Additionally, modification of the base can be done in parallel to modification to other regions of the G-quadruplex, allowing for numerous possible routes to nanoparticle functionalization.

However, many types of guanine base analogs have been observed to destabilize the G-quadruplex structure (111,112). Modification at the C8 position of the guanine base has received a fair amount of research attention (107). As discussed in Chapter 6, C8-modifications affect the stability of G-quadruplex formation in a manner dependent on the glycosidic conformation of the guanine base (25,113). This conformational dependence limits the ability to functionalize at the C8-position, with only substitutions to guanines in a *syn*-conformation being effectively incorporated.

By comparison, the N2-position of the guanine base has received far less research attention (122,123,267-269). In principle, chemical substitution of a single amino proton at the N2-position does not disrupt the Hoogsteen bonding of the G-tetrad. With this in mind, we set out to characterize the effects of a variety of N2-modified guanines bases on the formation and stability of G-quadruplex nanoparticles. Surprisingly, our results reveal that N2-modified guanines are potent stabilizers of the G-quadruplex architecture and can be substituted in a site-specific manner into a variety of locations on the G-quadruplex nanoparticle scaffold without disrupting nanoparticle formation. Furthermore, multiple N2-modifications may be incorporated simultaneously leading to highly stabilized G-quadruplex nanoparticles. We present the design of a “Ball-with-Hair” nanoparticle containing multiple “chain-like” N2-6-amino-hexyl-guanine chemistries serving as potential sites for nanoparticle functionalization.

7.2 Results and Discussion:

7.2.1: G-quadruplex nanoparticle formation of sequences containing single N2-guanine modifications

In our efforts to functionalize a G-quadruplex scaffold with N2-modified guanine bases, we systematically substituted N2-methyl-guanine (^{Met}G), N2-6-amino-hexyl-guanine (^{Hex}G), and N2-benzyl-guanine (^{Ben}G) into the G-quadruplex structure formed from the sequence d[T₂(G₃T₂A)₃G₃A] (Fig 7-1). This sequence has been previously shown to form a well-defined (3+1) G-quadruplex conformation with three strands oriented in one direction and one pointing in the opposite direction (28). Connecting the strands of the G-quadruplex are a fold-back propeller loop and two edge-wise loops. The orientation of the strands give rise to grooves of varying widths, into which the N2-modification of the guanine base would be projected (Fig 7-1B). Groove widths are classified as wide (W), medium (M), or narrow (N) based upon the geometry of the backbones which define the groove. We use an additional groove description to characterize the medium groove containing a fold-back propeller loop (P). The guanine-tetrad (G-tetrad) core of the molecule is characterized by 3 stacked guanine tetrads with guanine bases in both *syn* and

Figure 7-1: Comparison of experimental and computed **(A)** Schematic of the (3+1) hybrid G-quadruplex studied in this work. Groove widths and select guanine residues are labeled. Guanine bases are colored based on their *syn* (magenta) or *anti* (cyan) glycosidic conformation. **(B)** Example of a single-position N2-modified guanine substitution within the middle G-tetrad layer. Groove descriptions are shown. The 5'→3' strand directionality is indicated as into (x) and out of (•) the page. **(C)** Description of the N2-guanine modifications explored in this work.

Table 7-1: Sequences studied in this work

Name	Sequence								
Unmodified	TT	GGG	TTA	GGG	TTA	GGG	TTA	GGG	A
Met-3	TT	MGG	TTA	GGG	TTA	GGG	TTA	GGG	A
Met-4	TT	GMG	TTA	GGG	TTA	GGG	TTA	GGG	A
Met-5	TT	GGM	TTA	GGG	TTA	GGG	TTA	GGG	A
Met -9	TT	GGG	TTA	MGG	TTA	GGG	TTA	GGG	A
Met -10	TT	GGG	TTA	GMG	TTA	GGG	TTA	GGG	A
Met -11	TT	GGG	TTA	GGM	TTA	GGG	TTA	GGG	A
Met -15	TT	GGG	TTA	GGG	TTA	MGG	TTA	GGG	A
Met -16	TT	GGG	TTA	GGG	TTA	GMG	TTA	GGG	A
Met -17	TT	GGG	TTA	GGG	TTA	GGM	TTA	GGG	A
Met -21	TT	GGG	TTA	GGG	TTA	GGG	TTA	MGG	A
Met -22	TT	GGG	TTA	GGG	TTA	GGG	TTA	GMG	A
Met -23	TT	GGG	TTA	GGG	TTA	GGG	TTA	GGM	A
Hex-3	TT	HGG	TTA	GGG	TTA	GGG	TTA	GGG	A
Hex -4	TT	GHG	TTA	GGG	TTA	GGG	TTA	GGG	A
Hex -5	TT	GGH	TTA	GGG	TTA	GGG	TTA	GGG	A
Hex -9	TT	GGG	TTA	HGG	TTA	GGG	TTA	GGG	A
Hex -10	TT	GGG	TTA	GHG	TTA	GGG	TTA	GGG	A
Hex -11	TT	GGG	TTA	GGH	TTA	GGG	TTA	GGG	A
Hex -15	TT	GGG	TTA	GGG	TTA	HGG	TTA	GGG	A
Hex -16	TT	GGG	TTA	GGG	TTA	GHG	TTA	GGG	A
Hex -17	TT	GGG	TTA	GGG	TTA	GGH	TTA	GGG	A
Hex -21	TT	GGG	TTA	GGG	TTA	GGG	TTA	HGG	A
Hex -22	TT	GGG	TTA	GGG	TTA	GGG	TTA	GHG	A
Hex -23	TT	GGG	TTA	GGG	TTA	GGG	TTA	GGH	A
Ben-3	TT	BGG	TTA	GGG	TTA	GGG	TTA	GGG	A
Ben-4	TT	GBG	TTA	GGG	TTA	GGG	TTA	GGG	A
Ben-5	TT	GGB	TTA	GGG	TTA	GGG	TTA	GGG	A
Ben-9	TT	GGG	TTA	BGG	TTA	GGG	TTA	GGG	A
Ben-10	TT	GGG	TTA	GBG	TTA	GGG	TTA	GGG	A
Ben-11	TT	GGG	TTA	GGB	TTA	GGG	TTA	GGG	A
Ben-15	TT	GGG	TTA	GGG	TTA	BGG	TTA	GGG	A
Ben-16	TT	GGG	TTA	GGG	TTA	GBG	TTA	GGG	A
Ben-17	TT	GGG	TTA	GGG	TTA	GGB	TTA	GGG	A
Ben-21	TT	GGG	TTA	GGG	TTA	GGG	TTA	BGG	A
Ben-22	TT	GGG	TTA	GGG	TTA	GGG	TTA	GBG	A
Ben-23	TT	GGG	TTA	GGG	TTA	GGG	TTA	GGB	A

The position of N2-modified-guanines are indicated as such: ^{Met}G (**M**), ^{Hex}G (**H**), ^{Ben}G (**B**)

anti glycosidic conformations. The diverse structural features of this (3+1) folding topology provide an ideal scaffold to test the functionalization of G-quadruplex nanoparticles through N2-modifications.

The N2-modifications tested in this study were chosen to explore a variety of functional-group geometries. They include small (^{Met}G), bulky-aromatic (^{Ben}G) and long chain-like

(^{Hex}G) chemistries. The ability of modified sequences to form G-quadruplex nanoparticles was evaluated by monitoring the imino proton region of ¹H NMR spectra and by CD spectroscopy. G-quadruplex nanoparticles were studied in a variety of salt concentrations including “low salt” (1 mM KPi with 1 mM KCl), “moderate salt” (10 mM KPi with 10 mM KCl) and “physiological salt” (10 mM KPi with 130 mM KCl) environments in order to aid in the comparison of thermal stabilities of different constructs and to investigate nanoparticle formation in different salt concentrations (Appendix II – Protocols – Chapter 7).

Our study explored a total of 36 sequences containing single-position N2-modifications (Table 7-1). All modified sequences demonstrated G-quadruplex formation as determined by the presence of distinct peaks in the imino proton region of NMR spectra. Of these sequences, 30 were observed to fold into a single major species and adopted the same (3+1) conformation as the parent sequence based on similarity of NMR and CD spectra (Fig 7-2 and Table 7-2). This included 12 of the 12 sequences tested for the ^{Met}G chemistry, 8 for ^{Hex}G, and 10 for ^{Ben}G. Classification by groove type revealed that modified sequences containing substitution into the *N*, *P*, *W* and *M* grooves maintained the (3+1) conformation in 6, 7, 8, and 9 sequences, respectively, out of the 9 modifications that were tested per groove. Select N2-modifications were also tested in a parallel propeller type G-quadruplex scaffold, with G-quadruplex formation observed via imino proton NMR spectra (Fig 7-3), suggesting that N2-guanine modifications can be successfully incorporated into a variety of G-quadruplex structures.

The combination of NMR and CD data suggest that N2-modifications do not inhibit G-quadruplex formation, as all sequences were observed to adopt G-quadruplex structures. As hypothesized, most N2-modified guanines substitutions were successfully introduced

Figure 7-2: G-quadruplex nanoparticles containing single N2-modifications: (A) NMR and (B) CD spectra of representative N2-modified sequences for each of the ^{Met}G, ^{Hex}G, and ^{Ben}G chemistries that maintained the original (3+1) folding topology. Each imino proton resonances in (A) is indicated by a dot (•) (C) Melting curves of these sequences observed by UV absorbance at 295 nm in “moderate salt” conditions (10 mM KPi with 10 mM KCl). Spectra of the unmodified sequence are shown for reference.

without perturbing the native (3+1) conformation. The smaller ^{Met}G substitutions are less disruptive to the native (3+1) conformation than ^{Ben}G or ^{Hex}G substitutions. It is not surprising that modifications into the *N* or *P* grooves were more disruptive to the native G-quadruplex conformation than those into *M* and *W* grooves, as modification into the *M* and *W* grooves are expected to be less obstructed. We note that N2-modified guanine substitutions into positions 5 and 16 of the (3+1) hybrid G-quadruplex are not well tolerated, with ^{Hex}G and ^{Ben}G modifications inducing a mixture of conformations and ^{Met}G

Table 7-2: Biophysical characterization of sequences

Sequence	NMR ^a		
	Single Major Species	Notable Minor Population	Multiple Major Species
Unmodified	•		
Met-3	•		
Met-4	•		
Met-5		•	
Met-9	•		
Met-10	•		
Met-11	•		
Met-15	•		
Met-16		•	
Met-17	•		
Met-21		•	
Met-22	•		
Met-23		•	
Hex-3	•		
Hex-4	•		
Hex-5			•
Hex-9	•		
Hex-10			•
Hex-11	•		
Hex-15	•		
Hex-16			•
Hex-17	•		
Hex-21			•
Hex-22	•		
Hex-23	•		
Ben-3		•	
Ben-4	•		
Ben-5			•
Ben-9	•		
Ben-10	•		
Ben-11	•		
Ben-15	•		
Ben-16			•
Ben-17		•	
Ben-21	•		
Ben-22	•		
Ben-23		•	

[a] Determination of the same (3+1) conformation being adopted is based off a qualitative analysis of the amount of minor conformation present in NMR spectra and the similarity of NMR and CD spectra between the modified and unmodified sequences.

[b] The $\pm$ values indicated the hysteresis observed between heating and cooling curves in UV melting experiments

modifications producing a minor species in NMR spectra. Upon examination of the NMR solution structure of the native sequence, it is possible that modification into position 5 may clash with the propeller loop situated in that groove. There is no clear structural explanation for why position 16 is not well tolerated. It is important to note that multiple

Figure 7-3: ^1H NMR spectra of sequences containing single $^{\text{Hex}}\text{G}$ modifications to the sequence $\text{d}[\text{T}_2(\text{G}_4\text{T})_4]$. The position of $^{\text{Hex}}\text{G}$ modification is indicated in the sequence by a red “H”. Black dots indicate imino proton resonances of the monomer G-quadruplex.

species in solution can arise from the destabilization of the (3+1) structure and/or from the stabilization of an alternative G-quadruplex structure.

7.2.2: N2-modified G-quadruplexes display enhanced thermal stability

We proceeded to investigate how N2-guanine modifications affect the thermal stability of G-quadruplex nanoparticles in a “moderate salt” environment. We examined the stability of all sequences that maintained the native (3+1) G-quadruplex structure. Thermal denaturing studies were performed to determine the melting temperature (T_m) of the unmodified and N2-modified sequences by monitoring the 295 nm UV absorption as a function of temperature (258). Remarkably, all sequences that maintained the original (3+1) G-quadruplex conformation demonstrated enhanced thermal stability (Table 7-3), with a change in melting temperature (ΔT_m) observed in the range of $+2.0^\circ\text{C}$ to $+13.2^\circ\text{C}$ for each single-modification. Average ΔT_m values of 4.6°C , 6.0°C , and 6.6°C were obser-

Table 7-3. Change in melting temperature (°C) upon single-position N2-modifications

Modification Position	Groove ^[a]	Met	Hex	Ben
3	<i>M</i>	5.3	9.1	6.2
4	<i>P</i>	5.7	8.2	8.5
5	<i>P</i>	4.0	-	-
9	<i>P</i>	8.3	9.1	13.2
10	<i>W</i>	4.1	-	6.9
11	<i>W</i>	3.4	4.6	7.4
15	<i>N</i>	5.5	4.4	3.0
16	<i>N</i>	3.1	-	-
17	<i>W</i>	2.8	2.0	4.5
21	<i>N</i>	5.2	-	7.9
22	<i>M</i>	5.4	7.8	5.1
23	<i>M</i>	2.5	3.1	2.8

^a“Moderate salt” conditions were used (10 mM KPi with 10 mM KCl).

[a] The notation describing groove geometry is as shown in Figure 7-1

ved upon modification with ^{Met}G, ^{Hex}G, and ^{Ben}G respectively. Further classification of ΔT_m values by groove type reveal average ΔT_m values of 8.1°C, 5.3°C, 4.9°C, and 4.5°C for the *P*, *M*, *N*, and *W* grooves respectively.

The highly-stabilizing nature of N2-guanine modification into G-quadruplex is an important finding of this work. The extent of stabilization is chemistry-dependent, with the N2-modified guanines tested here ranked as ^{Ben}G > ^{Hex}G > ^{Met}G. Stabilizing effects are also position-dependent as modifications to certain residues, 4 and 9 for example, are observed to be highly stabilizing while other positions, such as 17 and 23, show moderate T_m enhancement. It was unexpected that N2-modifications into positions 4 and 9 of the *P* groove yielded some of the most stable structures, as modifications at these positions could potentially clash with the loop occupying the groove. Conversely, substitutions into the *W* groove at position 17 yielded some of the most modestly stabilized sequences. These observations suggest that the stabilizing properties of N2-modifications may be due to favorable interactions with neighboring structural elements, including the neighboring sugar-phosphate backbone. It is important to note that modifications replacing one of the amino protons with a bulkier chemistry should freeze the rotation about the N2-position,

as supported by the emergence of sharp peaks in the region of 9.0-10.0 ppm in NMR spectra of most modified sequences.

7.2.3: Design of highly stabilized G-quadruplex nanoparticles

The stabilizing properties of single N2-guanine modifications into the (3+1) G-quadruplex scaffold led us to pursue the design of highly stabilized G-quadruplex nanoparticles. We suspected that multiple N2-guanine substitutions would further enhance the stability of the molecular scaffold while simultaneously generating multiple sites to potentially functionalize the molecule. A number of sequences were synthesized containing a variety of N2-modifications in numerous positions based on the results of our single substitution studies (Table 7-4).

We designed a sequence “N2-Same-Groove” containing two ^{Met}G modifications into the W groove at position 10 and 11 to examine if multiple N2-modified guanines could be substituted at neighboring positions within the same groove. The sequence “N2-Different-Groove” tests the incorporation of two very stabilizing modifications into two separate grooves, with two ^{Hex}G substitutions into the M and P grooves at positions 3 and 9. We

Table 7-4. Thermal stability of sequences containing multiple N2-modifications

	Sequence ^[a]	T _m ^b	ΔT _m
Unmodified	TT GGG TTA GGG TTA GGG TTA GGG A	46.3 ± 0.3	-
N2-One-Groove	TT GGG TTA G ^{MM} TTA GGG TTA GGGA	53.4 ± 1.0	7.2
N2-Two-Groove	TT ^H GGG TTA ^H GGG TTA GGG TTA GGG A	65.2 ± 0.4	18.9
N2-All-Groove	TT ^H GGG TTA ^H G ^M TTA ^M GG TTA GGG A	73.8 ± 0.2	27.6
N2-Ball-with-Hair	TT ^H ^H G TTA ^H ^H G TTA ^H ^H G TTA ^G ^H ^H A	94.0 ± 0.1 ^c	47.8
N2-SS8	TT ^H GGG TTA ^B ^G ^B TTA ^M ^G ^M TTA ^B ^G ^B A	85.0 ± 0.6	38.7
N2-SS10	TT ^H ^B G TTA ^B ^M ^B TTA ^M ^G ^M TTA ^B ^M ^H A	91.9 ± 0.2 ^d	45.6

Samples were examined in “Low Salt” conditions (1 mM KPi with 1 mM KCl).

[a] The position of N2-modified guanines is boxed with the following notation: “M” for ^{Met}G; “H” for ^{Hex}G; “B” for ^{Ben}G.

[b] ± indicate the hysteresis between heating and cooling curves in CD melting experiments

[c] Incomplete melting transition observed. T_m determined by single base line correction with high temperature baseline set to a constant of ≈0 absorbance.

[d] Incomplete melting transition observed. T_m determined by 50% of maximum signal due to lack of a low temperature baseline.

design a sequence “N2-Same-Groove” containing two ^{Met}G modifications into the *W* groove at position 10 and 11 to examine if multiple N2-modified guanines could be substituted at neighboring positions within the same groove. The sequence “N2-Different-Groove” tests the incorporation of two very stabilizing modifications into two separate grooves, with two ^{Hex}G substitutions into the *M* and *P* grooves at positions 3 and 9 respectively. A substitution of one N2-modified guanine per groove was investigated by two ^{Hex}G modifications at positions 3 and 9 and two ^{Met}G substitutions at 11 and 15 in the sequence “N2-All-Groove”. We also pursued a more ambitious design in the sequence “N2-Ball-with-Hair” containing eight ^{Hex}G modifications into positions 3, 4, 9, 11, 15, 17, 22 and 23. The “Ball-with-Hair” nanoparticle is designed to contain many long and flexible N2-hexyl-chemistries capable of extending outwards from the surface of the G-quadruplex offering potential sites for functionalization. In addition, we report on the design of N2-SS8 and N2-SS10 sequences containing multiple N2-modifications.

Designed sequences were tested in a “low salt” solution in order to lower the T_m values, maximizing the range of T_m values that could be observed for comparing the stability of different constructs. The designed sequences described in Table 7-4 were determined to maintain the (3+1) G-quadruplex conformation by analysis of ¹H NMR and CD spectra, which were highly similar to the parent sequence (Fig. 7-4A-B). The thermal stability of designed sequences was examined through melting experiments by monitoring the CD signal at 290 nm (270). As desired, all of the sequences tested containing multiple N2-modifications displayed highly increased thermal stability (Fig 7-4C). The N2-One-Groove, N2-Two-Groove, and N2-All-Groove sequences displayed ΔT_m values of +7.2°C, +18.9°C and +27.6°C respectively, roughly equivalent to the sum of ΔT_m values gained from individual modifications. The N2-Ball-with-Hair sequence demonstrated the highest thermal stability, with a ΔT_m of +47.8°C and a melting temperature of ~94°C.

Figure 7-4: Design of highly stable G-quadruplex nanoparticles containing multiple N2-modifications: (A) NMR and (B) CD spectra of select nanoparticles including the N2-Ball with Hair construct (orange). Each imino proton resonances in (A) is indicated with a dot (•). (C) Melting curves of designed sequences observed by CD at 290 nm in “low salt” (1 mM KPi with 1 mM KCl) conditions. Spectra of the unmodified sequence are shown for reference.

We further tested the stability of the N2-Ball-with-Hair sequence in “physiological salt” conditions similar to those experienced in a cellular environment. Temperature dependent CD spectra show more than 80% signal intensity observed at 95 $^{\circ}\text{C}$ (Fig 7-5), suggesting the “Ball-with-Hair” G-quadruplex nanoparticle is highly stable in physiological K^{+} conditions.

Figure 7-5: Design of a HexG modified “Ball-with-hair” G-quadruplex nanoparticle: **(A)** NMR spectra of N2-Ball-with-Hair sequence in “low” and “physiological” salt conditions shows the same structure is formed. **(B)** Temperature dependent CD spectra demonstrate the incredible stability of the “Ball-with-hair” nanoparticle in physiological salt conditions. **(C)** Projection of CD intensity at 290 nm over varying temperatures.

The ability of multiple N2-modifications to stabilize the G-quadruplex without disrupting the folding topology demonstrates the great potential of these tools. Such chemistries can be used to decorate the G-quadruplex scaffold in a variety of ways, including a Ball-with-Hair-like fashion allowing multiple sites for functionalization (Fig 7-6). Previous studies have shown that modifications by Met^{G} or other bulky N2-modifications can enhance the

Figure 7-6: Design of a ^{Hex}G modified “Ball-with-hair” G-quadruplex nanoparticle: **(A)** Surface representation of G-quadruplex molecule (grey) modified with a N2-6-amino-hexyl group (red) in the position G3 illustrates the location and dimension of the ^{Hex}G modification with respect to the *M* groove. **(B)** Illustrative model of the “Ball with Hair” G-quadruplex nanoparticle formed by the N2-Ball with Hair sequence containing eight ^{Hex}G modifications.

inhibitory properties of biologically active G-quadruplex based drugs (122,123), demonstrating the value of N2-modifications in pharmacologically relevant G-quadruplexes. Additionally, the modification of an entire tetrad by ^{Met}G substitutions has been reported to stabilize a tetramer G-quadruplex with anti-HIV activity (123).

7.3 Conclusions:

Our current research reports on the versatility of N2-modifications in a structurally diverse (3+1) G-quadruplex scaffold. Our results indicate that single substitutions of N2-modified guanines are potent stabilizers of the G-quadruplex architecture and we show the ability to decorate this molecular scaffold with a variety of chemistries, including long chain-like chemistries that can be used as a bridge to functionalize this molecular scaffold. Experimentally, we design a “Ball with Hair” type nanoparticle through the addition of eight ^{Hex}G modifications to our 3-layered G-quadruplex scaffold yielding a remarkable increase in thermal stability of ~48°C without disrupting the conformation of the G-quadruplex nanoparticle. Our study demonstrates the design of highly stable G-quadruplex based nanoparticle systems, which are efficiently self-folding, ion sensitive, easily synthesized, and functionalizeable in a spatially-specific manner.

Chapter 8:

Summary and Future Work

8.1 Summary

We have demonstrated that unique G-tetrad stacking geometries exist within the core and at the stacking interface of G-quadruplex DNA. The geometries of interfacial G-tetrad orientations from experimental structures are estimated to be energetically different by up to 12 kcal/mol. We show that current MM force field, popularly used for simulations of nucleic acid, well-represents the relative interaction of stacked G-tetrad systems. MD simulations of parallel G-quadruplex systems demonstrate different accessible G-tetrad geometries for 5'-5' and 3'-3' stacked orientations. These results suggest favorability of G-tetrad orientations may be a key driver of the experimentally observed preference of G-quadruplexes to stack in a 5'-5' manner.

The ground state electronic properties of stacked G-tetrads vary greatly depending on their stacking geometry and thus the geometry adopted by stacked guanines bases. We demonstrate the *5/6-ring* stacking mode commonly found at the interface between 5'-5' stacked G-quadruplex systems is far superior to other stacking orientations in both the

extent of charge delocalization and speed of charge propagation through the system. Electron hole transfer rates for this geometry were at least an order of magnitude greater than other geometries explored.

Incorporation of LNA-modified guanosine (^{LNA}G) into G-quadruplex was found to generally be tolerated based on the glycosidic conformation of the base, with *anti*-position substitutions being tolerated and *syn*-position substitutions perturbing the native (3+1) G-quadruplex fold. However, we identify a scenario of both a *syn*-tolerated and *anti*-destabilizing ^{LNA}G modification. We discover a novel limitation of ^{LNA}G incorporation, with substitution prior to short loops being highly deleterious to G-quadruplex formation. The High-resolution NMR structure of a (3+1) G-quadruplex containing a single ^{LNA}G modification demonstrates local backbone rearrangement upstream of the nucleotide suggesting a possible structural mechanism for the incompatibility of ^{LNA}G modification prior to short loops.

We report on the versatile nature of 2'-deoxy-2'-fluoro-ribo-guanosine (^FG) and 2'-deoxy-2'-fluoro-arabinoguanosine (^{FANA}G) nucleotides, capable of being substituted into *anti*-conformation guanines in many structural environments without disrupting the G-quadruplex folding topology. This is in contrast to ^{LNA}G which has limitations. ^{FANA}G demonstrates stabilizing properties while ^FG demonstrates a mixture of modestly stabilizing and destabilizing properties. Incorporation of both of these nucleotides allows for the isolation of specific folding topologies from a mixture of conformers, or allows for the complete alteration of G-quadruplex folding topology in a polymorphic sequence.

The C8-modified chemistries 8-bromo-guanine (^{Br}G), 8-O-methyl-guanine (^{Omet}G), 8-amino-guanine (^{Am}G), and 8-oxo-guanine (^{Oxo}G) demonstrate a preference for *syn*-

position substitutions. Furthermore, we demonstrate the site-specific and chemical-specific nature of these modifications. Generally, modifications may be ranked $^{\text{Br}}\text{G} > ^{\text{Omet}}\text{G} > ^{\text{Am}}\text{G}$ in their ability to stabilizing the G-quadruplex scaffold. $^{\text{Oxo}}\text{G}$ substitutions are observed to be deleterious to G-quadruplex formation. The modification-induced change in NMR chemical shift of modified G-tetrad is reproduced using QM methods. The up-field shifting (decreased p.p.m) of imino proton resonances correspond to an increase in hydrogen bond length and vice versa.

The N2-modifications are shown to be highly-stabilizing with a single modification stabilizing between 2.0-13.2°C degrees. Multiple substitutions allow the design of a G-quadruplex based nanoparticle with an increased thermal stability of +50°C and a melting temperature of >95°C in physiological salt conditions. This designed nanoparticle contains multiple sites for functionalization. We present this engineered “Ball-with-Hair” G-quadruplex-based nano-scaffold as a useful tool with a variety of potential applications.

8.2 Future Work

Our computational studies of G-tetrad stacks have demonstrated large variability in the energy and electronic properties of different stacking orientations. Recent work from our laboratory and others has shown that the fluorescence emission of G-quadruplexes is enhanced and red-shifted by the stacking of individual G-quadruplex units, but only for certain sequences (271). Knowledge of the G-tetrad stacking orientations at the interface of these dimers leads us to believe that G-tetrad stacking geometry may play a large role in the fluorescent properties of these molecules. With this in mind, we plan to initiate a computational investigation of the excited state properties of different G-tetrad stacking orientations. A better understanding of how G-tetrad geometry affects excited state properties could aid in the designing G-quadruplex-based sensors and conductive devices.

Our work on alternative nucleic acid chemistries has enabled a better understanding of how to effectively apply guanosine analogs into the G-tetrad core of the G-quadruplex. We demonstrate that sugar-modified guanines ^{LNA}G, and ^{FANA}G are generally potent stabilizers of *anti*-position guanines. Alternatively, C8-modified guanine bases are potent *syn*-position stabilizers. Detailed knowledge of how to effectively incorporate these chemical tools into G-quadruplex nucleic acids will allow the design new folding topologies currently unrealized by conventional chemistries. The engineering of such molecules would be of great structural interest and could enable the building of novel G-quadruplex architectures for nanotechnology applications.

We demonstrate the effective incorporation of non-natural chemistries into G-quadruplexes, including highly-stabilizing N2-modified guanines as a novel method for nanoparticle functionalization. Combining the knowledge from our systematic studies

presented in within, we can combine different types of guanosine analogs in parallel to engineer G-quadruplexes with enhanced biological activity and novel applications in the area of nanotechnology. From a pharmacological perspective, G-quadruplex based aptamers of biological interest can be designed, with various guanine analogs being incorporates. The effects of these modifications on enzymatic degradation, cellular uptake, and *in vivo* activity can be investigated. From a nanotechnology perspective, the incorporation of multiple types of guanine analogs will allow us to control the assembly of nanoscale architectures and tune the physical properties of such assemblies.

Bibliography

1. Watson, J.D. and Crick, F.H.C. (1953) Molecular structure of nucleic Acids - a structure for deoxyribose nucleic acid. *Nature*, **171**, 737-738.
2. Wilkins, M.H.F., Stokes, A.R. and Wilson, H.R. (1953) Molecular structure of deoxypentose nucleic acids. *Nature*, **171**, 738-740.
3. Franklin, R.E. and Gosling, R.G. (1953) Molecular configuration in sodium thymonucleate. *Nature*, **171**, 740-741.
4. Bang, I. (1910) Examination of the guanyle acid. *Biochemische Zeitschrift*, **26**, 293-311.
5. Gellert, M., Lipsett, M.N. and Davies, D.R. (1962) Helix formation by guanylic acid. *Proc Natl Acad Sci USA*, **48**, 2013-2018.
6. Wang, Y. and Patel, D.J. (1993) Solution structure of the human telomeric repeat d[AG3(T2AG3)3] G-tetraplex. *Structure*, **1**, 263-282.
7. Laughlan, G., Murchie, A.I.H., Norman, D.G., Moore, M.H., Moody, P.C.E., Lilley, D.M.J. and Luisi, B. (1994) The high-resolution crystal-structure of a parallel-stranded guanine tetraplex. *Science*, **265**, 520-524.
8. Smith, F.W. and Feigon, J. (1992) Quadruplex structure of oxytricha telomeric DNA oligonucleotides. *Nature*, **356**, 164-168.
9. Sen, D. and Gilbert, W. (1988) Formation of parallel 4-stranded complexes by guanine-rich motifs in DNA and its implications for meiosis. *Nature*, **334**, 364-366.
10. Patel, D.J., Phan, A.T. and Kuryavyi, V. (2007) Human telomere, oncogenic promoter and 5'-UTR G-quadruplexes: diverse higher order DNA and RNA targets for cancer therapeutics. *Nucleic Acids Res*, **35**, 7429-7455.
11. Paeschke, K., Bochman, M.L., Garcia, P.D., Cejka, P., Friedman, K.L., Kowalczykowski, S.C. and Zakian, V.A. (2013) Pif1 family helicases suppress genome instability at G-quadruplex motifs. *Nature*, **497**, 458-462.
12. Biffi, G., Tannahill, D., McCafferty, J. and Balasubramanian, S. (2013) Quantitative visualization of DNA G-quadruplex structures in human cells. *Nat Chem*, **5**, 182-186.
13. Fry, M. (2007) Tetraplex DNA and its interacting proteins. *Front Biosci*, **12**, 4336-4351.
14. Palm, W. and de Lange, T. (2008) How shelterin protects mammalian telomeres. *Annu Rev Genet*, **42**, 301-334.
15. Zakian, V.A. (1989) Structure and function of telomeres. *Annu Rev Genet*, **23**, 579-604.
16. Blackburn, E.H. (1991) Structure and function of telomeres. *Nature*, **350**, 569-573.
17. Greider, C.W. and Blackburn, E.H. (1985) Identification of a specific telomere terminal transferase activity in Tetrahymena extracts. *Cell*, **43**, 405-413.
18. Greider, C.W. and Blackburn, E.H. (1987) The telomere terminal transferase of tetrahymena is a ribonucleoprotein enzyme with 2 kinds of primer specificity. *Cell*, **51**, 887-898.
19. Autexier, C. and Lue, N.F. (2006) The structure and function of telomerase reverse transcriptase. *Annu Rev Biochem*, **75**, 493-517.
20. Kim, N.W., Piatyszek, M.A., Prowse, K.R., Harley, C.B., West, M.D., Ho, P.L.C., Coviello, G.M., Wright, W.E., Weinrich, S.L. and Shay, J.W. (1994) Specific association of human telomerase activity with immortal cells and cancer. *Science*, **266**, 2011-2015.
21. Parkinson, G.N., Lee, M.P.H. and Neidle, S. (2002) Crystal structure of parallel quadruplexes from human telomeric DNA. *Nature*, **417**, 876-880.
22. Wang, Y. and Patel, D.J. (1992) Guanine residues in D(T2AG3) and D(T2G4) form parallel-stranded potassium cation stabilized G-quadruplexes with antiglycosidic torsion angles in solution. *Biochemistry*, **31**, 8112-8119.
23. Phan, A.T. and Patel, D.J. (2003) Two-repeat human telomeric d(TAGGGTTAGGGT) sequence forms interconverting parallel and antiparallel G-quadruplexes in solution: distinct topologies, thermodynamic properties, and folding/unfolding kinetics. *J Am Chem Soc*, **125**, 15021-15027.
24. Zhang, N., Phan, A.T. and Patel, D.J. (2005) (3 + 1) Assembly of three human telomeric repeats into an asymmetric dimeric G-quadruplex. *J Am Chem Soc*, **127**, 17277-17285.

25. Xu, Y., Noguchi, Y. and Sugiyama, H. (2006) The new models of the human telomere d[AGGG(TTAGGG)(3)] in K⁺ solution. *Bioorg Med Chem*, **14**, 5584-5591.
26. Ambrus, A., Chen, D., Dai, J., Bialis, T., Jones, R.A. and Yang, D. (2006) Human telomeric sequence forms a hybrid-type intramolecular G-quadruplex structure with mixed parallel/antiparallel strands in potassium solution. *Nucleic Acids Res*, **34**, 2723-2735.
27. Phan, A.T., Luu, K.N. and Patel, D.J. (2006) Different loop arrangements of intramolecular human telomeric (3+1) G-quadruplexes in K⁺ solution. *Nucleic Acids Res*, **34**, 5715-5719.
28. Luu, K.N., Phan, A.T., Kuryavyi, V., Lacroix, L. and Patel, D.J. (2006) Structure of the human telomere in K⁺ solution: an intramolecular (3 + 1) G-quadruplex scaffold. *J Am Chem Soc*, **128**, 9963-9970.
29. Dai, J., Punchihewa, C., Ambrus, A., Chen, D., Jones, R.A. and Yang, D. (2007) Structure of the intramolecular human telomeric G-quadruplex in potassium solution: a novel adenine triple formation. *Nucleic Acids Res*, **35**, 2440-2450.
30. Matsugami, A., Xu, Y., Noguchi, Y., Sugiyama, H. and Katahira, M. (2007) Structure of a human telomeric DNA sequence stabilized by 8-bromoguanosine substitutions, as determined by NMR in a K⁺ solution. *FEBS J*, **274**, 3545-3556.
31. Dai, J., Carver, M., Punchihewa, C., Jones, R.A. and Yang, D. (2007) Structure of the Hybrid-2 type intramolecular human telomeric G-quadruplex in K⁺ solution: insights into structure polymorphism of the human telomeric sequence. *Nucleic Acids Res*, **35**, 4927-4940.
32. Phan, A.T., Kuryavyi, V., Luu, K.N. and Patel, D.J. (2007) Structure of two intramolecular G-quadruplexes formed by natural human telomere sequences in K⁺ solution. *Nucleic Acids Res*, **35**, 6517-6525.
33. Lim, K.W., Amrane, S., Bouaziz, S., Xu, W.X., Mu, Y.G., Patel, D.J., Luu, K.N. and Phan, A.T. (2009) Structure of the Human Telomere in K⁺ Solution: A Stable Basket-Type G-Quadruplex with Only Two G-Tetrad Layers. *J Am Chem Soc*, **131**, 4301-4309.
34. Huppert, J.L. and Balasubramanian, S. (2007) G-quadruplexes in promoters throughout the human genome. *Nucleic Acids Res*, **35**, 406-413.
35. Huppert, J.L. (2008) Hunting G-quadruplexes. *Biochimie*, **90**, 1140-1148.
36. Eddy, J. and Maizels, N. (2006) Gene function correlates with potential for G4 DNA formation in the human genome. *Nucleic Acids Res*, **34**, 3887-3896.
37. Balasubramanian, S., Hurley, L.H. and Neidle, S. (2011) Targeting G-quadruplexes in gene promoters: a novel anticancer strategy? *Nat Rev Drug Discov*, **10**, 261-275.
38. Bugaut, A. and Balasubramanian, S. (2012) 5'-UTR RNA G-quadruplexes: translation regulation and targeting. *Nucleic Acids Res*, **40**, 4727-4741.
39. Huppert, J.L., Bugaut, A., Kumari, S. and Balasubramanian, S. (2008) G-quadruplexes: the beginning and end of UTRs. *Nucleic Acids Res*, **36**, 6260-6268.
40. Arora, A., Dutkiewicz, M., Scaria, V., Hariharan, M., Maiti, S. and Kurreck, J. (2008) Inhibition of translation in living eukaryotic cells by an RNA G-quadruplex motif. *Rna*, **14**, 1290-1296.
41. Beaudoin, J.D. and Perreault, J.P. (2010) 5'-UTR G-quadruplex structures acting as translational repressors. *Nucleic Acids Res*, **38**, 7022-7036.
42. Bonnal, S., Schaeffer, C., Creancier, L., Clamens, S., Moine, H., Prats, A.C. and Vagner, S. (2003) A single internal ribosome entry site containing a G quartet RNA structure drives fibroblast growth factor 2 gene expression at four alternative translation initiation codons. *J Biol Chem*, **278**, 39330-39336.
43. Raymond, E., Sun, D., Chen, S.F., Windle, B. and VonHoff, D.D. (1996) Agents that target telomerase and telomeres. *Curr Opin Biotech*, **7**, 583-591.
44. Zahler, A.M., Williamson, J.R., Cech, T.R. and Prescott, D.M. (1991) Inhibition of telomerase by G-quartet DNA structures. *Nature*, **350**, 718-720.
45. Neidle, S. and Parkinson, G. (2002) Telomere maintenance as a target for anticancer drug discovery. *Nat Rev Drug Discov*, **1**, 383-393.
46. Monchaud, D. and Teulade-Fichou, M.P. (2008) A hitchhiker's guide to G-quadruplex ligands. *Org Biomol Chem*, **6**, 627-636.
47. Maizels, N. and Gray, L.T. (2013) The g4 genome. *Plos Genet*, **9**, e1003468.
48. Randall, A. and Griffith, J.D. (2009) Structure of long telomeric RNA transcripts: the G-rich RNA forms a compact repeating structure containing G-quartets. *J Biol Chem*, **284**, 13980-13986.

49. Martadinata, H. and Phan, A.T. (2013) Structure of human telomeric RNA (TERRA): stacking of two G-quadruplex blocks in K(+) solution. *Biochemistry*, **52**, 2176-2183.
50. Schoeftner, S. and Blasco, M.A. (2008) Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. *Nat Cell Biol*, **10**, 228-236.
51. Cahoon, L.A. and Seifert, H.S. (2009) An Alternative DNA Structure Is Necessary for Pilin Antigenic Variation in *Neisseria gonorrhoeae*. *Science*, **325**, 764-767.
52. Lopes, J., Piazza, A., Bermejo, R., Kriegsman, B., Colosio, A., Teulade-Fichou, M.P., Foiani, M. and Nicolas, A. (2011) G-quadruplex-induced instability during leading-strand replication. *EMBO J*, **30**, 4033-4046.
53. Piazza, A., Serero, A., Boule, J.B., Legoix-Ne, P., Lopes, J. and Nicolas, A. (2012) Stimulation of gross chromosomal rearrangements by the human CEB1 and CEB25 minisatellites in *saccharomyces cerevisiae* depends on G-quadruplexes or Cdc13. *Plos Genet*, **8**, e1003033.
54. Davis, J.T. and Spada, G.P. (2007) Supramolecular architectures generated by self-assembly of guanosine derivatives. *Chem Soc Rev*, **36**, 296-313.
55. Williamson, J.R. (1993) Guanine quartets. *Curr Opin Struc Biol*, **3**, 357-362.
56. Hud, N.V., Smith, F.W., Anet, F.A.L. and Feigon, J. (1996) The selectivity for K⁺ versus Na⁺ in DNA quadruplexes is dominated by relative free energies of hydration: A thermodynamic analysis by H-1 NMR. *Biochemistry*, **35**, 15383-15390.
57. Gonzalez-Rodriguez, D., van Dongen, J.L.J., Lutz, M., Spek, A.L., Schenning, A.P.H.J. and Meijer, E.W. (2009) G-quadruplex self-assembly regulated by Coulombic interactions. *Nat Chem*, **1**, 151-155.
58. Pinnavaia, T.J., Marshall, C.L., Mettler, C.M., Fisk, C.I., Miles, H.T. and Becker, E.D. (1978) Alkali-metal ion specificity in solution ordering of a nucleotide, 5'-Guanosine monophosphate. *J Am Chem Soc*, **100**, 3625-3627.
59. Cang, X.H., Sponer, J. and Cheatham, T.E. (2011) Explaining the varied glycosidic conformational, G-tract length and sequence preferences for anti-parallel G-quadruplexes. *Nucleic Acids Res*, **39**, 4499-4512.
60. Smith, F.W., Lau, F.W. and Feigon, J. (1994) D(G(3)T(4)G(3)) Forms an asymmetric diagonally looped dimeric quadruplex with guanosine 5'-Syn-Syn-Anti and 5'-Syn-Anti-Anti N-glycosidic conformations. *P Natl Acad Sci USA*, **91**, 10546-10550.
61. Webba da Silva, M. (2007) Geometric formalism for DNA quadruplex folding. *Chemistry*, **13**, 9738-9745.
62. Phillips, K., Dauter, Z., Murchie, A.I.H., Lilley, D.M.J. and Luisi, B. (1997) The crystal structure of a parallel-stranded guanine tetraplex at 0.95 angstrom resolution. *J Mol Biol*, **273**, 171-182.
63. Hazel, P., Parkinson, G.N. and Neidle, S. (2006) Topology variation and loop structural homology in crystal and simulated structures of a bimolecular DNA quadruplex. *J Am Chem Soc*, **128**, 5480-5487.
64. Parkinson, G.N., Ghosh, R. and Neidle, S. (2007) Structural basis for binding of porphyrin to human telomeres. *Biochemistry*, **46**, 2390-2397.
65. Nielsen, J.T., Arar, K. and Petersen, M. (2009) Solution structure of a locked nucleic acid modified quadruplex: introducing the V4 folding topology. *Angew Chem Int Ed Engl*, **48**, 3099-3103.
66. Neidle, S. (2008) *Principles of Nucleic Acid Structure*. Elsevier Inc.
67. Phan, A.T. (2010) Human telomeric G-quadruplex: structures of DNA and RNA sequences. *FEBS J*, **277**, 1107-1117.
68. Kerwin, S.M. (2000) G-quadruplex DNA as a target for drug design. *Curr Pharm Design*, **6**, 441-471.
69. Boer, D.R., Canals, A. and Coll, M. (2009) DNA-binding drugs caught in action: the latest 3D pictures of drug-DNA complexes. *Dalton T*, **3**, 399-414.
70. Nanjunda, R., Musetti, C., Kumar, A., Ismail, M.A., Farahat, A.A., Wang, S.M., Sissi, C., Palumbo, M., Boykin, D.W. and Wilson, W.D. (2012) Heterocyclic dications as a new class of telomeric G-quadruplex targeting agents. *Curr Pharm Design*, **18**, 1934-1947.
71. Islam, B., Sgobba, M., Laughton, C., Orozco, M., Sponer, J., Neidle, S. and Haider, S. (2013) Conformational dynamics of the human propeller telomeric DNA quadruplex on a microsecond time scale. *Nucleic Acids Res*, **41**, 2723-2735.
72. Haider, S., Parkinson, G.N. and Neidle, S. (2008) Molecular dynamics and principal components analysis of human telomeric quadruplex multimers. *Biophys J*, **95**, 296-311.
73. Martadinata, H., Heddi, B., Lim, K.W. and Phan, A.T. (2011) Structure of long human telomeric RNA (TERRA): G-quadruplexes formed by four and eight UUAGGG repeats are stable building

- blocks. *Biochemistry*, **50**, 6455-6461.
74. Phan, A.T., Kuryavyi, V., Ma, J.B., Faure, A., Andreola, M.L. and Patel, D.J. (2005) An interlocked dimeric parallel-stranded DNA quadruplex: a potent inhibitor of HIV-1 integrase. *P Natl Acad Sci USA*, **102**, 634-639.
 75. Zhang, N., Gorin, A., Majumdar, A., Kettani, A., Chernichenko, N., Skripkin, E. and Patel, D.J. (2001) V-shaped scaffold: a new architectural motif identified in an A x (G x G x G x G) pentad-containing dimeric DNA quadruplex involving stacked G(anti) x G(anti) x G(anti) x G(syn) tetrads. *J Mol Biol*, **311**, 1063-1079.
 76. Tuerk, C. and Gold, L. (1990) Systematic evolution of ligands by exponential enrichment - rna ligands to bacteriophage-T4 DNA-Polymerase. *Science*, **249**, 505-510.
 77. Ellington, A.D. and Szostak, J.W. (1990) In vitro selection of RNA molecules that bind specific ligands. *Nature*, **346**, 818-822.
 78. Gatto, B., Palumbo, M. and Sissi, C. (2009) Nucleic acid aptamers based on the G-quadruplex structure: therapeutic and diagnostic potential. *Curr Med Chem*, **16**, 1248-1265.
 79. Bock, L.C., Griffin, L.C., Latham, J.A., Vermaas, E.H. and Toole, J.J. (1992) Selection of single-stranded-DNA molecules that bind and inhibit human thrombin. *Nature*, **355**, 564-566.
 80. Macaya, R.F., Schultze, P., Smith, F.W., Roe, J.A. and Feigon, J. (1993) Thrombin-binding DNA aptamer forms a unimolecular quadruplex structure in solution. *P Natl Acad Sci USA*, **90**, 3745-3749.
 81. Padmanabhan, K., Padmanabhan, K.P., Ferrara, J.D., Sadler, J.E. and Tulinsky, A. (1993) The structure of alpha-thrombin inhibited by a 15-mer single-stranded DNA aptamer. *J Biol Chem*, **268**, 17651-17654.
 82. Avino, A., Fabrega, C., Tintore, M. and Eritja, R. (2012) Thrombin binding aptamer, more than a simple aptamer: chemically modified derivatives and biomedical applications. *Curr Pharm Design*, **18**, 2036-2047.
 83. Do, N.Q., Lim, K.W., Teo, M.H., Heddi, B. and Phan, A.T. (2011) Stacking of G-quadruplexes: NMR structure of a G-rich oligonucleotide with potential anti-HIV and anticancer activity. *Nucleic Acids Res*, **39**, 9448-9457.
 84. Jing, N.J., Sha, W., Li, Y.D., Xiong, W.J. and Tweardy, D.J. (2005) Rational drug design of G-quartet DNA as anti-cancer agents. *Curr Pharm Design*, **11**, 2841-2854.
 85. Bates, P.J., Laber, D.A., Miller, D.M., Thomas, S.D. and Trent, J.O. (2009) Discovery and development of the G-rich oligonucleotide AS1411 as a novel treatment for cancer. *Exp Mol Pathol*, **86**, 151-164.
 86. Dempsey, L.A., Sun, H., Hanakahi, L.A. and Maizels, N. (1999) G4 DNA binding by LR1 and its subunits, nucleolin and hnRNP D, a role for G-G pairing in immunoglobulin switch recombination. *J Biol Chem*, **274**, 1066-1071.
 87. Hanakahi, L.A., Sun, H. and Maizels, N. (1999) High affinity interactions of nucleolin with G-G-paired rDNA. *J Biol Chem*, **274**, 15908-15912.
 88. Bates, P.J., Kahlon, J.B., Thomas, S.D., Trent, J.O. and Miller, D.M. (1999) Antiproliferative activity of G-rich oligonucleotides correlates with protein binding. *J Biol Chem*, **274**, 26369-26377.
 89. Wyatt, J.R., Vickers, T.A., Roberson, J.L., Buckheit, R.W., Klimkait, T., Debaets, E., Davis, P.W., Rayner, B., Imbach, J.L. and Ecker, D.J. (1994) Combinatorially selected guanosine-quartet structure is a potent inhibitor of human-immunodeficiency-virus envelope-mediated cell-fusion. *P Natl Acad Sci USA*, **91**, 1356-1360.
 90. de Soultrait, V.R., Lozach, P.Y., Altmeyer, R., Tarrago-Litvak, L., Litvak, S. and Andreola, M.L. (2002) DNA aptamers derived from HIV-1 RNase H inhibitors are strong anti-integrase agents. *J Mol Biol*, **324**, 195-203.
 91. Marsh, T.C. and Henderson, E. (1994) G-Wires - Self-assembly of a telomeric oligonucleotide, D(GGGGTTGGGG), into large superstructures. *Biochemistry*, **33**, 10718-10724.
 92. Marsh, T.C., Vesenska, J. and Henderson, E. (1995) A new DNA nanostructure, the G-wire, imaged by scanning probe microscopy. *Nucleic Acids Res*, **23**, 696-700.
 93. Krishnan, Y. and Simmel, F.C. (2011) Nucleic acid based molecular devices. *Angew Chem Int Edit*, **50**, 3124-3156.
 94. Lv, L., Guo, Z.J., Wang, J.H. and Wang, E.K. (2012) G-quadruplex as signal transducer for biorecognition events. *Curr Pharm Design*, **18**, 2076-2095.
 95. Chinnapen, D.J.F. and Sen, D. (2007) Towards elucidation of the mechanism of UV1C, a

- deoxyribozyme with photolyase activity. *J Mol Biol*, **365**, 1326-1336.
96. Li, T., Wang, E.K. and Dong, S.J. (2008) G-quadruplex-based DNAzyme for facile colorimetric detection of thrombin. *Chem Commun*, **31**, 3654-3656.
97. Li, T., Dong, S.J. and Wang, E. (2009) Label-free colorimetric detection of aqueous mercury ion (Hg²⁺) using Hg²⁺-modulated G-quadruplex-based DNAzymes. *Anal Chem*, **81**, 2144-2149.
98. Teller, C. and Willner, I. (2010) Functional nucleic acid nanostructures and DNA machines. *Curr Opin Biotech*, **21**, 376-391.
99. Li, Y.F. and Sen, D. (1997) Toward an efficient DNAzyme. *Biochemistry*, **36**, 5589-5599.
100. Travascio, P., Li, Y.F. and Sen, D. (1998) DNA-enhanced peroxidase activity of a DNA aptamer-hemin complex. *Chem Biol*, **5**, 505-517.
101. Nakayama, S. and Sintim, H.O. (2009) Colorimetric split G-quadruplex probes for nucleic acid sensing: Improving reconstituted DNAzyme's catalytic efficiency via probe remodeling. *J Am Chem Soc*, **131**, 10320-10333.
102. Kosman, J. and Juskowiak, B. (2011) Peroxidase-mimicking DNAzymes for biosensing applications: A review. *Anal Chim Acta*, **707**, 7-17.
103. Kawasaki, A.M., Casper, M.D., Freier, S.M., Lesnik, E.A., Zounes, M.C., Cummins, L.L., Gonzalez, C. and Cook, P.D. (1993) Uniformly modified 2'-deoxy-2'-fluoro phosphorothioate oligonucleotides as nuclease-resistant antisense compounds with high affinity and specificity for RNA targets. *J Med Chem*, **36**, 831-841.
104. Wahlestedt, C., Salmi, P., Good, L., Kela, J., Johnsson, T., Hokfelt, T., Broberger, C., Porreca, F., Lai, J., Ren, K.K. *et al.* (2000) Potent and nontoxic antisense oligonucleotides containing locked nucleic acids. *P Natl Acad Sci USA*, **97**, 5633-5638.
105. Kalota, A., Karabon, L., Swider, C.R., Viazovkina, E., Elzagheid, M., Damha, M.J. and Gewirtz, A.M. (2006) 2'-deoxy-2'-fluoro-beta-D-arabinonucleic acid (2'F-ANA) modified oligonucleotides (ON) effect highly efficient, and persistent, gene silencing. *Nucleic Acids Res*, **34**, 451-461.
106. Peng, C.G. and Damha, M.J. (2007) G-quadruplex induced stabilization by 2'-deoxy-2'-fluoro-D-arabinonucleic acids (2'F-ANA). *Nucleic Acids Res*, **35**, 4977-4988.
107. Sagi, J. (2013) G-quadruplexes incorporating modified constituents: a review. *J Biomol Struct Dyn*.
108. Demple, B., Jacobsson, A., Olsson, M., Robins, P. and Lindahl, T. (1982) Repair of Alkylated DNA in Escherichia-Coli - Physical-Properties of O-6-Methylguanine-DNA Methyltransferase. *J Biol Chem*, **257**, 13776-13780.
109. Gannett, P.M. and Sura, T.P. (1993) Base-Pairing of 8-Oxoguanosine and 8-Oxo-2'-Deoxyguanosine with 2'-Deoxyadenosine, 2'-Deoxycytosine, 2'-Deoxyguanosine, and Thymidine. *Chem Res Toxicol*, **6**, 690-700.
110. Bernstein, B.E., Meissner, A. and Lander, E.S. (2007) The mammalian epigenome. *Cell*, **128**, 669-681.
111. Gros, J., Rosu, F., Amrane, S., De Cian, A., Gabelica, V., Lacroix, L. and Mergny, J.L. (2007) Guanines are a quartet's best friend: impact of base substitutions on the kinetics and stability of tetramolecular quadruplexes. *Nucleic Acids Res*, **35**, 3064-3075.
112. Mekmaysy, C.S., Petraccone, L., Garbett, N.C., Ragazzon, P.A., Gray, R., Trent, J.O. and Chaires, J.B. (2008) Effect of O6-methylguanine on the stability of G-quadruplex DNA. *J Am Chem Soc*, **130**, 6710-6711.
113. Dias, E., Battiste, J.L. and Williamson, J.R. (1994) Chemical probe for glycosidic conformation in Telomeric DNAs. *J Am Chem Soc*, **116**, 4479-4480.
114. Esposito, V., Randazzo, A., Piccialli, G., Petraccone, L., Giancola, C. and Mayol, L. (2004) Effects of an 8-bromodeoxyguanosine incorporation on the parallel quadruplex structure [d(TGGGT)]₄. *Org Biomol Chem*, **2**, 313-318.
115. Virgilio, A., Esposito, V., Randazzo, A., Mayol, L. and Galeone, A. (2005) 8-Methyl-2'-deoxyguanosine incorporation into parallel DNA quadruplex structures. *Nucleic Acids Res*, **33**, 6188-6195.
116. Gros, J., Avino, A., de la Osa, J.L., Gonzalez, C., Lacroix, L., Perez, A., Orozco, M., Eritja, R. and Mergny, J.L. (2008) 8-amino guanine accelerates tetramolecular G-quadruplex formation. *Chem Commun*, **25**, 2926-2928.
117. Tran, P.L., Virgilio, A., Esposito, V., Citarella, G., Mergny, J.L. and Galeone, A. (2011) Effects of 8-methylguanine on structure, stability and kinetics of formation of tetramolecular quadruplexes. *Biochimie*, **93**, 399-408.

118. Xu, Y. and Sugiyama, H. (2006) Formation of the G-quadruplex and i-motif structures in retinoblastoma susceptibility genes (Rb). *Nucleic Acids Res*, **34**, 949-954.
119. Benz, A. and Hartig, J.S. (2008) Redesigned tetrads with altered hydrogen bonding patterns enable programming of quadruplex topologies. *Chem Commun*, **34**, 4010-4012.
120. Phan, A.T., Kuryavyi, V., Gaw, H.Y. and Patel, D.J. (2005) Small-molecule interaction with a five-guanine-tract G-quadruplex structure from the human MYC promoter. *Nat Chem Biol*, **1**, 167-173.
121. Lim, K.W., Lacroix, L., Yue, D.J.E., Lim, J.K.C., Lim, J.M.W. and Phan, A.T. (2010) Coexistence of two distinct G-quadruplex conformations in the hTERT promoter. *J Am Chem Soc*, **132**, 12331-12342.
122. He, G.X., Krawczyk, S.H., Swaminathan, S., Shea, R.G., Dougherty, J.P., Terhorst, T., Law, V.S., Griffin, L.C., Coutre, S. and Bischofberger, N. (1998) N-2- and C-8-substituted oligodeoxynucleotides with enhanced thrombin inhibitory activity in vitro and in vivo. *J Med Chmy*, **41**, 2234-2242.
123. Koizumi, M., Akahori, K., Ohmine, T., Tsutsumi, S., Sone, J., Kosaka, T., Kaneko, M., Kimura, S. and Shimada, K. (2000) Biologically active oligodeoxyribonucleotides. Part 12: N2-methylation of 2'-deoxyguanosines enhances stability of parallel G-quadruplex and anti-HIV-1 activity. *Bioorg Med Chem Lett*, **10**, 2213-2216.
124. Petersen, M. and Wengel, J. (2003) LNA: a versatile tool for therapeutics and genomics. *Trends Biotechnol*, **21**, 74-81.
125. Pradhan, D., Hansen, L.H., Vester, B. and Petersen, M. (2011) Selection of G-quadruplex folding topology with LNA-modified human telomeric sequences in K⁺ Solution. *Chem-Eur J*, **17**, 2405-2413.
126. Randazzo, A., Esposito, V., Ohlenschlager, O., Ramachandran, R. and Mayol, L. (2004) NMR solution structure of a parallel LNA quadruplex. *Nucleic Acids Res*, **32**, 3083-3092.
127. Nielsen, J.T., Arar, K. and Petersen, M. (2006) NMR solution structures of LNA (locked nucleic acid) modified quadruplexes. *Nucleic Acids Res*, **34**, 2006-2014.
128. Pasternak, A., Hernandez, F.J., Rasmussen, L.M., Vester, B. and Wengel, J. (2011) Improved thrombin binding aptamer by incorporation of a single unlocked nucleic acid monomer. *Nucleic Acids Res*, **39**, 1155-1164.
129. Tang, C.F. and Shafer, R.H. (2006) Engineering the quadruplex fold: Nucleoside conformation determines both folding topology and molecularity in guanine quadruplexes. *J Am Chem Soc*, **128**, 5966-5973.
130. Sacca, B., Lacroix, L. and Mergny, J.L. (2005) The effect of chemical modifications on the thermal stability of different G-quadruplex-forming oligonucleotides. *Nucleic Acids Res*, **33**, 1182-1192.
131. Zaitseva, M., Kaluzhny, D., Shchylkina, A., Borisova, O., Smirnov, I. and Pozmogova, G. (2010) Conformation and thermostability of oligonucleotide d(GGTTGGTGTGGTTGG) containing thiophosphoryl internucleotide bonds at different positions. *Biophys Chem*, **146**, 1-6.
132. Datta, B., Schmitt, C. and Armitage, B.A. (2003) Formation of a PNA(2)-DNA(2) hybrid quadruplex. *J Am Chem Soc*, **125**, 4111-4118.
133. Datta, B., Bier, M.E., Roy, S. and Armitage, B.A. (2005) Quadruplex formation by a guanine-rich PNA oligomer. *J Am Chem Soc*, **127**, 4199-4207.
134. Krishnan-Ghosh, Y., Stephens, E. and Balasubramanian, S. (2004) A PNA(4) quadruplex. *J Am Chem Soc*, **126**, 5944-5945.
135. Xu, Y., Suzuki, Y. and Komiyama, M. (2009) Click chemistry for the identification of G-quadruplex structures: discovery of a DNA-RNA G-quadruplex. *Angew Chem Int Edit*, **48**, 3281-3284.
136. Sponer, J., Gabb, H.A., Leszczynski, J. and Hobza, P. (1997) Base-base and deoxyribose-base stacking interactions in B-DNA and Z-DNA: A quantum-chemical study. *Biophys J*, **73**, 76-87.
137. Hobza, P. and Sponer, J. (2002) Toward true DNA base-stacking energies: MP2, CCSD(T), and complete basis set calculations. *J Am Chem Soc*, **124**, 11802-11808.
138. Dabkowska, I., Gonzalez, H.V., Jurecka, P. and Hobza, P. (2005) Stabilization energies of the hydrogen-bonded and stacked structures of nucleic acid base pairs in the crystal geometries of CG, AT, and AC DNA steps and in the NMR geometry of the 5'-d(GCGAAGC)-3' hairpin: Complete basis set calculations at the MP2 and CCSD(T) levels. *J Phys Chem A*, **109**, 1131-1136.
139. Sponer, J., Jurecka, P., Marchan, I., Luque, F.J., Orozco, M. and Hobza, P. (2006) Nature of base stacking: Reference quantum-chemical stacking energies in ten unique B-DNA base-pair steps. *Chem-Eur J*, **12**, 2854-2865.

140. Sponer, J., Riley, K.E. and Hobza, P. (2008) Nature and magnitude of aromatic stacking of nucleic acid bases. *Phys Chem Chem Phys*, **10**, 2595-2610.
141. Svozil, D., Hobza, P. and Sponer, J. (2010) Comparison of intrinsic stacking energies of ten unique dinucleotide steps in A-RNA and B-DNA duplexes. Can we determine correct order of stability by quantum-chemical calculations? *J Phys Chem B*, **114**, 1191-1203.
142. Gu, J.D. and Leszczynski, J. (2000) A remarkable alteration in the bonding pattern: An HF and DFT study of the interactions between the metal cations and the Hoogsteen hydrogen-bonded G-tetrad. *J Phys Chem A*, **104**, 6308-6313.
143. Gu, J.D. and Leszczynski, J. (2002) Origin of Na⁺/K⁺ selectivity of the guanine tetraplexes in water: The theoretical rationale. *J Phys Chem A*, **106**, 529-532.
144. Meyer, M., Hocquet, A. and Suhnel, J. (2005) Interaction of sodium and potassium ions with sandwiched cytosine-, guanine-, thymine-, and uracil-base tetrads. *J Comput Chem*, **26**, 352-364.
145. Deng, J.P., Xiong, Y. and Sundaralingam, M. (2001) X-ray analysis of an RNA tetraplex (UGGGGU)₄ with divalent Sr²⁺ ions at subatomic resolution (0.61 angstrom). *P Natl Acad Sci USA*, **98**, 13665-13670.
146. Caceres, C., Wright, G., Gouyette, C., Parkinson, G. and Subirana, J.A. (2004) A thymine tetrad in d(TGGGGT) quadruplexes stabilized with Tl⁺/Na⁺ ions. *Nucleic Acids Res*, **32**, 1097-1102.
147. Kondo, J., Adachi, W., Umeda, S., Sunami, T. and Takenaka, A. (2004) Crystal structures of a DNA octaplex with I-motif of G-quartets and its splitting into two quadruplexes suggest a folding mechanism of eight tandem repeats. *Nucleic Acids Res*, **32**, 2541-2549.
148. Lee, M.P.H., Parkinson, G.N., Hazel, P. and Neidle, S. (2007) Observation of the coexistence of sodium and calcium ions in a DNA G-quadruplex ion channel. *J Am Chem Soc*, **129**, 10106-10107.
149. Creze, C., Rinaldi, B., Haser, R., Bouvet, P. and Gouet, P. (2007) Structure of a d(TGGGGT) quadruplex crystallized in the presence of Li⁺ ions. *Acta Crystallogr D Biol Crystallogr*, **63**, 682-688.
150. Parkinson, G.N., Cuenca, F. and Neidle, S. (2008) Topology conservation and loop flexibility in quadruplex-drug recognition: Crystal structures of inter- and intramolecular telomeric DNA quadruplex-drug complexes. *J Mol Biol*, **381**, 1145-1156.
151. Matsugami, A., Ouhashi, K., Kanagawa, M., Liu, H., Kanagawa, S., Uesugi, S. and Katahira, M. (2001) An intramolecular quadruplex of (GGA)₄ triplet repeat DNA with a G:G:G:G tetrad and a G(A):G(A):G(A):G heptad, and its dimeric interaction. *J Mol Biol*, **313**, 255-269.
152. Liu, H., Matsugami, A., Katahira, M. and Uesugi, S. (2002) A dimeric RNA quadruplex architecture comprised of two G:G(A):G:G(A) hexads, G:G:G:G tetrads and UUUU loops. *J Mol Biol*, **322**, 955-970.
153. Matsugami, A., Okuizumi, T., Uesugi, S. and Katahira, M. (2003) Intramolecular higher order packing of parallel quadruplexes comprising a G:G:G:G tetrad and a G(A):G(A):G(A):G heptad of GGA triplet repeat DNA. *J Biol Chem*, **278**, 28147-28153.
154. Mashima, T., Matsugami, A., Nishikawa, F., Nishikawa, S. and Katahira, M. (2009) Unique quadruplex structure and interaction of an RNA aptamer against bovine prion protein. *Nucleic Acids Res*, **37**, 6249-6258.
155. Martadinata, H. and Phan, A.T. (2009) Structure of propeller-type parallel-stranded RNA G-quadruplexes, formed by human telomeric RNA sequences in K⁺ solution. *J Am Chem Soc*, **131**, 2570-2578.
156. Collie, G.W., Haider, S.M., Neidle, S. and Parkinson, G.N. (2010) A crystallographic and modelling study of a human telomeric RNA (TERRA) quadruplex. *Nucleic Acids Res*, **38**, 5569-5580.
157. Mukundan, V.T., Do, N.Q. and Phan, A.T. (2011) HIV-1 integrase inhibitor T30177 forms a stacked dimeric G-quadruplex structure containing bulges. *Nucleic Acids Res*, **39**, 8984-8991.
158. Sponer, J. and Spackova, N. (2007) Molecular dynamics simulations and their application to four-stranded DNA. *Methods*, **43**, 278-290.
159. Cang, X.H., Sponer, J. and Cheatham, T.E. (2011) Insight into G-DNA structural polymorphism and folding from sequence and loop connectivity through free energy analysis. *J Am Chem Soc*, **133**, 14270-14279.
160. Lavery, R., Zakrzewska, K., Sun, J.S. and Harvey, S.C. (1992) A comprehensive classification of nucleic-Acid structural families based on strand direction and base-pairing. *Nucleic Acids Res*, **20**, 5011-5016.
161. Boys, S.F. and Bernardi, F. (1970) Calculation of small molecular interactions by differences of

- separate total energies - Some procedures with reduced errors. *Mol Phys*, **19**, 553-566.
162. Campbell, N.H., Parkinson, G.N., Reszka, A.P. and Neidle, S. (2008) Structural basis of DNA quadruplex recognition by an acridine drug. *J Am Chem Soc*, **130**, 6722-6724.
 163. Collie, G.W., Sparapani, S., Parkinson, G.N. and Neidle, S. (2011) Structural basis of telomeric RNA quadruplex-acridine ligand recognition. *J Am Chem Soc*, **133**, 2721-2728.
 164. Clark, G.R., Pytel, P.D., Squire, C.J. and Neidle, S. (2003) Structure of the first parallel DNA quadruplex-drug complex. *J Am Chem Soc*, **125**, 4066-4067.
 165. Pan, B.C., Xiong, Y., Shi, K. and Sundaralingam, M. (2003) Crystal structure of a bulged RNA tetraplex at 1.1 angstrom resolution: Implications for a novel binding site in RNA tetraplex. *Structure*, **11**, 1423-1430.
 166. Pan, B.C., Xiong, Y., Shi, K., Deng, J.P. and Sundaralingam, M. (2003) Crystal structure of an RNA purine-rich tetraplex containing adenine tetrads: Implications for specific binding in RNA tetraplexes. *Structure*, **11**, 815-823.
 167. Pan, B.C., Xiong, Y., Shi, K. and Sundaralingam, M. (2003) An eight-stranded helical fragment in RNA crystal structure: Implications for tetraplex interaction. *Structure*, **11**, 825-831.
 168. Pan, B.C., Shi, K. and Sundaralingam, M. (2006) Crystal structure of an RNA quadruplex containing inosine tetrad: Implications for the roles of NH₂ group in purine tetrads. *J Mol Biol*, **363**, 451-459.
 169. Horvath, M.P. and Schultz, S.C. (2001) DNA G-quartets in a 1.86 Å resolution structure of an *Oxytricha nova* telomeric protein-DNA complex. *J Mol Biol*, **310**, 367-377.
 170. Haider, S., Parkinson, G.N. and Neidle, S. (2002) Crystal structure of the potassium form of an *Oxytricha nova* G-quadruplex. *J Mol Biol*, **320**, 189-200.
 171. Haider, S.M., Parkinson, G.N. and Neidle, S. (2003) Structure of a G-quadruplex-ligand complex. *J Mol Biol*, **326**, 117-125.
 172. Gill, M.L., Strobel, S.A. and Loria, J.P. (2006) Crystallization and characterization of the thallium form of the *Oxytricha nova* G-quadruplex. *Nucleic Acids Res*, **34**, 4506-4514.
 173. Campbell, N.H., Patel, M., Tofa, A.B., Ghosh, R., Parkinson, G.N. and Neidle, S. (2009) Selectivity in ligand recognition of G-quadruplex loops. *Biochemistry*, **48**, 1675-1680.
 174. Campbell, N.H., Smith, D.L., Reszka, A.P., Neidle, S. and O'Hagan, D. (2011) Fluorine in medicinal chemistry: beta-fluorination of peripheral pyrrolidines attached to acridine ligands affects their interactions with G-quadruplex DNA. *Org Biomol Chem*, **9**, 1328-1331.
 175. Pan, B., Shi, K. and Sundaralingam, M. (2006) Base-tetrad swapping results in dimerization of RNA quadruplexes: Implications for formation of the i-motif RNA octaplex. *P Natl Acad Sci USA*, **103**, 3130-3134.
 176. Wang, Y. and Patel, D.J. (1994) Solution structure of the *Tetrahymena* telomeric repeat d(T₂G₄)₄ G-tetraplex. *Structure*, **2**, 1141-1156.
 177. Balkwill, G.D., Garner, T.P., Williams, H.E. and Searle, M.S. (2009) Folding topology of a bimolecular DNA quadruplex containing a stable mini-hairpin motif within the diagonal loop. *J Mol Biol*, **385**, 1600-1615.
 178. Lim, K.W., Lacroix, L., Yue, D.J., Lim, J.K., Lim, J.M. and Phan, A.T. (2010) Coexistence of two distinct G-quadruplex conformations in the hTERT promoter. *J Am Chem Soc*, **132**, 12331-12342.
 179. Cornell, W.D., Cieplak, P., Bayly, C.I., Gould, I.R., Merz, K.M., Ferguson, D.M., Spellmeyer, D.C., Fox, T., Caldwell, J.W. and Kollman, P.A. (1995) A second generation force field for the simulation of proteins, nucleic acids, and organic molecules. *J Am Chem Soc*, **117**, 3181-3198.
 180. Martadinata, H., Heddi, B., Lim, K.W. and Phan, A.T. (2011) Structure of long human telomeric RNA (TERRA): G-quadruplexes formed by four and eight UUAGGG repeats are stable building blocks. *Biochemistry*, **50**, 6455-6461.
 181. Kato, Y., Ohyama, T., Mita, H. and Yamamoto, Y. (2005) Dynamics and thermodynamics of dimerization of parallel G-quadruplexed DNA formed from d(TTAG_n) (n=3-5). *J Am Chem Soc*, **127**, 9980-9981.
 182. Do, N.Q. and Phan, A.T. (2012) Monomer-dimer equilibrium for the 5'-5' stacking of propeller-type parallel-stranded G-quadruplexes: NMR structural study. *Chemistry*, **18**, 14752-14759.
 183. Yu, H.Q., Miyoshi, D. and Sugimoto, N. (2006) Characterization of structure and stability of long telomeric DNA G-quadruplexes. *J Am Chem Soc*, **128**, 15461-15468.
 184. Xu, Y., Ishizuka, T., Kurabayashi, K. and Komiyama, M. (2009) Consecutive formation of G-quadruplexes in human telomeric-overhang DNA: a protective capping structure for telomere ends.

- Angew Chem Int Ed Engl*, **48**, 7833-7836.
185. Collie, G.W., Parkinson, G.N., Neidle, S., Rosu, F., De Pauw, E. and Gabelica, V. (2010) Electrospray mass spectrometry of telomeric RNA (TERRA) reveals the formation of stable multimeric G-quadruplex structures. *J Am Chem Soc*, **132**, 9328-9334.
 186. Merino, E.J., Boal, A.K. and Barton, J.K. (2008) Biological contexts for DNA charge transport chemistry. *Curr Opin Chem Biol*, **12**, 229-237.
 187. Schuster, G.B. (2004) *Long-Range Charge Transfer in DNA, I and II; Topics in Current Chemistry*. Springer.
 188. Genereux, J.C. and Barton, J.K. (2010) Mechanisms for DNA charge transport. *Chem Rev*, **110**, 1642-1662.
 189. Venkatramani, R., Keinan, S., Balaeff, A. and Beratan, D.N. (2011) Nucleic acid charge transfer: Black, white and gray. *Coord Chem Rev*, **255**, 635-648.
 190. Siri Wong, K. and Voityuk, A.A. (2012) Electron transfer in DNA. *Wires Comput Mol Sci*, **2**, 780-794.
 191. Voityuk, A.A., Siri Wong, K. and Rosch, N. (2001) Charge transfer in DNA. Sensitivity of electronic couplings to conformational changes. *Phys Chem Chem Phys*, **3**, 5421-5425.
 192. Troisi, A. and Orlandi, G. (2002) Hole migration in DNA: a theoretical analysis of the role of structural fluctuations. *J Phys Chem B*, **106**, 2093-2101.
 193. von Feilitzsch, T., Tuma, J., Neubauer, H., Verdier, L., Haselsberger, R., Feick, R., Gurzadyan, G., Voityuk, A.A., Griesinger, C. and Michel-Beyerle, M.E. (2008) Chromophore/DNA interactions: Femto- to nanosecond spectroscopy, NMR structure, and electron transfer theory. *J Phys Chem B*, **112**, 973-989.
 194. Grozema, F.C., Tonzani, S., Berlin, Y.A., Schatz, G.C., Siebbeles, L.D.A. and Ratner, M.A. (2008) Effect of structural dynamics on charge transfer in DNA hairpins. *J Am Chem Soc*, **130**, 5157-5166.
 195. Hatcher, E., Balaeff, A., Keinan, S., Venkatramani, R. and Beratan, D.N. (2008) PNA versus DNA: Effects of structural fluctuations on electronic structure and hole-transport mechanisms. *J Am Chem Soc*, **130**, 11752-11761.
 196. Berlin, Y.A., Voityuk, A.A. and Ratner, M.A. (2012) DNA base pair stacks with high electric conductance: A systematic structural search. *ACS Nano*, **6**, 8216-8225.
 197. Kotlyar, A.B., Borovok, N., Molotsky, T., Cohen, H., Shapir, E. and Porath, D. (2005) Long, monomolecular guanine-based nanowires. *Adv Mater*, **17**, 1901-1905.
 198. Borovok, N., Iram, N., Zikich, D., Ghabboun, J., Livshits, G.I., Porath, D. and Kotlyar, A.B. (2008) Assembling of G-strands into novel tetra-molecular parallel G4-DNA nanostructures using avidinbiotin recognition. *Nucleic Acids Res*, **36**, 5050-5060.
 199. Shapir, E., Sagiv, L., Molotsky, T., Kotlyar, A.B., Di Felice, R. and Porath, D. (2010) Electronic structure of G4-DNA by scanning tunneling spectroscopy. *J Phys Chem C*, **114**, 22079-22084.
 200. Davis, J.T. (2004) G-quartets 40 years later: From 5'-GMP to molecular biology and supramolecular chemistry. *Angew Chem Int Edit*, **43**, 668-698.
 201. Choi, J., Park, J., Tanaka, A., Park, M.J., Jang, Y.J., Fujitsuka, M., Kim, S.K. and Majima, T. (2013) Hole trapping of G-quartets in a G-quadruplex. *Angew Chem Int Edit*, **52**, 1134-1138.
 202. Delaney, S. and Barton, J.K. (2003) Charge transport in DNA duplex/quadruplex conjugates. *Biochemistry*, **42**, 14159-14165.
 203. de Champdore, M., De Napoli, L., Montesarchio, D., Piccialli, G., Caminal, C., Mulazzani, Q.G., Navacchia, M.L. and Chatgililoglu, C. (2004) Excess electron transfer in G-quadruplex. *Chem Commun*, **15**, 1756-1757.
 204. Ndlebe, T. and Schuster, G.B. (2006) Long-distance radical cation transport in DNA: horizontal charge hopping in a dimeric quadruplex. *Org Biomol Chem*, **4**, 4015-4021.
 205. Huang, Y.C., Cheng, A.K.H., Yu, H.Z. and Sen, D. (2009) Charge conduction properties of a parallel-stranded DNA G-quadruplex: Implications for chromosomal oxidative damage. *Biochemistry*, **48**, 6794-6804.
 206. Cohen, H., Sapir, T., Borovok, N., Molotsky, T., Di Felice, R., Kotlyar, A.B. and Porath, D. (2007) Polarizability of G4-DNA observed by electrostatic force microscopy measurements. *Nano Lett*, **7**, 981-986.
 207. Šponer, J. and Lankas, F. (2006) *Computational studies of RNA and DNA*. Springer, Dordrecht.
 208. Cavallari, M., Calzolari, A., Garbesi, A. and Di Felice, R. (2006) Stability and migration of metal ions in G4-wires by molecular dynamics simulations. *J Phys Chem B*, **110**, 26337-26348.

209. Calzolari, A., Di Felice, R., Molinari, E. and Garbesi, A. (2004) Electron channels in biomolecular nanowires. *J Phys Chem B*, **108**, 2509-2515.
210. Di Felice, R., Calzolari, A., Garbesi, A., Alexandre, S.S. and Soler, J.M. (2005) Strain-dependence of the electronic properties in periodic quadruple helical G4-wires. *J Phys Chem B*, **109**, 22301-22307.
211. Woiczikowski, P.B., Kubar, T., Gutierrez, R., Cuniberti, G. and Elstner, M. (2010) Structural stability versus conformational sampling in biomolecular systems: Why is the charge transfer efficiency in G4-DNA better than in double-stranded DNA? *J Chem Phys*, **133**, 035103.
212. Karsisiotis, A.I., Hessari, N.M., Novellino, E., Spada, G.P., Randazzo, A. and da Silva, M.W. (2011) Topological characterization of nucleic acid G-quadruplexes by UV absorption and circular dichroism. *Angew Chem Int Edit*, **50**, 10645-10648.
213. Voityuk, A.A. and Rosch, N. (2002) Fragment charge difference method for estimating donor-acceptor electronic coupling: Application to DNA pi-stacks. *J Chem Phys*, **117**, 5607-5616.
214. Voityuk, A.A. (2012) Electronic coupling for charge transfer in donor-bridge-acceptor systems. Performance of the two-state FCD model. *Phys Chem Chem Phys*, **14**, 13789-13793.
215. Ridley, J. and Zerner, M. (1973) Intermediate Neglect of Differential Overlap Technique for Spectroscopy - Pyrrole and Azines. *Theor Chim Acta*, **32**, 111-134.
216. Voityuk, A.A. (2006) Assessment of semiempirical methods for the computation of charge transfer in DNA pi-stacks. *Chem Phys Lett*, **427**, 177-180.
217. Marcus, R.A. and Sutin, N. (1985) Electron Transfers in Chemistry and Biology. *Biochimica Et Biophysica Acta*, **811**, 265-322.
218. Davis, W.B., Hess, S., Naydenova, I., Haselsberger, R., Ogrodnik, A., Newton, M.D. and Michel-Beyerle, M.E. (2002) Distance-dependent activation energies for hole injection from protonated 9-amino-6-chloro-2-methoxyacridine into duplex DNA. *J Am Chem Soc*, **124**, 2422-2423.
219. Siriwong, K., Voityuk, A.A., Newton, M.D. and Rosch, N. (2003) Estimate of the reorganization energy for charge transfer in DNA. *J Phys Chem B*, **107**, 2595-2601.
220. LeBard, D.N., Lilichenko, M., Matyushov, D.V., Berlin, Y.A. and Ratner, M.A. (2003) Solvent reorganization energy of charge transfer in DNA hairpins. *J Phys Chem B*, **107**, 14509-14520.
221. Kubar, T. and Elstner, M. (2009) Solvent reorganization energy of hole transfer in DNA. *J Phys Chem B*, **113**, 5653-5656.
222. Haaima, G., Hansen, H.F., Christensen, L., Dahl, O. and Nielsen, P.E. (1997) Increased DNA binding and sequence discrimination of PNA oligomers containing 2,6-diaminopurine. *Nucleic Acids Res*, **25**, 4639-4643.
223. Diaz-Mochon, J.J., Bialy, L., Watson, J., Sanchez-Martin, R.M. and Bradley, M. (2005) Synthesis and cellular uptake of cell delivering PNA-peptide conjugates. *Chem Commun*, **26**, 3316-3318.
224. Paul, A., Sengupta, P., Krishnan, Y. and Ladame, S. (2008) Combining G-quadruplex targeting motifs on a single peptide nucleic acid scaffold: A hybrid (3+1) PNA-DNA bimolecular quadruplex. *Chem-Eur J*, **14**, 8682-8689.
225. Singh, S.K., Nielsen, P., Koshkin, A.A. and Wengel, J. (1998) LNA (locked nucleic acids): synthesis and high-affinity nucleic acid recognition. *Chem Commun*, **4**, 455-456.
226. Sorensen, J.J., Nielsen, J.T. and Petersen, M. (2004) Solution structure of a dsDNA : LNA triplex. *Nucleic Acids Res*, **32**, 6078-6085.
227. Vester, B., Lundberg, L.B., Sorensen, M.D., Babu, B.R., Douthwaite, S. and Wengel, J. (2002) LNAzymes: Incorporation of LNA-type monomers into DNAzymes markedly increases RNA cleavage. *J Am Chem Soc*, **124**, 13682-13683.
228. Randazzo, A., Esposito, V., Ohlenschlager, O., Ramachandran, R., Virgilio, A. and Mayol, L. (2005) Structural studies on LNA quadruplexes. *Nucleos Nucleot Nucl*, **24**, 795-800.
229. Dominick, P.K. and Jarstfer, M.B. (2004) A conformationally constrained nucleotide analogue controls the folding topology of a DNA G-quadruplex. *J Am Chem Soc*, **126**, 5050-5051.
230. Pedersen, E.B., Nielsen, J.T., Nielsen, C. and Filichev, V.V. (2011) Enhanced anti-HIV-1 activity of G-quadruplexes comprising locked nucleic acids and intercalating nucleic acids. *Nucleic Acids Res*, **39**, 2470-2481.
231. Bonifacio, L., Church, F.C. and Jarstfer, M.B. (2008) Effect of locked-nucleic acid on a biologically active G-quadruplex. A structure-activity relationship of the thrombin aptamer. *Int J Mol Sci*, **9**, 422-433.
232. Adrian, M., Heddi, B. and Phan, A.T. (2012) NMR spectroscopy of G-quadruplexes. *Methods*, **57**, 11-

- 24.
233. Crnugelj, M., Sket, P. and Plavec, J. (2003) Small change in a G-rich sequence, a dramatic change in topology: New dimeric G-quadruplex folding motif with unique loop orientations. *J Am Chem Soc*, **125**, 7866-7871.
234. Vorlickova, M., Kejnovska, I., Sagi, J., Renciuik, D., Bednarova, K., Motlova, J. and Kypr, J. (2012) Circular dichroism and guanine quadruplexes. *Methods*, **57**, 64-75.
235. Avino, A., Mazzini, S., Ferreira, R., Gargallo, R., Marquez, V.E. and Eritja, R. (2012) The effect on quadruplex stability of North-nucleoside derivatives in the loops of the thrombin-binding aptamer. *Bioorg Med Chem*, **20**, 4186-4193.
236. He, G.X., Williams, J.P., Postich, M.J., Swaminathan, S., Shea, R.G., Terhorst, T., Law, V.S., Mao, C.T., Sueoka, C., Coutre, S. *et al.* (1998) In vitro and in vivo activities of oligodeoxynucleotide-based thrombin inhibitors containing neutral formacetal linkages. *J Med Chem*, **41**, 4224-4231.
237. Mendelboun Raviv, S., Horvath, A., Aradi, J., Bagoly, Z., Fazakas, F., Batta, Z., Muszbek, L. and Harsfalvi, J. (2008) 4-thio-deoxyuridylate-modified thrombin aptamer and its inhibitory effect on fibrin clot formation, platelet aggregation and thrombus growth on subendothelial matrix. *J Thromb Haemost*, **6**, 1764-1771.
238. Bishop, J.S., Guy-Caffey, J.K., Ojwang, J.O., Smith, S.R., Hogan, M.E., Cossum, P.A., Rando, R.F. and Chaudhary, N. (1996) Intramolecular G-quartet motifs confer nuclease resistance to a potent anti-HIV oligonucleotide. *J Biol Chem*, **271**, 5698-5703.
239. Koizumi, M., Koga, R., Hotoda, H., Ohmine, T., Furukawa, H., Agatsuma, T., Nishigaki, T., Abe, K., Kosaka, T., Tsutsumi, S. *et al.* (1998) Biologically active oligodeoxyribonucleotides. Part 11: The least phosphate-modification of quadruplex-forming hexadeoxyribonucleotide TGGGAG, bearing 3- and 5-end-modification, with anti-HIV-1 activity. *Bioorg Med Chem*, **6**, 2469-2475.
240. Kuwasaki, T., Hatta, M., Takeuchi, H. and Takaku, H. (2003) Inhibition of human immunodeficiency virus 1 replication in vitro by a self-stabilized oligonucleotide with 2'-O-methyl-guanosine-uridine quadruplex motifs. *J Antimicrob Chemother*, **51**, 813-819.
241. Urata, H., Kumashiro, T., Kawahata, T., Otake, T. and Akagi, M. (2004) Anti-HIV-1 activity and mode of action of mirror image oligodeoxynucleotide analogue of Zintevir. *Biochem Biophys Res Commun*, **313**, 55-61.
242. Deleavey, G.F., Watts, J.K., Alain, T., Robert, F., Kalota, A., Aishwarya, V., Pelletier, J., Gewirtz, A.M., Sonenberg, N. and Damha, M.J. (2010) Synergistic effects between analogs of DNA and RNA improve the potency of siRNA-mediated gene silencing. *Nucleic Acids Res*, **38**, 4547-4557.
243. Pallan, P.S., Greene, E.M., Jicman, P.A., Pandey, R.K., Manoharan, M., Rozners, E. and Egli, M. (2011) Unexpected origins of the enhanced pairing affinity of 2'-fluoro-modified RNA. *Nucleic Acids Res*, **39**, 3482-3495.
244. Martin-Pintado, N., Yahyaee-Anzahae, M., Deleavey, G.F., Portella, G., Orozco, M., Damha, M.J. and Gonzalez, C. (2013) Dramatic effect of furanose c2' substitution on structure and stability: directing the folding of the human telomeric quadruplex with a single fluorine atom. *J Am Chem Soc*, **135**, 5344-5347.
245. Watts, J.K., Martin-Pintado, N., Gomez-Pinto, I., Schwartzentruber, J., Portella, G., Orozco, M., Gonzalez, C. and Damha, M.J. (2010) Differential stability of 2' F-ANA.RNA and ANA.RNA hybrid duplexes: roles of structure, pseudohydrogen bonding, hydration, ion uptake and flexibility. *Nucleic Acids Res*, **38**, 2498-2511.
246. Heddi, B. and Phan, A.T. (2011) Structure of human telomeric DNA in crowded solution. *J Am Chem Soc*, **133**, 9824-9833.
247. Phan, A.T., Kuryavyi, V., Gaw, H.Y. and Patel, D.J. (2005) Small-molecule interaction with a five-guanine-tract G-quadruplex structure from the human MYC promoter. *Nat Chem Biol*, **1**, 167-173.
248. Hu, L.Y., Lim, K.W., Bouaziz, S. and Phan, A.T. (2009) Giardia Telomeric Sequence d(TAGGG)(4) Forms Two Intramolecular G-Quadruplexes in K⁺ Solution: Effect of Loop Length and Sequence on the Folding Topology. *J Am Chem Soc*, **131**, 16824-16831.
249. Xu, Y., Noguchi, Y. and Sugiyama, H. (2006) The new models of the human telomere d[AGGG(TTAGGG)(3)] in K⁺ solution. *Bioorgan Med Chem*, **14**, 5584-5591.
250. Dumas, A. and Luedtke, N.W. (2010) Cation-mediated energy transfer in G-quadruplexes revealed by an internal fluorescent probe. *J Am Chem Soc*, **132**, 18004-18007.
251. Grollman, A.P. and Moriya, M. (1993) Mutagenesis by 8-oxoguanine - an enemy within. *Trends Genet*,

- 9, 246-249.
252. David, S.S., O'Shea, V.L. and Kundu, S. (2007) Base-excision repair of oxidative DNA damage. *Nature*, **447**, 941-950.
253. Tan, X.Z., Suzuki, N., Johnson, F., Grollman, A.P. and Shibutani, S. (1999) Mutagenic properties of the 8-amino-2'-deoxyguanosine DNA adduct in mammalian cells. *Nucleic Acids Res*, **27**, 2310-2314.
254. Venkatarangan, L., Sivaprasad, A., Johnson, F. and Basu, A.K. (2001) Site-specifically located 8-amino-2'-deoxyguanosine: thermodynamic stability and mutagenic properties in *Escherichia coli*. *Nucleic Acids Res*, **29**, 1458-1463.
255. Shrivastav, N., Li, D.Y. and Essigmann, J.M. (2010) Chemical biology of mutagenesis and DNA repair: cellular responses to DNA alkylation. *Carcinogenesis*, **31**, 59-70.
256. Luu, K.N., Phan, A.T., Kuryavyi, V., Lacroix, L. and Patel, D.J. (2006) Structure of the human telomere in K⁺ solution: An intramolecular (3+1) G-quadruplex scaffold. *J Am Chem Soc*, **128**, 9963-9970.
257. Gray, D.M., Wen, J.D., Gray, C.W., Repges, R., Repges, C., Raabe, G. and Fleischhauer, J. (2008) Measured and calculated CD spectra of G-quartets stacked with the same or opposite polarities. *Chirality*, **20**, 431-440.
258. Mergny, J.L., Phan, A.T. and Lacroix, L. (1998) Following G-quartet formation by UV-spectroscopy. *FEBS Letters*, **435**, 74-78.
259. Phan, A.T. and Patel, D.J. (2002) A site-specific low-enrichment N-15, C-13 isotope-labeling approach to unambiguous NMR spectral assignments in nucleic acids. *J Am Chem Soc*, **124**, 1160-1161.
260. Hehre, W.J., Radom, L., Schleyer, P.V. and Pople, J.A. (1986) *Ab Initio Molecular Orbital Theory*. Wiley-Interscience.
261. de la Osa, J.P., Gonzalez, C., Gargallo, R., Rueda, M., Cubero, E., Orozco, M., Avino, A. and Eritja, R. (2006) Destabilization of quadruplex DNA by 8-aminoguanine. *Chembiochem*, **7**, 46-48.
262. Kumar, G.A. and McAllister, M.A. (1998) Theoretical investigation of the relationship between proton NMR chemical shift and hydrogen bond strength. *J Org Chem*, **63**, 6968-6972.
263. Barfield, M., Dingley, A.J., Feigon, J. and Grzesiek, S. (2001) A DFT study of the interresidue dependencies of scalar J-coupling and magnetic shielding in the hydrogen-bonding regions of a DNA tripler. *J Am Chem Soc*, **123**, 4014-4022.
264. Dingley, A.J., Peterson, R.D., Grzesiek, S. and Feigon, J. (2005) Characterization of the cation and temperature dependence of DNA quadruplex hydrogen bond properties using high-resolution NMR. *J Am Chem Soc*, **127**, 14466-14472.
265. Koizumi, M., Koga, R., Hotoda, H., Momota, K., Ohmine, T., Furukawa, H., Agatsuma, T., Nishigaki, T., Abe, K., Kosaka, T. *et al.* (1997) Biologically active oligodeoxyribonucleotides - IX. Synthesis and anti-HIV-1 activity of hexadeoxyribonucleotides, TGGGAG, bearing 3'- and 5'-end-modification. *Bioorgan Med Chem*, **5**, 2235-2243.
266. Juskowiak, B. (2006) Analytical potential of the quadruplex DNA-based FRET probes. *Anal Chim Acta*, **568**, 171-180.
267. Liu, X.Y., Kwan, I.C.M., Wang, S.N. and Wu, G. (2006) G-quartet formation from an N-2-modified guanosine derivative. *Organic Letters*, **8**, 3685-3688.
268. Kaucher, M.S. and Davis, J.T. (2006) N2, C8-disubstituted guanosine derivatives can form G-quartets. *Tetrahedron Lett*, **47**, 6381-6384.
269. Martic, S., Liu, X.Y., Wang, S.N. and Wu, G. (2008) Self-assembly of N-2-modified guanosine derivatives: Formation of discrete G-octamers. *Chem-Eur J*, **14**, 1196-1204.
270. Rachwal, P.A. and Fox, K.R. (2007) Quadruplex melting. *Methods*, **43**, 291-301.
271. Dao, N.T., Haselsberger, R., Michel-Beyerle, M.E. and Phan, A.T. (2013) Excimer Formation by Stacking G-Quadruplex Blocks. *Chemphyschem*, **14**, 2667-2671.
272. Moller, C. and Plesset, M.S. (1934) Note on an approximation treatment for many-electron systems. *Phys Rev*, **46**, 0618-0622.
273. Frisch, M.J.T., G. W.; Schlegel, H. B.; Scuseria, G. E.; Robb, M. A.; Cheeseman, J. R.; Montgomery, Jr., J. A.; Vreven, T.; Kudin, K. N.; Burant, J. C.; Millam, J. M.; Iyengar, S. S.; Tomasi, J.; Barone, V.; Mennucci, B.; Cossi, M.; Scalmani, G.; Rega, N.; Petersson, G. A.; Nakatsuji, H.; Hada, M.; Ehara, M.; Toyota, K.; Fukuda, R.; Hasegawa, J.; Ishida, M.; Nakajima, T.; Honda, Y.; Kitao, O.; Nakai, H.; Klene, M.; Li, X.; Knox, J. E.; Hratchian, H. P.; Cross, J. B.; Adamo, C.; Jaramillo, J.; Gomperts, R.; Stratmann, R. E.; Yazyev, O.; Austin, A. J.; Cammi, R.; Pomelli, C.; Ochterski, J. W.; Ayala, P. Y.

- Morokuma, K.; Voth, G. A.; Salvador, P.; Dannenberg, J. J.; Zakrzewski, V. G.; Dapprich, S.; Daniels, A. D.; Strain, M. C.; Farkas, O.; Malick, D. K.; Rabuck, A. D.; Raghavachari, K.; Foresman, J. B.; Ortiz, J. V.; Cui, Q.; Baboul, A. G.; Clifford, S.; Cioslowski, J.; Stefanov, B. B.; Liu, G.; Liashenko, A.; Piskorz, P.; Komaromi, I.; Martin, R. L.; Fox, D. J.; Keith, T.; Al-Laham, M. A.; Peng, C. Y.; Nanayakkara, A.; Challacombe, M.; Gill, P. M. W.; Johnson, B.; Chen, W.; Wong, M. W.; Gonzalez, C.; and Pople, J. A.; (2003). Gaussian, Inc., Pittsburgh PA, pp. and references therein.
274. Hobza, P., Schneider, B., Carsky, P. and Zahradnik, R. (1986) The Superposition Error Problem - the (Hf)₂ and (H₂O)₂ Complexes at the Scf and Mp2 Levels. *Theochem-J Mol Struc*, **31**, 377-385.
 275. Case, D.A., Cheatham, T.E., Darden, T., Gohlke, H., Luo, R., Merz, K.M., Onufriev, A., Simmerling, C., Wang, B. and Woods, R.J. (2005) The Amber biomolecular simulation programs. *J Comput Chem*, **26**, 1668-1688.
 276. Perez, A., Marchan, I., Svozil, D., Sponer, J., Cheatham, T.E., Loughton, C.A. and Orozco, M. (2007) Refinement of the AMBER force field for nucleic acids: Improving the description of alpha/gamma conformers. *Biophys J*, **92**, 3817-3829.
 277. Dupradeau, F.Y., Pigache, A., Zaffran, T., Savineau, C., Lelong, R., Grivel, N., Lelong, D., Rosanski, W. and Cieplak, P. (2010) The R.E.D. tools: advances in RESP and ESP charge derivation and force field library building. *Phys Chem Chem Phys*, **12**, 7821-7839.
 278. Jorgensen, W.L., Chandrasekhar, J., Madura, J.D., Impey, R.W. and Klein, M.L. (1983) Comparison of simple potential functions for simulating liquid water. *J Chem Phys*, **79**, 926-935.
 279. Berendsen, H.J.C., Postma, J.P.M., Vangunsteren, W.F., Dinola, A. and Haak, J.R. (1984) Molecular-dynamics with coupling to an external bath. *J Chem Phys*, **81**, 3684-3690.
 280. Vangunsteren, W.F. and Berendsen, H.J.C. (1977) Algorithms for macromolecular dynamics and constraint dynamics. *Mol Phys*, **34**, 1311-1327.
 281. Darden, T., York, D. and Pedersen, L. (1993) Particle mesh ewald - an N.Log(N) method for ewald sums in large systems. *J Chem Phys*, **98**, 10089-10092.
 282. Plateau, P. and Gueron, M. (1982) Exchangeable proton NMR without base-line distortion, Using new strong-pulse sequences. *J Am Chem Soc*, **104**, 7310-7311.
 283. Phan, A.T., Gueron, M. and Leroy, J.L. (2001) Investigation of unusual DNA motifs. *Method Enzymol*, **338**, 341-371.
 284. Frisch, M.J., Trucks, G.W., Schlegel, H.B., Scuseria, G.E., Robb, M.A., Cheeseman, J.R., Montgomery, J.A., Jr., Vreven, T., Kudin, K.N., Burant, J.C. *et al.* 2004. Gaussian 03, Rev. e.01. Gaussian, Wallingford, CT.
 285. Ditchfield, R. (1974) Self-consistent perturbation-theory of diamagnetism .1. Gauge-invariant LCAO Method for NMR chemical-shifts. *Mol Phys*, **27**, 789-807.

Appendix I – List of Publications

Lech, C.J., Phan, A.T. (2014) Ball with hair: Functionalization of extremely stable G-quadruplex nanoparticles through N2-guanine modifications. (in review)

Li, Z.*, Lech, C.J.*, Phan, A.T. (2014) Sugar-modified G-quadruplexes: effects of LNA-, 2'-F-RNA- and 2'-F-ANA-guanosine chemistries on G-quadruplex structure and stability. *Nucleic Acids Research*, [in press]. (* Denotes co-first authorship)

Lech, C.J., Phan, A.T., Michel-Beyerle, M., Voityuk, A.A. (2013) Electron-hole transfer in G-quadruplexes with different tetrad stacking geometries: A combined QM and MD study. *Journal of Physical Chemistry B*, 117, 9851-9856

Lech, C.J., Heddi, B., Phan, A.T. (2013) Guanine base stacking in G-quadruplex Nucleic Acids. *Nucleic Acids Research*, 41 (3). 2034-2046.

Lech, C.J.*, Zhe, Li.*, Heddi, B., Phan, A.T. (2012) 2'-F-ANA-guanosine and 2'-F-guanosine as powerful tools for structural manipulation of G-quadruplexes. *Chemical Communications*. 48. 11425-11427. (* Denotes co-first authorship)

Lech, C.J., Lim, J.K.C., Lim, J.M.W., Amrane, S., Heddi, B., Phan, A.T. (2011) Effects of Site-Specific Guanine C8-Modifications on an intramolecular DNA G-quadruplex. *Biophysical Journal*. 101. 1987-1996.

Appendix II – Protocols

Chapter 2: Fundamentals of G-quadruplexes: Base Stacking and High-Order Assemblies

Model Tetrads

Tetrad models were created by first performing QM geometry optimization of a single G-tetrad at the MP2/6-31(d,p) level (260,272). Optimized structures consisted of planar G-tetrads displaying C₄-symmetry stabilized by two centrally located cations, located above and below the tetrad plane. Optimized tetrads were then used to create models used for single-point energy calculations which contain two parallel centered planar G-tetrads with a single, central K⁺ ion. The geometries of stacked planar tetrads were varied by their separation (d_{tet}) and relative rotation (θ_{tet}).

Structural Characterization

A survey of G-quadruplex structures published in the Protein Data Bank was carried out using the following keywords: quadruplex(es); tetrad(s); tetraplex(es). Search results of X-ray crystallographic structures of G-quadruplexes containing neighboring G-tetrads as of 16th June, 2011 are presented. A selection of NMR G-quadruplex solution structures containing stacked G-tetrads is also presented. The relative rotation (θ_{tet}) and separation (d_{tet}) of individual pairs of stacked guanine bases in experimentally determined structures were quantified by comparison with a library of stacked guanines taken from our models of stacked tetrads with geometries varying in rotation and separation with increments of 0.05° and 0.005 Å, respectively. The characterization of guanines within G-quadruplexes involved the alignment of over 1000 experimentally determined stacked guanine pairs (from 40 crystallographic and 16 NMR PDB structures) to a library of model stacked guanines. The pair of model stacked guanines that best fit a given experimental pair was determined by the lowest RMSD (C,N,O atoms) alignment of the guanine bases. A single RMSD minimum was observed in rotation-separation space for every guanine pair cataloged. Stacked guanines within G-tetrads tend to be parallel although G-tetrads are not always planar. Characterization of individual stacked base separations is an accurate measure of inter-base stacking distance and is relevant to our computational study involving planar and parallel G-tetrad stacks. Structural characterization code was written using MATLAB software (version R2008B, The Mathworks Inc.). Structural visualization was performed using PyMOL molecular visualization software (version 0.99, <http://www.pymol.org/>).

Figure A2-1: Experimental Characterization Procedure: Stacked guanine bases from experimental tetrads are fit to stacked guanines from a library of model stacked guanine tetrads. The relative rotation (θ_{tet}) and separation ($dtet$) are determined by the model stacked guanine pair that provides the lowest RMSD fit to the experimental guanine pair.

Quantum Mechanical Computations

QM computations were carried out using the Gaussian 03 software (273). Single-point interaction energy calculations used to create energy landscapes were performed at the MP2 level (272) using the modified split valance basis set 6-31G*(0.25) (274) in which the polarization d-function exponent from the standard 6-31G* basis set (260) is changed from 0.8 to 0.25 for first row atoms (Carbon, Nitrogen, Oxygen) as well as for Potassium ions. Previous works by Hobza et al. (137) and Šponer et al. (140) have demonstrated the 6-31G*(0.25) basis set to be an inexpensive choice for modeling stacking interactions. The 6-31G*(0.25) basis set was further tested here.

Energy landscapes were determined for both same-polarity and opposite-polarity stacked G-tetrads. Single-point interaction energy was computed over a tetrad separation distance (d_{tet}) of 3.0-3.6 Å with a 0.1 Å resolution and a relative rotation angle (θ_{tet}) of 0° – 90° with 5° resolution. In total, 266 single-point interaction energy calculations were performed at the MP2/6-31G*(0.25) level. Single-point energy calculations were also performed for average geometries of base stacking modes observed in crystal structures at the MP2/6-311+G(2d,2p) level (260,272). Interaction energies (ΔE) in this work have been determined using the counterpoise procedure (161) to correct for basis set superposition error treating each tetrad layer and central ion as individual fragments.

Molecular Mechanics and Dynamic Computations

MM and MD computations were performed with the AMBER 10 software (275) using the force field of Cornell et al. (179) with the Parmbsc0 force field modification (276) commonly employed for nucleic acid simulations. The intermolecular terms effecting the energy landscape of MM computed stacked G-tetrads include Van der Waals and electrostatic terms as described by the original force field of Cornell et al. (179). Single-point energy calculations used to generate energy landscapes were performed using the same models employed in QM computation. Energy landscape calculations were performed in vacuum with no cutoff used for non-bonded interactions. Force field parameters for isolated guanines used in our models were derived by standard AMBER protocols. RESP partial atomic charges were calculated at HF/6-31G* level using the R.E.D. software (277).

Molecular dynamic simulations of stacked G-quadruplexes were generated from an NMR based model of the sequence d[TT(G₃T)₄] (182). Flanking ends were removed to generate a model for the d[(G₃T)₃G₃] sequence used in our simulations. The chemistry of strand termini were modified to reflect that observed in experimentally synthesized oligonucleotides with the 3'-end capped with a C3'-O3'-H chemistry and the 5'-end capped with a C5'-O5'-H chemistry. Two individual G-quadruplex units were then manually stacked at their 5'-5' and 3'-3' stacking. The relative rotation of these units was varied in ~30° intervals to generate 3 unique starting structures for each of the 5'-5' and 3'-3' stacking models. Starting structures ranged over two of the four possible stacking quadrants. K⁺ ions were manually added between tetrads in the core and at the interface of stacked G-quadruplexes. Additional K⁺ ions (for a total of 28) were added to neutralize the systems. The systems were solvated with a TIP3P (278) water truncated octahedral box with water molecules ranging in number from 5,500-7,500 over the models explored.

Periodic boundary conditions were used in our simulations. Pressure and temperature were held constant at 1 bar and 300K respectively according to the Berendsen algorithm (279). A 2-fs time step was used while constraining covalent hydrogen bonds using SHAKE (280). The particle mesh Ewald (PME) method (281) was used to calculate long range electrostatic interactions. Non-bonded interactions were calculated within a 9 Å cutoff in our MD simulations and the list of non-bonded pairs was updated and the center-of-mass motion was removed at 10 ps intervals. Systems underwent a series of initial constrained minimizations and equilibration dynamics. Systems were first minimized with harmonic potential position restraints (25 kcal.mol⁻¹.Å⁻²) on DNA atoms and ions within the G-quadruplex core/interface over 500 steps of steepest decent minimization followed by 500 step of conjugated gradient minimization. Systems were then heated from 100 to 300K over 10 ps and equilibrated at 300K for 90 ps under constant volume while maintaining 25 kcal.mol⁻¹.Å⁻² position restraints on above mentioned atoms. Systems underwent further steps of minimization and equilibration in which the positional restraints were gradually reduced to 5, 4, 3, 2, 1, and 0.5 kcal.mol⁻¹.Å⁻². We take the start of simulations as the first segment of unrestrained dynamic simulation. Structures were examined every 25 ps in the first 3 ns and every 100 ps thereafter. The guanines at the interface were compared to model stacked guanines of variable separation and relative rotation. All four stacked guanine pairs at the interface were cataloged for each snap shot and the average rotational values are presented.

Chapter 3: Fundamentals of G-quadruplexes: Charge Transport

Molecular Dynamics simulations

Molecular Dynamics simulations (MD) were performed with the AMBER program using the Parmbsc0 correction to the Cornell et al. force field (179,275,276) in explicit solvent in the presence of neutralizing K^+ ions described previously (see Chapter 2 Methods and Materials). The G-tetrad stacking modes adopted by a given G-quadruplex are dependent on structural conformation and the ability to form stacked dimers. Considering this, we explore multiple MD systems in order to obtain data on all 5 of the previously observed G-tetrad stacking modes: 1) Complexes of parallel G-quadruplexes stacked in different orientations were examined. G-quadruplex structures formed by the $d[(G_3T)_3G_3]$ sequences were derived from an NMR based model (182). Simulations of 200 ns were run for various orientations of stacked G-quadruplexes. 2) Simulations of the (2+2) anti-parallel G-quadruplex structure formed by the sequence $d[(G_4T_4G_4)_2]$ were performed using the same protocol with a simulation time of 5ns. The starting structure for the simulation of $d[(G_4T_4G_4)_2]$ was taken from a crystal structure ID 1JRN (170).

Quantum Chemical Calculations

G-tetrad stacks examined at the QM level were taken from 1ns segments of above mentioned MD trajectories. Snapshots were analyzed at 1ps intervals leading to 1000 unique G-tetrad stacks for each G-tetrad stacking mode. G-tetrad models were created by stripping away backbone/sugar atoms from the G-quadruplex and isolating a single stack G-tetrads including the coordinated K^+ ion from MD snapshots. The C1' atom of the sugar moiety was replaced by a hydrogen atom with the bond length adjusted to 1.08 Å. The structures were examined using the INDO/S method (215). As shown previously, the INDO/S scheme allows one to properly describe electronic coupling of stacked nucleobases (216). For each G-tetrad stack, eight electronic states of the lowest energy corresponding to the radical cation were analyzed. These states were represented by 8 highest occupied MOs (HOMOs) of the neutral species (HOMO→HOMO-7) and are associated with each of the eight guanine bases in the G-tetrad stack.

Several types of control calculations were performed. To test the effects of electrostatic contributions from solvent on coupling matrix elements, models with and without electrostatic embedding were treated. Point charges taken from the Cornell et al. force field were placed at the coordinates of atoms in the fully represented systems consisting of nucleobases and the sugar-phosphate backbone hydrated in water and K^+ ions. We also tested the effects of excluding the coordinating K^+ ion from the stack. It was found that while these changes affected the energies of the hole states, they did not significantly affect the electronic coupling of stacked guanines.

Chapter 4: Engineering the Sugar Backbone: Locked Nucleic Acid

DNA sample preparation

All DNA sequences used in this study were synthesized on a Applied Biosystems 394 DNA synthesizer (Foster City, CA, USA). ^{LNA}G phosphoramidite was purchased from ScienceWerke (Singapore). Deuterium-labeled guanine phosphoramidites was purchased from Glen Research (Sterling, VA, USA). After purification, samples were dialyzed successively against de-ionised water, KCl solution (25 mM) and de-ionised water again. After lyophilization, DNA was dissolved and stored in potassium phosphate (KPi) buffer (pH 7, 10% D₂O and 90% H₂O). The concentrations of KPi were 5 mM for the PS-series series and 20 mM for the HT-series. DNA concentration is expressed in strand molarity with the extinction coefficient of modified sequences approximated to that of the unmodified sequence.

Circular Dichroism spectroscopy

Circular Dichroism (CD) experiments were performed on JASCO-815 spectropolarimeter (Tokyo, Japan) using 1-cm path length quartz cuvette. CD spectra were taken at 20°C in the range of 220-320 nm and accumulated over 3 scans for the PS-series and 10 scans for the HT-series. Spectra were base-line corrected and zero-corrected at 320 nm. CD spectra were normalized by strand concentration determined by concurrent UV absorbance measurements. Samples for the PS-series were prepared by diluting with 1 mM KPi buffer to obtain DNA concentration of 3 µM and KPi concentration of 1.1 mM. Samples for the HT-series series were prepared by diluting with 20 mM KPi buffer to a DNA concentration of 10 µM.

Circular Dichroism melting

CD melting was performed for PS-series and loop-elongated samples by monitoring CD intensity at 260 nm against temperature over a range of 45-95°C. CD melting was performed by stabilizing the sample at 95°C for 20 minutes and then cooling at rate of 0.2°C/min. Samples were then stabilized at 45°C, and heated up again to 95°C at the same rate. For several samples, this range was extended to a lower temperature. T_m values were obtained by baseline normalization of CD melting curves and calculation of the fraction folded. Presented T_m values are an average between cooling and heating curves.

UV Melting

UV melting experiments were performed on a JASCO V-650 UV-Vis spectrophotometer (Tokyo, Japan). UV melting experiments were done for HT-series samples by monitoring UV absorbance at 295 nm (258) over a temperature range of 14-83°C using a 1-cm path length quartz cuvette. For several samples, this range was extended to a lower temperature. UV melting was done by first stabilizing at 83°C for 30 minutes. Samples were cooled down to 14°C at rate of 0.1°C/min, stabilized, and heated up to 83°C at the same rate. Melting curves were normalized by baseline correction to obtain fraction folded curves (270). T_m values presented are an average over cooling and heating curves. Samples for the HT-series were prepared by diluting with 20 mM KPi to obtain a DNA concentration of 10 µM.

NMR Spectroscopy

NMR experiments were performed on 600 or 700 MHz Bruker NMR spectrometer at 25°C using a jump and return-type water suppression pulse sequence (282,283). Chemical shift was calibrated by DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) in NMR buffer. Prior to recording NMR spectra, samples were annealed by heating at 100°C for 5 minutes and cooled down slowly overnight. Samples of PS-series contained 0.15 mM DNA in 5 mM KPi buffer, while samples of HT-series contained 0.1-0.2 mM DNA in the 20 mM KPi buffer. For 2D NMR experiments, the DNA concentration was ~1.5 mM.

Structural Calculation

NMR structural computation was performed by the XPLOR-NIH program (40). Distance restraints, dihedral restraints, hydrogen bond restraints and planarity restraints were used to compute the structure of the HT-L4 sequence. Dihedral angle restraints for χ were imposed on all guanines except for ^{LNA}G4. Dihedral angle restraints for ϵ were imposed on all G-tetrad dinucleotide steps except for G3-to-^{LNA}G4 and ^{LNA}G4-to-G5 steps. Distance restraints were deduced from NOESY experiments at different mixing times. Through the structural refinement and computational procedures described, we present the 10 lowest energy structures from an original ensemble of 100. Structures were visualized using PyMOL software.

Structural Data Deposition

The coordinates of the 10 lowest energy NMR solution structures of the (3+1) G-quadruplex formed by the d[T₂G(^{LNA}G)GT₂A(G₃T₂A)₂G₃A] sequence (HT-L4) will be deposited into the Protein Data Bank upon publication.

Chapter 5: Engineering the Sugar Backbone: 2'-F-Guanosine and 2'-F-ANA-Guanosine Modifications

DNA sample preparation

Oligonucleotides were chemically synthesized using an Applied Biosystems 394 DNA/RNA synthesizer (Foster City, CA, U.S.A.). Phosphoramidites of 2'-deoxy-2'-fluoro-guanosine and 2'-deoxy-2'-fluoroarabino-guanosine were purchased from Glen Research (Sterling, VA, U.S.A.). Oligonucleotides were then purified using a Poly-Pak cartridge (Glen Research) following standard protocol. Purified samples were dialyzed successively against water, KCl solution, and water. Samples were then lyophilized and dissolved in a solution of 5 mM KCl and 5 mM potassium phosphate (pH 7) for the HT-series sequences and 1 mM potassium phosphate (pH 7) for the PS-series sequences, containing 10% D₂O and 20 μ M 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS).

NMR spectroscopy

One-dimensional ¹H NMR spectra of unmodified and modified sequences were recorded on a 600 or 700 MHz Bruker spectrometer (Billerica, MA, USA) at 25°C. Samples were heated to 100°C for 5 minutes and allowed to slowly cooled overnight prior to NMR and subsequent CD studies. DNA strand concentration was 0.05 mM for the PS-series samples and in the range of 0.05-0.4 mM for the HT-series samples. Spectra were recorded using a jump-and-return-type pulse sequence for water suppression (282,283). Chemical shifts were internally referenced using DSS.

Circular dichroism

CD spectra were taken using a JASCO-815 spectropolarimeter (Tokyo, Japan). DNA strand concentration was 4-6 μ M. Spectra were recorded at 20°C in a wavelength range of 220-320 nm and averaged over 10 accumulations. Spectra were zero corrected at 320 nm and normalized for concentration by concurrent UV absorbance measurements.

UV denaturing experiments

Thermal denaturation was monitored using a JASCO V-650 UV-visible spectrophotometer (Tokyo, Japan). DNA strand was concentration of 4-6 μ M. For the PS-series sequences, samples were first cooled from 89 to 42°C at a rate of 0.2°C/min, then allowed to equilibrate for 10 minutes and heated at the same rate over the same range. The HT-series sequences were treated in a similar manner between the range of 83°C and 19°C. This range was extended for certain sequences in order to monitor the complete transition. The UV absorbance of samples was recorded at 295 nm and corrected by subtracting the absorbance at 320 nm as well as baseline corrected with a reference cell. Melting curves were then normalized to create folded fraction curves (258,270). The melting temperature (T_m) is determined to be the temperature at which half of the sample is in the folded state.

CD denaturing experiments

Thermal denaturation was monitored using CD spectroscopy to confirm UV transitions for some select samples. Experiments were recorded in a similar manner to UV experiments. The CD intensity was recorded at 260 nm for the PS-series sequences and 295 nm for the HT-series sequences and corrected by subtracting the intensity at 320 nm. CD melting experiments were normalized in a manner similar to UV melting experiments.

Chapter 6: Engineering the Base: C8-modified Guanines

DNA sample preparation

The unlabeled and the site-specific low-enrichment (2% ^{15}N -labeled) DNA oligonucleotides were chemically synthesized on an ABI 394 DNA synthesizer and purified as previously described (259). Phosphoramidites of guanine derivatives were purchased from Glen Research. ^{15}N -labeled phosphoramidites were purchased from Cambridge Isotope. Samples were dialyzed successively against KCl solution and against water.

UV spectroscopy

Experiments were performed on a Varian 300 Cary Bio UV-Vis spectrophotometer. Samples contained 2-10 μM DNA in a solution of 20 mM potassium phosphate (pH 7). The thermal stability for each sequence was determined by recording the absorbance at 295 nm (258). Heating and cooling (15-88°C) was performed at a rate of 0.5°C/min. Quantitative melting temperature (T_m) data presented represents an average over an annealing cycle. Hysteresis of less than 1.5°C was observed for all samples.

CD spectroscopy

Experiments were performed on a JASCO-815 spectropolarimeter. Samples contained 2-10 μM DNA in a solution of 20 mM potassium phosphate (pH 7). Samples were heated to 90°C for 5 minutes and left to cool down to room temperature. CD spectra were monitored at 20°C from 220 to 320 nm and averaged over 10 accumulation cycles. Data were zero-corrected at 320 nm and finally normalized by strand concentration determined by concurrent UV absorbance measurements at 260 nm.

NMR spectroscopy

Experiments were performed on 600 and 700 MHz Bruker NMR spectrometers at 25°C using a jump-and-return-based water suppression pulse sequence (282,283). DNA concentration was typically 0.2-0.7 mM. Samples contained KCl (70 or 150 mM) and 20 mM potassium phosphate (pH 7). We also monitored several sequences in a low salt condition (only 20 mM potassium phosphate) and did not observe significant changes in NMR spectra. NMR chemical shifts were referenced with 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). Resonances were assigned unambiguously for G_δ imino protons for most sequences using site-specific low-enrichment labeling (259).

Ab initio computation

Quantum mechanical calculations were performed using the GAUSSIAN 03 software (284). Molecular geometries were optimized at the Hartree-Fock level using four standard split-valance basis sets including 6-31G(d), 6-31G(d,p), 6-31++G(d,p), and 6-311++G(d,p) (260). Tight optimization criteria were imposed for optimized geometries. NMR chemical shifts were calculated using the GIAO method (285) at the Hartree-Fock level using the above mentioned standard split-valance basis sets as well as the 6-311+G(d) basis set (260).

Models consisted of four guanines arranged in a planar tetrad with two centrally located potassium ions placed at a distance of 1.7 Å above and below the tetrad plane. Initial guanines were arranged within the $Z=0$ plane allowing the tetrad to maintain planarity during optimization. The unmodified models underwent initial geometry optimization resulting in a C_4 symmetry with identical hydrogen-bond geometry. A single guanine was modified at the C8 position by adding a modification group (Bromo-, Amino-, or O-methyl-) within the plane of the base. In the case of $^{O_{\text{met}}}\text{G}$, a *cis* conformation of the $\text{N}_7\text{-C}_8\text{-O}_R\text{-C}_R$ bond was chosen. This was done to maintain planarity and was thought to be a more likely than the *trans* conformation, which may crash with sugar-phosphate backbone in the G-quadruplex structure. Geometry

optimization was again performed after substitution of a single guanine with a C8-modified guanine derivative. NMR chemical shift calculations were carried out on the unmodified and modified models.

A total of 16 individual molecular geometries (Unmodified, Bromo-, Amino-, and O-methyl-containing models each optimized with 4 different basis sets) were calculated. The geometries of modified tetrads were compared to the geometries of the unmodified tetrads as presented in Tables 5 and 6. A total of 80 individual chemical shifts (16 individual geometries evaluated with 5 different basis sets for NMR chemical shift calculations) were computed..

Chapter 7: Engineering the Base: N2-modified Guanines

Synthesis and sample preparation

DNA samples were synthesized on an Applied Biosystems 394 DNA Synthesizer (Foster City, CA U.S.A.) and purified following Glen Research protocols. The modified phosphoramidite N2-6-amino-hexyl-guanine was purchased from Glen Research (Sterling, VA U.S.A.). Modified phosphoramidites N2-methyl-guanine and N2-benzyl-guanine were purchased from Berry & Associates (Dexter, MI U.S.A.). Purified DNA samples were dialyzed against H₂O, ~30 mM KCl, and H₂O before being dried and dissolved in solution containing KCl, potassium phosphate (KPi) buffer (pH 7), 20 μ M 4,4-dimethyl-4-silapentane-1-sulfonic acid used as a chemical shift reference in NMR, and containing 10% D₂O. Concentrations of KPi and KCl varied to generate varying salt conditions, including “low salt” (1 mM KPi + 1 mM KCl), “moderate salt” (10 mM KPi + 10 mM KCl) and “physiological Salt” (10 mM KPi + 130 mM KCl).

NMR spectroscopy

Spectra were obtained on a 600 MHz Bruker spectrometer (Billerica, MA U.S.A.) at a temperature of 25°C using a jump-and-return type water suppression pulse sequence (282). Samples were annealed prior to recording of NMR spectra by heating to 100°C for 2 minutes and allowed to cool slowly at room temperature. Chemical shifts were internally referenced with D.S.S.

CD spectroscopy

Spectra were recorded on a JASCO-815 spectropolarimeter (Tokyo, Japan). Samples contained a DNA strand concentration of 4-5 μ M. Spectra were recorded at 20°C in the range of 220-320 nm and averaged over 10 scans. Spectra were normalized through subtracting by the CD absorbance at 320 nm and dividing by concentration of the sample determined by concurrent UV absorption measurements. Samples were annealed prior to recording of CD spectra by heating to 100°C and allowed to cool slowly at room temperature.

CD melting experiments

CD melting experiments were performed for the unmodified sequence and for sequences containing multiple N2-modifications. Samples contained 4-5 μ M DNA by strand concentration in 1 mM KCl and 1 mM KPi. Samples were heated to 95°C (98°C for N2-Ball-with-Hair sample) and cooled to 15°C at a rate of 0.2°C/min. Samples were then heated in a similar manner. Data was recorded every 0.5°C at 290 nm and corrected by subtraction at 320 nm. Normalized melting curves are used to determine the melting temperature (T_m) of modified sequences (270), which is taken as an average of the heating and cooling curves. Hysteresis in a heating/cooling cycle was observed to be below 2°C over all samples.

UV melting experiments

Melting experiments were performed on a JASCO V-650 UV-visible spectrophotometer (Tokyo, Japan). Samples containing ~5 μ M DNA strand concentration were first heated to 83°C and cooled to 14°C at a rate of 0.2 °C/min. The absorbance was sampled every 0.5°C at 295 nm and 320 nm. At the end of the cooling cycle, samples were held for 10 minutes before being heated back to 83°C at the same rate. Melting curves were normalized first by subtraction at 320 nm and then further processed to create fraction folded curves (258). Melting temperatures (T_m) reported represent an average over heating and cooling cycles. Hysteresis of < 2°C (generally < 1.5°C) was observed over all sequences.

Modeling

Models of Hex-3 and N2-Ball-with-Hair structures were built based on a NMR model of the unmodified (3+1) sequence (PDB ID 2GKU) (28). Guanine bases containing N2-6-amino-hexyl

modifications in a straight orientation were fit to guanine bases within the NMR structure. The dihedral angles around the connection of the guanine base to the N2-6-amino-hexyl functional group (Guanine(C2)-Guanine(N2)-Hex(C1)-Hex(C2)) were then manually varied to project the N2-6-amino-hexyl into the groove. Illustrative models were visualized in Pymol.

Appendix III – PDB Survey

Table A3-1. Summary and statistics of crystallographic structures cataloged in Chapter 2.

PDB	Sequence	Description	Res. (Å)	Statistics of Rotation (deg) / Separation (Å)					
				Core Stacking			Interface Stacking		
				Type	N	Mean(σ)	Type	N	Mean(σ)
1KF1	d[AG ₃ (T ₂ A G ₃) ₃]	Telomeric DNA Intramolecular Parallel Propeller Quadruplex – K ⁺	2.10	A/A	8	61.7(0.9) 3.42(0.08)	5'-5' 5/6-ring	2	56.2(1.9) 3.38(0.05)
1K8P	d[^{Br} UAG ₃ ^{Br} UTAG ₃ T] ₂	Telomeric DNA Bimolecular Parallel Propeller Quadruplex – K ⁺	2.40	A/A	8	60.2(3.6) 3.32(0.06)	-	-	-
2HRI	d[TAG ₃ T ₂ A G ₃] ₂	Telomeric DNA Bimolecular Parallel Propeller Quadruplex – K ⁺ + porphyrin TMPyP4	2.09	A/A	8	63.4(2.7) 3.36(0.05)	3'-3' 6-ring	2	46.0(0.9) 3.43(0.01)
3CCO	d[TAG ₃ T ₂ A G ₃ T] ₂	Telomeric DNA Bimolecular Parallel Propeller Quadruplex – K ⁺ + tetra- substituted naphthalene diimide	2.20	A/A	4	63.0(10.1) 3.42(0.15)	5'-5' 5-ring	1	86.5(-) 3.46(-)
3CDM	d[TA(G ₃ T ₂ A) ₃ G ₃]	Telomeric DNA Intramolecular Parallel Propeller Quadruplex – K ⁺ + tetra- substituted naphthalene diimide	2.10	A/A	16	62.3(2.3) 3.36(0.06)	-	-	-
3CE5	d[TAG ₃ T ₂ A G ₃ T] ₂	Telomeric DNA Bimolecular Parallel Propeller Quadruplex – K ⁺ + BRACO-19	2.50	A/A	8	63.1(1.2) 3.46(0.11)	-	-	-
3QCR	d[TAG ₃ T ₂ A G ₃] ₂	Telomeric DNA Bimolecular Parallel Propeller Quadruplex – K ⁺ + acridine ligand	3.2	A/A	4	60.6(0.7) 3.20(0.12)	-	-	-
3IBK	r[^{5Br} UAG ₃ U ₂ AG ₃ U] ₂	Telomeric RNA Bimolecular Parallel Propeller Quadruplex – K ⁺	2.20	A/A	8	60.9(4.9) 3.40(0.08)	-	-	-
3MIJ	r[UAG ₃ U ₂ AG ₃ U] ₂	Telomeric RNA Bimolecular Parallel Propeller Quadruplex – K ⁺ + acridine ligand	2.60	A/A	4	63.6(4.1) 3.40(0.11)	-	-	-
2AVH	d[G ₄ T ₃ G ₄] ₂	DNA Bimolecular (2+2 ↑•↓•↑•↓) Edgewise quadruplex – K ⁺	1.50	S/A	4	81.8(1.6) 3.47(0.07)	-	-	-
				A/S	2	30.4(2.1) 3.66(0.05)			
2AVJ	d[G ₄ ^{Br} UT ₂ G ₄] ₂	DNA Bimolecular (2+2 ↑•↓•↑•↓) and (2+2 ↑•↑•↓•↓) Edgewise quadruplex – K ⁺	2.39	S/A	24	83.2(2.5) 3.46(0.09)	Mixed 5'- 3'	4	26.2(1.5) 3.50(0.08)
				A/S	12	29.0(1.5) 3.51(0.08)	Partial 6-ring		

1JPQ	d[G ₄ ^{Br} UT ₃ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓•↓) Diagonal quadruplex – K ⁺	DNA (2+2)	1.60	S/A	8	83.0(2.0) 3.48(0.12)	-	-	-
					A/S	4	29.5(1.3) 3.50(0.05)			
1JRN	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓•↓) Diagonal quadruplex – K ⁺	DNA (2+2)	2.00	S/A	16	82.7(2.9) 3.47(0.14)	-	-	-
					A/S	8	29.1(0.9) 3.53(0.08)			
2HBN	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓•↓) Diagonal quadruplex – Tl ⁺	DNA (2+2)	1.55	S/A	16	82.8 (2.7) 3.49(0.12)	-	-	-
					A/S	8	29.2(1.9) 3.57(0.04)			
2GWQ	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓•↓) Diagonal quadruplex – K ⁺	DNA (2+2)	2.00	S/A	32	83.1(1.0) 3.46(0.10)	-	-	-
					A/S	16	29.5(1.6) 3.54(0.07)			
2GWE	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓•↓) Diagonal quadruplex – K ⁺	DNA (2+2)	2.20	S/A	48	83.0(2.8) 3.44(0.13)	-	-	-
					A/S	24	29.3(1.6) 3.55(0.08)			
1L1H	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓•↓) Diagonal quadruplex – K ⁺ + acridine ligand	DNA (2+2)	1.75	S/A	8	83.1(2.1) 3.42(0.10)	-	-	-
					A/S	4	28.8(1.1) 3.56(0.07)			
3EM2	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓•↓) Diagonal quadruplex – K ⁺ + acridine ligand	DNA (2+2)	2.30	S/A	8	84.0(3.0) 3.42(0.09)	-	-	-
					A/S	4	28.7(1.6) 3.57(0.05)			
3EQW	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓•↓) Diagonal quadruplex – K ⁺ + acridine ligand	DNA (2+2)	2.20	S/A	8	83.9(2.6) 3.43(0.08)	-	-	-
					A/S	4	28.6(1.2) 3.53(0.06)			
3ERU	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓•↓) Diagonal quadruplex – K ⁺ + acridine ligand	DNA (2+2)	2.00	S/A	8	83.8(2.5) 3.43(0.08)	-	-	-
					A/S	4	28.9(0.9) 3.55(0.08)			
3ES0	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓•↓) Diagonal quadruplex – K ⁺ + acridine ligand	DNA (2+2)	2.20	S/A	8	83.8(1.5) 3.46(0.06)	-	-	-
					A/S	4	29.0(1.0) 3.56(0.04)			

3ET8	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓• Diagonal quadruplex – K ⁺ + acridine ligand	DNA (2+2)	2.45	S/A	8	82.7(2.4) 3.52(0.09)	-	-	-
					A/S	4	29.9(1.6) 3.59(0.08)			
3EUI	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓• Diagonal quadruplex – K ⁺ + acridine ligand	DNA (2+2)	2.20	S/A	16	84.2(1.9) 3.47(0.10)	-	-	-
					A/S	8	30.3(0.7) 3.55(0.08)			
3EUM	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓• Diagonal quadruplex – K ⁺ + acridine ligand	DNA (2+2)	1.78	S/A	8	83.5(1.9) 3.45(0.08)	-	-	-
					A/S	4	29.0(0.8) 3.49(0.04)			
3NYP	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓• Diagonal quadruplex – K ⁺ + acridine ligand	DNA (2+2)	1.18	S/A	8	84.6(2.3) 3.60(0.07)	-	-	-
					A/S	4	28.1(1.1) 3.71(0.04)			
3NZ7	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Bimolecular ↑•↑•↓•↓• Diagonal quadruplex – K ⁺ + acridine ligand	DNA (2+2)	1.10	S/A	8	83.8(2.0) 3.44(0.07)	-	-	-
					A/S	4	29.0(0.9) 3.56(0.03)			
2GW0	d[<i>TG</i> ₄ <i>T</i>] ₄	Tetramolecular parallel DNA quadruplex – Na ⁺ / Ca ⁺²		1.55	A/A	24	60.6(3.0) 3.35(0.06)	5'-5' 5-ring	4	81.5(0.7) 3.49 (0.04)
244D	d[<i>TG</i> ₄ <i>T</i>] ₄	Tetramolecular parallel DNA quadruplex – Na ⁺		1.20	A/A	48	60.6(2.8) 3.41(0.05)	5'-5' 5-ring	8	83.0(0.4) 3.50 (0.02)
352D	d[<i>TG</i> ₄ <i>T</i>] ₄	Tetramolecular parallel DNA quadruplex – Na ⁺		0.95	A/A	48	60.9(3.1) 3.34(0.05)	5'-5' 5-ring	8	82.7(0.5) 3.42(0.03)
100K	d[<i>TG</i> ₄ <i>T</i>] ₄	Tetramolecular parallel DNA quadruplex – Na ⁺ + daunomycin ligand		1.17	A/A	12	59.1(4.2) 3.27(0.04)	-	-	-
1S45	d[<i>TG</i> ₄ <i>T</i>] ₄	Tetramolecular parallel DNA quadruplex – Na ⁺ /Ti ⁺		2.20	A/A	24	61.8(2.6) 3.42(0.09)	5'-5' 5-ring	4	84.5(0.7) 3.59(0.02)
1S47	d[<i>TG</i> ₄ <i>T</i>] ₄	Tetramolecular parallel DNA quadruplex – Na ⁺ /Ti ⁺		2.50	A/A	36	62.5(3.3) 3.45(0.16)	5'-5' 5-ring	4	85.1(1.8) 3.60(0.07)
1V3P	d[G ⁵¹ CGA GAGC] ₄	Octamolecular quadruplex – K ⁺	DNA	2.30	-	-	-	5'-5' 5/6-ring	1	57.1(-) 3.47(-)
1P79	[r(UGUG ₂ U)] ₄	Tetramolecular parallel Bulged RNA quadruplex – K ⁺	RNA	1.10	S/A	1	77.7(-) 3.40(-)	-	-	-
					A/A	1	61.9(-) 3.41(-)			

2AWE	r[U ^{8Br} GGU GU] ₄	Tetramolecular parallel Bulged RNA quadruplex – K ⁺	2.10	S/A	8	82.2(0.8) 3.57(0.04)	-	-	-
				A/A B ^b	8	35.7(0.9) 3.53(0.06)			
2GRB	r[U ^{5Br} UGIG GU] ₄	Tetramolecular parallel RNA quadruplex – K ⁺	1.40	A/A	8	65.5(0.9) 3.52(0.04)	-	-	-
1J6S	[r(^{Br} UGAG zU)] ₄	Tetramolecular parallel RNA quadruplex – Ba ²⁺	1.40	A/A	4	65.7(0.5) 3.46(0.05)	-	-	-
1J8G	r[UG ₄ U] ₄	Tetramolecular parallel RNA quadruplex – Sr ²⁺	0.61	A/A	12	60.6(3.9) 3.31(0.04)	5'-5' 6-ring	2	37.2(0.7) 3.39(0.00)
1MDG	[r(U)d(^{Br} G) r(AG ₂ U)] ₄	Tetramolecular parallel RNA quadruplex – Na ⁺	1.50	A/A	1	64.5(-) 3.39(-)	-	-	-
2O4F	d[^{Br} TG ₄ T] ₄	Tetramolecular parallel DNA quadruplex – Na ⁺	1.50	A/A	24	61.2(2.6) 3.42(0.06)	5'-5' 5-ring	4	82.7(0.2) 3.63(0.03)

G-quadruplex structures included in this work were obtained by searching the Protein Data Bank using the following keywords: quadruplex(es); tetrad(s); tetraplex(es); G4. PDB Entries 1D59 and 1JB7 were not examined in this work. Statistics presented are for experimental stacked guanines pairs from a single PDB compared to model stacked guanine pairs.

^a PDB Entries from work not yet published.

^b *Anti/Anti* Stacking present with a one residue bulge.

Table A3-2. Summary and statistics of NMR G-quadruplex structures cataloged in Chapter 2.

PDB	Sequence	Description	Statistics of Rotation (deg) / Separation (Å)					
			Core Stacking			Interface Stacking		
			Type	N	Mean(σ)	Type	N	Mean(σ)
139D	d[T ₂ G ₄ T] ₄	DNA Intermolecular Parallel Quadruplex – K ⁺	A/A	12	60.1(5.0) 3.17(0.08)	-	-	-
143D	d[AG ₃ (T ₂ AG ₃) ₃]	DNA Intramolecular Telomeric (2+2 $\uparrow\bullet\downarrow\bullet\uparrow\bullet\downarrow$) Quadruplex – Na ⁺	S/A	4	79.0(5.3) 3.45(0.12)	-	-	-
			A/S	4	29.8(9.2) 3.74(0.10)			
148D	d[G ₂ T ₂ G ₂ TGTG ₂ T ₂ G ₂] ₂	DNA Intermolecular Thrombin aptamer (2+2 $\uparrow\bullet\downarrow\bullet\uparrow\bullet\downarrow$) Quadruplex – K ⁺	S/A	4	86.4(3.8) 3.55(0.57)	-	-	-
156D	d[G ₄ T ₄ G ₄] ₂	<i>Oxytricha</i> Intermolecular DNA (2+2 $\uparrow\bullet\uparrow\bullet\downarrow\bullet\downarrow$) Quadruplex – K ⁺	S/A	8	85.7(4.8) 3.38(0.37)	-	-	-
			A/S	4	31.7(8.4) 3.39(0.32)			
186D	d[(T ₂ G ₄) ₄]	<i>Oxytricha</i> Intarmolecular DNA (3+1) Quadruplex – Na ⁺	S/A	4	84.8(2.0) 3.38(0.25)	-	-	-
			A/A	3	63.9(10.8) 3.51(0.28)			
			S/S	1	15.1(-) 3.28(-)			
1A8N	d[G ₃ CT ₄ G ₃ C] ₂	<i>Human Chromosomal repeat</i> DNA (2+2 $\uparrow\bullet\uparrow\bullet\downarrow\bullet\downarrow$) Quadruplex – Na ⁺	S/A	4	88.3(1.5) 3.46(0.02)	-	-	-
1A8W	d[G ₃ CT ₄ G ₃ C] ₂	DNA (2+2 $\uparrow\bullet\uparrow\bullet\downarrow\bullet\downarrow$) Quadruplex – K ⁺	S/A	4	81.6(0.5) 3.61(0.03)	-	-	-
1AFF	d[T ₂ AG ₃] ₄	Tetrameric DNA Quadruplex –	S/A	4	84.0(10.1) 3.76(0.04)	-	-	-
			A/A	3	61.1(6.4) 3.38(0.09)			
2GKU	d[T ₂ (G ₃ T ₂ A) ₃ G ₃ A] ₃	Telomeric DNA (3+1) Quadruplex – K ⁺	S/S	1	30.1(-) 3.55(-)	-	-	-
			S/A	4	82.3(3.0) 3.57(0.18)			
			A/A	3	59.9(3.1) 3.33(0.14)			
2KZD	d[AG ₃ LAG ₄ CTG ₃ AG ₃ C] ₃	DNA (3+1) Quadruplex – K ⁺	S/S	1	28.2(-) 3.93(-)	-	-	-
			S/A	4	85.8(2.1) 3.93(0.15)			
				2				
2KAZ	d[G ₃ ACGTAGT G ₃] ₂	DNA (2+2 $\uparrow\bullet\uparrow\bullet\downarrow\bullet\downarrow$) Diagonal Quadruplex – K ⁺	A/A	2	64.4(1.74) 3.37(0.12)	-	-	-
			S/S	2	27.9(2.2) 3.53(0.21)			

				S/A	4	85.9(2.6) 3.42(0.08)						
1MYQ	d[(GGA) ₄]	DNA Propeller Quadruplex – K ⁺	Parallel	-	-	-	5'-5' 5/6-ring	4	60.1(7.5) 3.26(0.23)			
1MY9	r[G ₂ AG ₂ U ₄ G ₂ AG ₂]	RNA Propeller Quadruplex – K ⁺	Parallel	-	-	-	5'-5' 5-ring	3	84.2(2.4) 3.00(0.19)			
							5/6-ring	1	71.3(-) 3.93(-)			
1EEG	d[(GGA) ₂] ₂	DNA Parallel Quadruplex – Na ⁺	Biomolecular Propeller	-	-	-	5' ↔ 5' ND	-	-			
1OZ8	d[(GGA) ₈]	DNA Parallel Quadruplex Na ⁺ /K ⁺	Linked Dimeric Propeller	-	-	-	Linked 5' ↔ 5' 5/6-ring	4	61.7(5.0) 3.57(0.50)			
1JJP	d[G ₃ AG ₂ T ₃ G ₃ AT l ₂]	DNA Dimeric – Na ⁺	Interlocked Quadruplex	-	-	-	Locked 5-ring	-	-			
2RQJ	r[(GGA) ₄]	RNA Propeller Quadruplex – K ⁺	Parallel	-	-	-	5'-5' 5-ring	2	77.2(0.1) 3.26(0.11)			
							5/6-ring	2	68.5(0.2) 3.32(0.06)			
1Y8D	d[G ₄ TG ₃ AG ₂ AG ₃ T] ₂	DNA Dimeric – K ⁺	Interlocked Quadruplex	-	-	-	Locked 5-ring	-	-			
2LE6	d[GIGT(G ₃ T) ₃]	DNA Propeller Quadruplex – K ⁺	Parallel	-	-	-	5'-5' 5/6-ring	4	56.8(1.94) 3.37(0.04)			
2WCN	d[G(^{LNA} G)G(^{LNA} G) T ₄ G(^{LNA} G)G(^{LNA} G)]	DNA Quadruplex – K ⁺	V4 folded	-	-	-	Locked P 6-ring	-	-			

G-quadruplex structures included in this work cover a range of different folding topologies and include structures in which stacked guanine tetrads are presented at the interface between monomer G-quadruplexes. Statistics presented are for experimental stacked guanines pairs from a single PDB compared to model stacked guanine pairs.