Resistance of DNA G-quadruplex against oxidative stress

Wee, Xue Xin

2012

Wee, X. X. (2012). Resistance of DNA G-quadruplex against oxidative stress. Doctoral thesis, Nanyang Technological University, Singapore.

<https://hdl.handle.net/10356/50941>

<https://doi.org/10.32657/10356/50941>

**NANYANG
TECHNOLOGICAL
UNIVERSITY**

**RESISTANCE OF DNA G-QUADRUPLIX AGAINST OXIDATIVE
STRESS**

WEE XUE XIN

**SCHOOL OF PHYSICAL AND MATHEMATICAL SCIENCES
2012**

**RESISTANCE OF DNA G-QUADRUPLEX AGAINST OXIDATIVE
STRESS**

WEE XUE XIN

School of Physical and Mathematical Sciences

A thesis submitted to the Nanyang Technological University
in fulfilment of the requirement for the degree of
Master of Science

2012

Acknowledgments

I would like to express my gratitude to my supervisor Dr Shao Fangwei for her guidance over the whole journey of my Masters candidature. I was an amateur at research without any FYP experience during my undergraduate years. Despite knowing this, Dr Shao has given me the chance to work under her and supported me financially. Without any senior members during the earlier months of our lab establishment, she has also kindly given me the utmost attention and has coached me personally in all the basic lab techniques which are useful for my project.

I would also have to highlight that my current group members, Dr Wang Jintao, Xu Baochang, Wong Jiun Ru, Wu qiong, Xuan Shuguang, Lu Kaihui, Riandy Tanri and also previous group members (Dr Yun Wen, Fang Weiqin and Jasmine Lee) are definitely the most understanding and encouraging group members anyone can have. We have bonded well and their presence has made my postgraduate life much more fun-filled and exciting. I would like to express my heartfelt appreciation to them for all the help and guidance they have given me in one way or another.

I would also like to thank the teaching lab staff, Ms Seow Aihua and her crew for their technical assistance in the teaching lab. They have attended to me promptly whenever I met up with some problems from the machines.

Lastly, I would like to thank my family for their patience and love which has given me the courage to take onto this challenge and strength to persevere on.

List of Abbreviations

ATP	Adenosine 5' Triphosphate
CD	Circular Dichroism
DNA	Deoxyribonucleic Acid
DS	Duplex
GQ	Guanine-quadruplex
Iz	2,5-diamino-4H-imidazol-4-one
NI	5-nitro-4-guanidinohydantoin
Oz	2,2,4-triamino-5-(2H)-oxazolone
PAGE	Polyacrylamide Gel Electrophoresis
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
TA	Terephthalic Acid
TAOH	2-Hydroxyterephthalic Acid
T _m	Melting Temperature
Tris-Cl	Tris(hydroxymethyl)amino Methane with Hydrochloric Acid
³² P	Phosphorus 32

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Abstract

The existence of guanine quadruplex (GQ) in the genomic regions with biological significance is closely related to the inhibition of disease progression. Studies have found that GQs at the telomere overhang can inhibit the activity of overexpressed telomerase in the cancer cells. Furthermore, the formation of GQs at promoter regions of oncogenes is capable of regulating the transcription of malignant proteins in the diseased cell lines. The vulnerability of GQs towards oxidative damage is hence interesting to know as the motif concentrates twelve guanines, the genomic oxidation hotspot, into approximately 1 nm³ space. The survivability of the GQs under malignant/physiological ROS levels can be a key towards therapeutic methods against cancer. Furthermore, the rich topological structures distinguish the biological roles of the GQs. Hence, it is of great interests to explore whether and furthermore how the GQ topology would modulate their oxidative resistance. Reactive oxygen/nitrogen species (ROS/RNS) are constantly produced from normal cellular metabolism and inflammatory processes, and are key molecular triggers in cellular signalling pathways. Under pathological conditions such as cancer and cardiovascular diseases etc, oxidative stress, a hike in ROS level, often is presented in cellular compartments. In this thesis work, the oxidative damage in two G-rich genomic sequences, human telomere (HT) and the promoter of *cmyc* oncogene (cMYC), are interrogated by various ROS threats under both physiological and malignant levels. The resulting DNA damage on guanines and other nucleotides are revealed by alkaline labile strand cleavage and visualized on denature PAGE gel electrophoresis. For both HT and cMYC sequences, comparable guanine damages are observed from both the duplex structures and the GQs under physiological conditions. Folding into GQs does not increase the resistance against the ROS to the genomic DNA under interest comparing to the duplex when the oxidative stress is not stringent. Instead, GQs are found to be more chemically vulnerable under malignant conditions with high ROS levels.

Guanine damage and total strand break ups are more pronounced in both GQ under high concentrations of peroxynitrite and hydroxyl radicals. The high vulnerability of GQ towards oxidative stresses suggest a possibility that acceleration of strand breaks in GQ due to oxidative damage may be a key to telomeric shortening and malfunction of oncogenic promoters. The results here may reveal a new biofunction of GQs as a damage hotspot under oxidative threats, and suggests a new anticancer therapeutic strategy as to target GQ for photodynamic therapy.

1 Introduction

Guanine-quadruplexes (GQ) are a family of secondary structures of nucleic acids stabilized in the presence of monovalent cations. As its name suggests, GQs are formed by the guanine rich sequences. The guanine bases serve as the building blocks of the entire structure. Every four guanines are held together by hydrogen bonds and coordination to monovalent cations (i.e. K^+ , Na^+ , Li^+) to form planar guanine quartets (Figure 1A). These quartets/tetrads are stacked together and stabilize the four stranded GQ structures. Other bases present in the sequence are functionalized as loops or flanks.^{1 2 3}

1.1 G-Quadruplex Topologies

GQs have rich polymorphisms based upon the variations in the number of strands, strand direction, loop length/orientation, guanine conformation, and flanking bases at the sequence ends. Monomeric sequence and multiplex up to 4 strands can assemble a quadruplex. The strand direction can be either parallel or anti-parallel as shown in Figure 1B. The diagonal, propeller or lateral loops exist over a wide range of GQ topology (Figure 1C), while the guanine bases may also orientate themselves in the syn or anti conformations according to the deoxyribose as shown in Figure 1D.^{2 3}

Figure 1 Schematic view of GQ. A) Guanines held by Hoogsteen hydrogen bonds in a tetrad layer. B) Strand directions in the guanine quadruplex with loops. C) Intramolecular quadruplex with loops. D) Structure of anti and syn guanines conformation.^{4 5}

1.2 Biological Significance of GQs

GQs can be found in different biologically significant genomic areas. They have the potential to curb the development of diseases and are found to function uniquely.

Telomerase in charge of elongating the telomeric ends of the genome are found to be over expressed in 85% of cancer cells.⁶ This causes the cancer cell to be immortal as constant elongation of the telomeric ends prevents the natural cell death mechanism, apoptosis, from proceeding.^{7 8} The presence of GQ at the telomeric ends has shown inhibitory effects on telomerase activity presumably due to the blocking of the assembly of telomeric substrate and telomerase. Hence, the antiproliferation of cancer cells can be promoted by promoting and/or stabilizing telomeric quadruplexes.

Another well-established example is that the promoter regions of many oncogenes possess potential GQ folding sequences, which have shown regulatory effects on the transcription of the oncogenes via folding into quadruplex structures. The over expression of these oncogenes in cancer cells can be suppressed by the therapeutic molecules which stabilize the GQ structures. The phenomena are less pronounced in the cell lines with G to A mutations in the GQ folding sequence. This suggests that GQs at promoter regions are promising targets for the regulation of these undesirable transcriptions in the diseased cells.^{9 10 11}

Diseased cells have been known to possess elevated levels of reactive oxygen species leading to oxidative stress and mutations on the genome duplex DNA. The resistivity of GQ against these species is interesting to find out due to its biological functions. Furthermore, as guanines are the most easily oxidized nucleobases, the presence of close clustering guanine bases in the tetrad layers of the GQ may be a crucial hot spot towards oxidative genome damages.

1.3 Reactive Oxygen/nitrogen Species (ROS/RNS)

Chemical reactions on DNA such as oxidations are associated with development of diseases caused by ROS. ROS are by-products of cellular metabolism and inflammatory processes.^{12 13} Basal levels of ROS are necessary in the cells due to their involvement in cell signaling and regulatory processes.¹⁴ For example, superoxide and hydrogen peroxide can mediate the formation of lipid peroxides. Also, recent research has shown that an initial hike in the production of ROS can activate a series of cellular communication events which propels through different tissues and hence transmitting the signals over vast distances in both plants and animals.¹⁵

However, despite their role in the cell function, ROS can also be a threat to organisms. Excessive production of ROS from cellular metabolism and inflammatory conditions are usually controlled by biochemical antioxidants within the cell. The imbalance of the ROS and antioxidant causes oxidative stress on cells which leads to many diseases. Studies have shown an apparent correlation between the elevations of ROS levels in cells and serums, and the diseases such as cancer, cardiovascular and neural related illnesses.^{16 17 18}

1.3.1 ROS and Pathogenic Consequences

Developments of diseases such as cancer associated with macro-molecular damage caused by the ROS are illustrated by figure 2. Exposure of nucleic acids to ROS can cause severe damage in nucleic acids. The most abundant cancer related mutation is caused by the oxidation of the guanine bases to 8-oxo-guanine, which leads to a G to T transversion (Figure 3).¹⁹ The occurrence of such mutation at vital genes can result in the initiation and progression of cancer.

Figure 2 Biological roles of ROS in the pathological progress leading to human carcinoma.¹⁶

1.3.2 ROS and Guanine Damage

A) Guanine oxidation.

B) Hoogsteen hydrogen bonding between 8-oxo-guanine and adenine during replication.

Figure 3 A) Guanine oxidation to 8-oxo-guanine. B) 8-oxo-guanine and adenine bonded by Hoogsteen hydrogen bond during replication process, leading to G→T transversion.

- Hydroxyl Radicals

Hydroxyl radicals are produced from ionizing radiation or reduction of dioxygen in the cell during metabolic processes.^{12 13} Such processes include the widely known Fenton reaction whereby hydrogen peroxide is reduced by Fe^{2+} ions in the cell as shown below.

Though short-lived, the potency of hydroxyl radical is high and its damage on DNA bases has been studied extensively.^{20 21}

Figure 4 Oxidation of guanine by hydroxyl radical.²²

Hydroxyl radical mainly attacks C4, C5 and C8 of guanosine base as shown in Figure 4. The former two positions can undergo reverse reaction to regenerate the guanine base and avoid the permanent damage by obtaining an electron from the medium. However, OH adduct at C8 results in an intermediate with life time long enough to allow it to further react with environmental water and oxygen to yield the two major products, 8-oxo-guanine (8-oxo-G) and FAPy-G. 8-oxo-G is well-known by its ability to form base pair with adenine and sequentially introduce G-to-T mutation in genome (Figure 3). The two products are both labile to alkaline conditions and generate strand cleavage upon hot piperidine treatment.

Alternatively, hydroxyl radicals may attack the sugar moiety via hydrogen abstraction. The favorable site of attack (sugar vs base) varies with the experimental conditions. An excess of Fe^{2+} reductant in the Fenton reaction will favor the sugar attack leading to a direct strand scission while the absence of the excess reductant will cause base damage to be predominant.²²

- Peroxynitrite

Figure 5 Summary of oxidation and nitration products of peroxynitrite on guanine base.²³

Peroxynitrite is an anion RNS, and is often generated *in vivo* by superoxide and nitric oxide¹³ as shown below.

Peroxynitrite plays an essential part in cellular signaling. As a nitrogen containing adduct, peroxynitrite does not only cause oxidative damage to the nucleobase but also nitration modifications on it. Previous work from Tannenbaum, S.R and co-workers²³ have shown that majority of ONOO⁻ attacks occur to C8 as in the case of hydroxyl radical and generate 8-oxo-guanine as oxidative product and 8-NO₂-guanine as nitration product, respectively. It was debated that 8-oxo-guanine might not be a product of peroxynitrite oxidation. However, such controversy has been resolved as it is proven that the inconsistency in the appearance of this adduct is due to that peroxynitrite reacts more efficiently with 8-oxo-guanine rather than the guanine base and generates secondary oxidative products, such as 2,5-diamino-4H-imidazol-4-one (Iz) and 2,2,4-triamino-5-(2H)-oxazolone (Oz).²³

- **Hydrogen Peroxide**

The *in vivo* generation of hydrogen peroxide from superoxide is catalyzed by superoxide dismutases. Although hydrogen peroxide is almost the most abundant ROS species in the cell, the mechanism of hydrogen peroxide attack on nucleobases has not been well established, presumably due to its weak oxidizing power on them. Most studies on hydrogen peroxide oxidation are usually accompanied by the presence of transition metals such iron(II) or copper(II) and their resulting damages brought upon to the DNA bases are attributed to the production of hydroxyl radicals.^{24 25 26}

1.4 Objective

The guanine-rich GQs have shown promising potential in interfering the progression of diseased cell lines, which are constantly under the threats of over expressed oxidative stresses. The investigation on the chemical susceptibility of GQs towards oxidative damage is under high demands and is indispensable to the development of GQ-based anticancer therapeutics. Questions, such as whether the clustering of guanine bases in GQs would cause the G-rich sequences become more oxidation prone needs to be answered. More recent evidences suggest that a correlation between the topological structures and biological functions of GQs. It is also of great interest to explore the effects of folding topology, such as quartet stacking, loop orientation and guanine conformation, on the oxidative resistance of GQs. It has been revealed that UV irradiation causes guanine damage both in the absence and presence of photo oxidants. However, the chemical vulnerability of guanine bases in the duplex and quadruplex form was inconsistent, probably due to the difference in interaction mode between the photo oxidants and DNA structures.^{26 27 28 29} Our study here will interrogate the oxidative damage of two potential GO-folding sequences, HT and cMYC, induced by various cellular ROS/RNS at both physiological and malignant levels. The effects of secondary structures of DNA and GQ topology on the oxidative stability will be elucidated by comparing the damage pattern and yields of the two sequences folded into single stranded, double stranded or GQs. Hence, the result may provide new insights for the vulnerability of guanine rich genomic regions towards endogenous ROS and for biological roles of GQs in the pathogenic progresses.

2 Experimental

Two sequences were selected to be folded into quadruplex structure as shown in Table 1. Both DNA sequences were acquired from Sangon Biotech (Shanghai).

Table 1: DNA sequences folded into quadruplex. *Italic bases* are mutations to the wild type sequences.

DNA	Sequence
HT	5'AAAGGGTTAGGGTTAGGGTTAGGGA ^{3'}
cMYC	5'TGAGGGTGGGTAGGGTGGGTAA ^{3'}

The mutated sequences were chosen to form a single quadruplex in buffer containing 100mM KCl.^{30 31} NMR studies have shown that these 2 sequences are capable of forming at least 95% of a single anti-parallel (HT) or parallel (cMYC) quadruplex. The purity of sequences was checked with HPLC (Shimadzu Prominence UFLC system) C-18 column. The analysis was performed in 2 solvent mixtures namely acetonitrile (ACN) and 0.1M triethylammonium acetate buffer (TEAA) pH 7.0. The run method consists of 2% ACN for the 1st 30 minutes followed by an increment of ACN from 15%-30% over the next 5 minutes and lastly back to 2% ACN for another 5 minutes.

2.1 Annealing of DNA

DNA concentrations were determined using UV-Vis absorbance machine Cary-100 and calculated by Beer Lambert's law. After which, the DNA is annealed into duplex or quadruplex under the below conditions (Table 2).

Table 2: HT and cMYC complementary sequences and folding buffers. *Italic bases are mutations to the wild type sequences.*

	HT	cMYC
Complementary Strand	5' <i>TTCCCTAACCCTAACCCTAA</i> <i>CCCTTT</i> 3'	5' TTACCCACCCTACCCACCC TCA 3'
Single Strand Buffer	10mM Tris Cl, pH 7.7	10mM Tris Cl, pH7.7
Duplex Buffer	10mM Tris Cl and 100mM LiCl, pH 7.7	10mM Tris Cl and 100mM LiCl, pH7.7
Quadruplex Buffer	10mM Tris Cl and 100mM KCl pH 7.7	10mM Tris Cl and 100mM KCl pH 7.7

2.2 Circular Dichroism Measurements

CD analysis was done on Jasco J-810 CD Spectropolarimeter at 400nm to 200nm with 5mm pathlength quartz cuvettes. 3 μ M of DNA was used to validate the structures of single strand and quadruplex while 1.5 μ M of DNA was used for duplex. A 10 time accumulation was done for each measurement. The scan rate used was 100nm/min with a data pitch of 0.2nm.

2.3 UV-Melting Analysis

3 μ M of single strand and quadruplex DNA was used for UV-melting analysis while 1.5 μ M of duplex DNA was used. The analysis was generated using Beckman Coulter DU800 Spectro-Photometer equipped with T_m cuvette holder (1cm light path) and temperature controller. HT melting profile was monitored over a range of 20°C to 95°C while cMYC was monitored over a range of 50°C to 105°C. The melting profile for quadruplex was measured at 295nm and duplex was at 260nm. The ramp rate for temperature increment was 0.5°C per minute and the absorbance was taken every 1°C increment.

2.4 Radiolabelling

γ -³²P-Adenosine 5'-triphosphate (ATP) from Perkin Elmer, T4 polynucleotide kinase and buffer from New England Biolabs (NEB) and DNA in ultrapure water were incubated for 1 hour at 37°C. The labeled DNA was filtered with GE microspin 50 columns (cat no.: 27-5325-01) to remove any free ATP. A 15% denature polyacrylamide gel electrophoresis (PAGE) was ran for at least 1.5 hours at constant power of 16W to purify the labeled DNA. The gel was exposed onto Kodak green film for 30s in a cassette and printed with the X-ray Medical Film Developer machine PRO-200. The darkest band shown on the film was excised from the gel and extracted by 500 μ l of distilled water for at least 1.5 hours. The microtube was centrifuged and the extraction was filtered to remove small gel crumbs. The sample was speedvac to dry and re-dissolve with 100 μ l of water. The labeled DNA was desalted with GE microspin 50 columns and speed vac to dry.

100 μ l of distilled water was added to dissolve the labeled DNA. 1 μ l of the labeled DNA was tested against the Geiger counter, Ludlum Scaler ratemeter 2221, to determine the counts per minute per microliter of DNA.

2.5 Oxidant Preparations

Hydrogen Peroxide- 480 μ M or 2400 μ M of hydrogen peroxide stock was prepared from diluting 35% hydrogen peroxide in water. 2 μ l of the stock was added into 30 μ l of DNA sample.

Peroxynitrite (CalBiochem)- Exact concentration of peroxynitrite was determined by back calculations using Beer's Lambert Law. UV absorption was taken at 302nm while extinction coefficient was taken to be 1670M⁻¹ cm⁻¹. 9.6mM or 48mM of peroxynitrite was prepared from diluting the peroxynitrite stock into pH 10 NaOH solution. 0.5 μ l of 9.6mM / 48mM peroxynitrite was added into the 31.5 μ l of the DNA sample.

Hydroxyl radicals- 0.05M of Iron perchlorate (Sigma Aldrich) stock was prepared by dissolving it in water. Further dilutions by water were done to obtain 1.6mM iron perchlorate solution / 8mM of iron perchlorate solution. The iron perchlorate solutions were freshly prepared before the oxidation reactions. 35% hydrogen peroxide was diluted by water to achieve 160mM / 800mM of stock. 1 μ l of hydrogen peroxide stock was added into 30 μ l of DNA sample, vortex and spin, followed by 1 μ l of iron perchlorate stock.

2.6 Hydroxyl Radical Quantification

Hydroxyl radicals via Fenton reactions were quantified using terephthalic acid (TA) (Sigma Aldrich) trap by detecting fluorescence emission of product 2-hydroxyterephthalic acid (TAOH) (Sigma Aldrich)^{32 17 33}. TA stock was prepared in 2 equivalence of KOH in KCl and pH was adjusted to 7.7. 0.4-5.0mM of iron perchlorate stock and 40-500mM hydrogen peroxide stock were prepared in water. Solutions were added in this order: 1) TA stock 2) hydrogen peroxide stock 4) iron perchlorate stock. Hydrogen peroxide was added at 100 equivalence of iron perchlorate. Final buffer condition was either 4.25mM TA in 100mM KCl pH7.7 or 21.25mM TA in 100mM KCl pH7.7. Higher TA concentration was used to detect a higher range of hydroxyl radicals. Fluorescence emissions of the samples were measured immediately after the addition of iron perchlorate using an excitation and emission wavelength of 330nm and 425nm respectively with *Cary Eclipse Fluorescence Spectrophotometer*. TAOH standard curve was measured by dissolving TAOH in 10mM KPO₄ and 100mM KCl (final pH6.6/6.8).

2.7 Oxidation Reactions

4μM of non-labeled DNA and ³²P labeled DNA (~ 10000 CPM) were annealed to different secondary structures in corresponding buffers (Table 2). Respective oxidants of desired concentrations were added to the DNA samples making up a total volume of 32μl. Reactions were incubated at conditions listed in Table 3 and precipitated with biology grade cold ethanol. Piperidine samples were treated with 10% piperidine at 90°C for 25mins. Piperidine was removed by speed vac after the incubation. All samples were washed with 30μl of water before dissolving in bromophenol blue and xylene cyanol green dye mixture (Fermentas). The samples were then incubated at 90°C for 5 minutes prior to loading into a 20% denaturing PAGE gel. The gel was run at constant power of 30W.

Table 3: Oxidation conditions of different oxidants.

Oxidants	Incubation Conditions	Concentration/ μ M
Hydrogen peroxide	37°C 30 minutes	30
		150
Peroxynitrite	Room temperature 10 minutes	150
		750
Hydroxyl radicals	Room temperature	0.6
		2.6

3 Results and Discussion

3.1 Structural Characterization of the secondary structures of the HT and cMYC DNA sequences.

3.1.1 Circular Dichroism

Figure 6 CD spectrum of HT single strand (black) duplex (red) and quadruplex (blue)

Figure 7 CD spectrum of cMYC single strand (black) duplex (red) and quadruplex (blue)

GQs are known to produce specific wavelengths of maximum and minimum intensities when shined by an incident circularly polarized light. The maximum and minimum are caused by the asymmetric characteristics of the bases and glycosidic bonds in the quadruplex. Hence, anti-parallel and parallel quadruplexes share different characteristic CD spectra. An anti-parallel quadruplex usually has a positive peak at ~295nm, a negative maximum at ~260nm and a smaller negative peak at ~240nm. On the other hand, a parallel quadruplex has a positive maximum at ~260nm and a negative peak at ~240nm.^{2, 4} Hybrid type of quadruplexes such as the (3 + 1) anti-parallel adopts another set of characteristics bands, with a maximum at ~295nm, shoulder at ~260nm and small minimum at ~240nm.

Figure 6 shows HT DNA folded into a (3+1) anti-parallel quadruplex as seen from its characteristic maximum at 290nm, shoulder at 268nm and negative band at 240nm. The band at 290nm is due to the alternating guanine glycosidic bonds at the 3' and middle tetrad layer. The bands at 268nm and 240nm are probably originated from the non-alternating guanine glycosidic bonds of the 5' and middle tetrad layers. Here, the two positive peaks shift about 5~8 nm away from the typical maximum for (3+1) anti-parallel hybrid structure, which indicate the interactions between the two conformations in a stable GQ with dominant one topological structure.³¹ NMR study of this structure has proven its dominancy of more than 95% in 100mM KCl buffer.³¹

The CD spectra of cMYC sequence in Figure 7 indicate that the cMYC GQ folds into a parallel type quadruplex. A positive peak at 265nm and a negative peak at 245nm are consistent with previous studies.^{30 31} CD signals of cMYC GQ are distinct to those of single stranded and duplex cMYC DNA, implying that our buffer conditions are suitable for the formation of the desired secondary structures for the oxidation experiment in later sections.

3.1.2 UV melting

Figure 8 UV-melting profile for HT (empty) duplex and cMYC (shaded) duplex analyzed with sigmoidal fit

Figure 9 UV-melting profile for HT (empty) quadruplex and cMYC (shaded) quadruplex analyzed with sigmoidal fit

The thermodynamic stability of the secondary structures of nucleic acids can be determined by UV melting experiment. When single stranded oligonucleotides forms either duplex structures or other high order structures, such as four stranded GQ, hypochromism occurs to the characteristic peak of nucleobases at 260nm, due to the π stacking of the nucleobases inside the high order structures. In the case of GQ, a tail of UV spectra extends to long wavelength around 295nm. Hence, the hyperchromism at 295 nm and hypochromism at 260 nm are used to characterize the formation of GQ and duplex DNA in UV-melting experiment, respectively. Here, both HT and cMYC sequences show typical UV melting curves under 260 nm for duplex structure and 295 nm for GQ structure (Figure 8, 9). T_m of HT duplex and quadruplex are 68.4±0.2°C and 57.8±0.6°C respectively, while those of cMYC duplex and quadruplex are 70.1±0.0°C and more than 89.2±0.2°C, respectively. All T_m are consistent with the literature, hence, indicating the formation of the desire duplex and quadruplex topologies of both sequences under our buffer conditions.^{31 34}

3.2 Oxidative Damages of ROS on DNA secondary structures

3.2.1 The Type of Oxidative Damages

The two DNA sequences are treated with various ROS species after they are folded into desired secondary structures controlled by the buffer conditions. The resulting DNAs are submitted to alkaline treatment or pure water to reveal the nucleobase damage and direct strand break upon ROS oxidation. The damage patterns and yields are then revealed on denature PAGE. Figure 10 shows a typical gel image of the ROS induced oxidation on the duplex and GQ DNA. Under both circumstances, the lanes without piperidine treatment show no bands moving faster than the mother band on the top, while many bands with faster mobility show up in the lanes after piperidine treatment, which indicates the cleavage at corresponding base position. The same phenomena are observed across all of the experiments in this thesis work. The results indicate that the major damages induced by ROS are the oxidation of nucleobases which can only cause strand cleavage after alkaline treatment. The reaction on deoxyribose, which would cause direct strand break without piperidine treatment, is not prevailing if even does not occur here.

Lane	1	2	3	4	5	6	7	8	9
Oxidant				+	+	+	+	+	+
Piperidine			+				+	+	+
H ₂ O ₂ Control	+								

Figure 10 Denature PAGE gel electrophoresis of malignant concentration (2.6 μ M) hydroxyl radicals oxidation on cMYC duplex and quadruplex DNA with maxam gilbert sequencing. Lane 1 is hydrogen peroxide control with piperidine treatment. Lane 2 is control without oxidant and piperidine treatment. Lane 3 is control with piperidine treatment. Lanes 4-6 are samples treated with oxidant but not piperidine. Lanes 7-9 are samples treated with oxidant and piperidine.

3.2.2 Comparison between the Secondary Structures

Figure 11 Total guanine cleavage of cMYC duplex (empty), cMYC quadruplex (stripe), HT duplex (empty) and HT quadruplex (stripe) with malignant (red) and physiological (black) levels of hydrogen peroxide, peroxyntirite and hydroxyl radicals oxidations. Total cleavage percentage is obtained from the summation of normalized gel band intensities against the top band intensity from the same lane. All error bars <2%. Asterisk represents error bar <5%.

Figure 12 Total loop cleavage per base of cMYC duplex (empty), cMYC quadruplex (stripe), HT duplex (empty) and HT quadruplex (stripe) with malignant (red) and physiological (black) levels of hydrogen peroxide, peroxyntirite and hydroxyl radicals oxidations. Total cleavage percentage per loop base is obtained from the average of normalized loop bases gel band intensities against the top band intensity from the same lane. All error bars <0.2%.

- **GQ vs Duplex Guanine Damage**

Base cleavages on the DNA are observed when exposed to ROS under physiological and malignant conditions. Our data (Figure 11) shows that hydroxyl radicals have yielded the most pronounced amount of cleavages followed by peroxynitrite and lastly by hydrogen peroxide which is in line with the reactivity of the oxidants from previous studies.^{20 25} As only a slight amount of oxidative cleavage is incurred on all DNA structures by hydrogen peroxide, limited conclusions can be drawn from its oxidative damage reactions.

When exposed to malignant levels of peroxynitrite and hydroxyl radicals, more guanine oxidative damage is produced in cMYC than HT DNA regardless of the type of structure. This effect is especially distinctive for hydroxyl radical oxidation (Figure 11). Difference in total guanine damage between the 2 duplex sequences may be due to differing charge transfer efficiency. One of the factors governing the charge transfer efficiency is the length of thymine and adenine (TA) tracts in a sequence. The longer the tract the lower is the charge transfer efficiency as proven by Giese.³⁵ Higher charge efficiency will enable the formation of more guanine radical cation and more guanine cleavage in the duplex. As the HT sequence possesses a longer TA tract than cMYC, charge transfer efficiency is lower, hence, lesser cleavage is observed.

Regardless of the quadruplex topologies formed, the guanine bases are more vulnerable towards hydroxyl radicals and peroxynitrite than in their duplex forms when treated with malignant levels of the ROS. At physiological levels of hydroxyl radicals and peroxynitrite, the difference in guanine cleavages between that of the quadruplex and duplex is smaller. However, still show a trend whereby quadruplex guanines are less resistive against the ROS in most cases.

- **GQ vs Duplex Damage in the Loop**

Loop bases (i.e. adenine and thymine) in the HT and cMYC sequences are more vulnerable in the quadruplex than the duplex structure when exposed to malignant of both peroxynitrite and hydroxyl radicals (Figure 12). In general, the same trend has been observed for physiological levels of peroxynitrite and hydroxyl radicals as well.

3.2.3 Comparison between Topological GQ structures

- **Under Physiological ROS level**

As shown in Figure 11, similar damage pattern and yields are observed on both anti-parallel (HT) and parallel (cMYC) quadruplexes when two GQs are exposed to peroxynitrite and hydroxyl radicals under physiological conditions. Nucleobase oxidation by both ROS is not limited to guanines, but occurs to adenine and thymine bases in the loop too. The oxidative damage on the G-quartets of both structures is quite low and comparable to those on the loop bases. The result indicates that both topological folding of GQ can provide enough protection to the G-rich sequence from regular oxidative threats. The protection is beyond the gauge of normal cellular oxidation power and hence yields no difference between GQ polymorphism.

- **Under Malignant ROS level**

However, under increased levels of oxidants, the parallel cMYC quadruplex is more vulnerable than the anti-parallel HT quadruplex. Nucleobase damages not only occur at G-quartets but also at the loop bases in cMYC quadruplex. Although the G-quartet stacking in parallel cMYC quadruplex may provide more protection for the four guanines in the middle layer as shown in Figure 13, the reactive efficiency of ROS species to the parallel folding are significantly enhanced. Furthermore, the effects of topological folding on the damage of loop bases are more apparent. Almost under all the elevated ROS conditions observed here, the average cleavages of loop bases in cMYC quadruplex are significantly higher than those of HT quadruplex. This is not unexpected based upon the topological structures. Short, single base propeller loop and no diagonal loops are unique features and sometime necessary condition for parallel quadruplex. Both features prevent good π -stacking between the loop base and G-quartets, which is essential factor in the case of duplex DNA to protect the loop base from environmental oxidants. Hence, parallel folding in cMYC quadruplex diminishes the resistance of both guanines and loop bases against oxidative stresses to an extend more dramatically than the anti-parallel folding in HT quadruplex does. This trend is also presented in the comparison between duplex cMYC and HT sequences, which may due to the sequence dependence of charge transport in duplex DNA as discussed in section 3.2.2.

- **Outside G-quartet Specificity**

Figure 13 Comparison of 5'(black), middle (red) and 3' (blue) G-quartet layer cleavage for malignant (top) and physiological (bottom) levels of ROS. Total cleavage percentage is obtained from the summation of normalized gel band intensities against the top band intensity from the same lane.

The degree of cleavage of the middle tetrad layer of both parallel and anti-parallel quadruplex is much lower than its 5' and 3' layers when exposed to peroxynitrite at both physiological and malignant levels. However, for hydroxyl radical oxidations on the HT and cMYC quadruplexes, similar amount of damages among the 3 layers are observed for both concentrations. Also, though little cleavage is observed for all hydrogen peroxide oxidation reactions, a phenomenon similar to that of hydroxyl radical oxidation is seen to be applicable to those reactions as well. The occurrence of this observation may be attributed to the size of the ROS where a smaller ROS such as hydrogen peroxide and hydroxyl radical may enter into the middle tetrad layer through the grooves much more easily than the bigger peroxynitrite. Another possible reason for this observation can be due to the negative phosphate back bone shielding the middle tetrad layer from the negatively charged peroxynitrite by electrostatic repulsion.

3.2.4 Correlation to Biological Significance

Structural effects on the resistance of potential GQ folding sequences are observed here and suggest a correlation to their biological functions. Relatively to their duplex equivalents, both GQs have shown higher stability towards physiological than malignant levels of ROS. We suggest that the resistances of quadruplex and duplex are good enough to offset the ordinary oxidative threats in vivo and hence may not show apparent advantages over each other in the healthy cells.

Whereas, under many carcinoma and inflammation conditions, oxidative stresses are highly elevated by increasing local presences of ROS/RNS species. At malignant levels of ROS, our study indicates that the quadruplex chemical vulnerability towards the ROS explored is enhanced (Figure 11). The increments of oxidative cleavage for both parallel and anti-parallel quadruplexes over duplexes when treated with malignant levels of peroxynitrite anions and hydroxyl radicals suggest that formation of quadruplexes may reduce the chemical stability of the potential GQ-folding sequences and consequently compromise the genome stability in the cancer cell. This finding is consistent with the previous experiments from Majima and co-workers, where a higher amount of guanine damage in HT quadruplex than in HT duplex were observed when the oligonucleotides were subjected to the exposure of UVB irradiation.²⁷ The authors believe that this observation is due to the selective excitation of guanine bases and lower energy transfer efficiency from the excited guanine to cytosine and thymine in the HT quadruplex than HT duplex. This result also suggests the possibility of guanine oxidation in quadruplex may be the key towards telomeric shortening. The acceleration of cancer cells apoptosis may be predicted since the quadruplexes has a more drastic effect in lowering the overall telomeric stability than the duplex under malignant ROS levels. In addition, our observations have shown that the anti parallel HT quadruplex loops are less oxidation sensitive than the parallel cMYC quadruplex loops. This feature of the anti parallel quadruplex may serve as a protection to prevent over shortening of the telomeric regions.

On the other hand, the instability of cMYC quadruplex in the promoter regions can regulate the transcription of proteins in the cancer cell. With the presence of more chemically unstable quadruplex to promote cell apoptosis, the levels of the overly expressed *cmyc* oncogene in the cancer cells may be toned down. Furthermore, the parallel cMYC quadruplex loops are highlighted to be less chemically stable than the anti parallel quadruplex in 3.2.3, suggesting that the cMYC quadruplex is more oxidation sensitive. This feature of the cMYC quadruplex may cause the removal of the redundant *cmyc* oncogene to be more relentless.

Hence, like many other studies, our observations have highlighted the importance of stabilizing GQ as a therapeutic concept.

4 Conclusion

The work in this thesis has investigated the stability of two potential GQ folding sequences, human telomere and the promoter of *cmyc* oncogene, against the oxidative threats from various ROS, including hydrogen peroxide, peroxynitrite and hydroxyl radicals. Under both physiological and malignant relevant concentrations of ROS, the major damage in all the secondary structures occurs to the nucleobases, especially the guanines. No apparent direct strand break is observed. It is the oxidant, instead of the folding structures, that determines the damage type of the G-rich sequences under question.

Upon oxidation by ROS, both HT and cMYC sequences show more nucleobase damage in the quadruplex than in usual duplex form. The more chemically unstable the quadruplex is the higher chance of inducing cell apoptosis. As the over expression of telomeric ends are found in most cancer cells, this trend has enable HT quadruplex to regulate the telomeric regions by promoting apoptosis more easily than the duplex. Similarity for the cMYC quadruplex, its higher vulnerability towards ROS can also encourage cell apoptosis and removal of the *cmyc* oncogene that are overly expressed in cancer cells.

Topological structures also exhibit the ability to modulate the oxidative resistance of GQ against ROS threats. The parallel cMYC quadruplex is more sensitive towards the ROS at the loop regions in comparison to the anti parallel HT quadruplex. This suggests that the parallel cMYC quadruplex are more prone to encouraging apoptosis. Hence, the parallel cMYC quadruplex found in the promoter regions of the *cmyc* oncogene may in turn be less merciful in removing the redundant and overly expressed *cmyc* oncogene. HT quadruplex on the other hand, have to maintain a reasonable length of the telomere in the process of removing overly expressed telomeric ends. The shortening of telomeric ends has been found to be associated with aging and other diseases. Hence, the less chemical vulnerable anti parallel HT quadruplex may have been designed for this purpose.

Thus, the formation of quadruplex in cancer cell may be a key for the induction of cancer cell apoptosis which will curb the development of the disease.

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6 Appendix – Gel Electrophoresis

HT Hydrogen Peroxide Oxidation at Malignant and Physiological Concentration

Figure 14 Denature PAGE gel electrophoresis of malignant concentration (150 μ M) and physiological concentration (30 μ M) hydrogen peroxide oxidation on HT duplex and quadruplex strand DNA with maxam gilbert sequencing. Lane 1 is control with piperidine treatment for duplex. Lane 2 is control with piperidine treatment for quadruplex. Lanes 3-5 are duplex with oxidation and piperidine treatment. Lanes 6-8 are quadruplex with oxidation and piperidine treatment.

HT Peroxynitrite Oxidation at Malignant Concentration

Lane	1	2	3	4	5	6	7	8
Structure	Duplex	GQ	Duplex	Duplex	Duplex	GQ	GQ	GQ
Oxidant			+	+	+	+	+	+
Piperidine	+	+	+	+	+	+	+	+

Figure 15 Denature PAGE gel electrophoresis of malignant concentration (750 μ M) peroxynitrite oxidation on HT duplex and quadruplex strand DNA with maxam gilbert sequencing. Lane 1 is control with piperidine treatment for duplex. Lane 2 is control with piperidine treatment for quadruplex. Lanes 3-5 are duplex with oxidation and piperidine treatment. Lanes 6-8 are quadruplex with oxidation and piperidine treatment.

[illegible]

Lane	1	2	3	4	5	6	7	8
Oxidant			+	+	+	+	+	+
Piperidine		+				+	+	+

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HT Hydroxyl Radicals Oxidation at Malignant Concentration

Lane	1	2	3	4	5	6	7	8	9	10	11
Oxidant						+	+	+	+	+	+
Fe(ClO ₄) ₂ Control	+		+								
H ₂ O ₂ Control		+									
Piperidine		+	+		+				+	+	+

Figure 17 Denature PAGE gel electrophoresis of malignant concentration (2.6 μ M) hydroxyl radicals oxidation on HT duplex and quadruplex DNA with maxam gilbert sequencing. Lane 1 is iron perchlorate control without piperidine treatment. Lane 2 is hydrogen peroxide control with piperidine treatment. Lane 3 is iron perchlorate control with piperidine treatment. Lane 4 is control without oxidant and piperidine treatment. Lane 5 is control with piperidine treatment. Lanes 6-8 are samples treated with oxidant but not piperidine. Lanes 9-11 are samples treated with oxidant and piperidine.

HT Hydroxyl Radicals Oxidation at Physiological Concentration

Lane	1	2	3	4	5	6	7	8
Structure	Duplex	GQ	Duplex	Duplex	Duplex	GQ	GQ	GQ
Oxidant			+	+	+	+	+	+
Piperidine	+	+	+	+	+	+	+	+

Figure 18 Denature PAGE gel electrophoresis of physiological concentration (0.6 μ M) hydroxyl radicals oxidation on HT duplex and quadruplex DNA with maxam gilbert sequencing. Lane 1 is control with piperidine treatment for duplex. Lane 2 is control with piperidine treatment for quadruplex. Lanes 3-5 are duplex with oxidation and piperidine treatment. Lanes 6-8 are quadruplex with oxidation and piperidine treatment.

cMYC Hydrogen Peroxide Oxidation at Malignant and Physiological Concentrations

Lane	1	2	3	4	5	6	7	8
Structure	Duplex	GQ	Duplex	Duplex	Duplex	GQ	GQ	GQ
Oxidant			+	+	+	+	+	+
Piperidine		+				+	+	+

Figure 19 Denature PAGE gel electrophoresis of malignant concentration (150 μ M) and physiological concentration (30 μ M) hydrogen peroxide oxidation on cMYC duplex and quadruplex DNA with maxam gilbert sequencing. Lane 1 is control with piperidine treatment for duplex. Lane is 2 control with piperidine treatment for quadruplex. Lanes 3-5 are duplex with oxidation and piperidine treatment. Lanes 6-8 are quadruplex with oxidation and piperidine treatment

cMYC Peroxynitrite Oxidation at Malignant and Physiological Concentrations

Figure 20 Denature PAGE gel electrophoresis of malignant concentration (750µM) and physiological concentration (150µM) peroxynitrite oxidation on cMYC duplex and quadruplex strand DNA with maxam gilbert sequencing. Lane 1 is control with piperidine treatment for duplex. Lane 2 is control with piperidine treatment for quadruplex. Lanes 3-5 are duplex with oxidation and piperidine treatment. Lanes 6-8 are quadruplex with oxidation and piperidine treatment.

cMYC Hydroxyl Radicals Oxidation at Physiological Concentration

Lane	1	2	3	4	5	6	7	8	9	10	11
Oxidant						+	+	+	+	+	+
Fe(ClO ₄) ₂ Control	+		+								
H ₂ O ₂ Control		+									
Piperidine		+	+		+				+	+	+

Figure 21 Denature PAGE gel electrophoresis of physiological concentration (0.6μM) hydroxyl radical oxidation on cMYC duplex and quadruplex DNA with maxam gilbert sequencing. Lane 1 is iron perchlorate control without piperidine treatment. Lane 2 is hydrogen peroxide control with piperidine treatment. Lane 3 is iron perchlorate control with piperidine treatment. Lane 4 is control without oxidant and piperidine treatment. Lane 5 is control with piperidine treatment. Lanes 6-8 are samples treated with oxidant but not piperidine. Lanes 9-11 are samples treated with oxidant and piperidine.