Engineering of anti-cancer g-quadruplex nucleic acids and anti-bacterial protein fibers

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NANYANG
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**ENGINEERING OF
ANTI-CANCER G-QUADRUPLEX NUCLEIC ACIDS
AND ANTI-BACTERIAL PROTEIN FIBERS**

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**ENGINEERING OF
ANTI-CANCER G-QUADRUPLEX NUCLEIC ACIDS
AND ANTI-BACTERIAL PROTEIN FIBERS**

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School of Physical and Mathematical Sciences

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ABBREVIATIONS

1D	One-dimensional
2D	Two-dimensional
3'-(6-FAM)-CPG	[1-Dimethoxytrityloxy-3-[O-(N-carboxy-(di-O-pivaloyl-fluorescein)-3-aminopropyl)]-propyl-2-O-succinoyl-long chain alkylamino-CPG
A	Adenine
AFM	Atomic Force Microscopy
AgNPs	Silver nanoparticles
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
CD	Circular dichroism
C	Cytosine
CFU	Colony forming units
CPG	Controlled pore glass
D ₂ O	Deuterium oxide
DMEM	Dulbecco's modified eagle medium
DMT	N,N-Dimethyltryptamine
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
DNA	Deoxyribonucleic acid
DSS	4,4-dimethyl-4-silapentane-1-sulfonic acid
Duplex	Double helix
<i>E. coli</i>	Escherichia coli
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EDX	Energy-dispersive X-ray spectroscopy
ELPs	Elastin-like proteins
FAM	Fluorescein dye

FBS	Fetal bovine serum
FESEM	Field Emission Scanning Electron Microscope
FID	Free induction decay
FTIR	Fourier-transform infrared spectroscopy
G	Guanine
G4	G-quadruplex
G-tetrad	Guanine tetrad
GTP	Guanosine triphosphate
HMBC	Heteronuclear multiple-bond correlation
HSQC	Heteronuclear single-quantum correlation
JR	Jump-and-return
Kpi	Potassium phosphate
NCL	Nucleolin
NMR	Nuclear magnetic resonance
NOE	Nuclear overhauser effect
NOESY	Nuclear overhauser effect spectroscopy
μM	micromolar
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
OD	Optical density
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate-buffered saline
pM	picomolar
PP	Polypropylene
PPM	parts per million
PQS	Putative G4 forming sequences
RMSD	Root-mean-square deviation
RNA	Ribonucleic acid
<i>S. aureus</i>	Staphylococcus aureus
SAED	selected area electron diffraction
SELEX	Systematic Evolution of Ligands by Exponential enrichment
SDS	Sodium dodecyl sulfate
ssDNA	single strand DNA
T	Thymine

TBE	Tris/Borate/EDTA
TEM	Transmission electron microscopy
TFE	Trifluoroethanol
T _m	Melting temperature
TMS	Tetramethylsilane
TOCSY	Total correlation spectroscopy
UTC	Untreated cells
UTR	Untranslated region
UV	Ultraviolet

ABSTRACT

Functional synthetic biopolymers are widely used in therapeutic treatment and medical devices. This thesis consists of two parts focusing on nucleic acids and proteins respectively. In the first project, structural conformation and bioactivity of G-quadruplex-forming sequences, derived from a well-known aptamer *AGRO100*, was explored. Our findings on the novel G-quadruplex structures and their anti-proliferative activity against cancer cells might contribute in the development of oligonucleotides for therapeutic treatment. In the second project, a recombinant protein derived from elastin was synthesized and induced to self-assemble and form a fiber platform for silver nanoparticles formation. The silver-coated protein fibers exhibited the anti-bacterial properties on *E. coli* and *S. aureus*. This synthetic protein offers a novel biomaterial for an anti-bacterial coating in medical devices.

1. CHAPTER 1 - INTRODUCTION

Biopolymer is defined as a natural polymer occurred in living organisms. Biopolymer is constituted from many monomeric units connected by covalent bonds. Two common classes of biopolymers are polynucleotides and polypeptides whose monomers are nucleotides and amino acids, respectively. Exploration of these basic biopolymers helps to apply them as an agent for therapeutic treatment or a material for medical devices.

1.1. FUNCTIONAL POLYNUCLEOTIDES

1.1.1. Deoxyribonucleic acid (DNA)

DNA encodes the genetic information of living organisms, in which the growth, development and reproduction of an organism are determined by its genomic sequence ¹⁻². The structure of a DNA double helix was first described by Watson and Crick, whereby X-ray diffraction determined the structure as two DNA strands running in opposite direction with a helical conformation (Figure 1.1A) ³. Each polynucleotide chain is made up of repeated monomer called nucleotide. Each nucleotide unit comprises of a deoxyribose sugar centered between a nitrogenous base (either purine or pyrimidine base) and a phosphate group at 5'-end (Figure 1.1B). The nucleotides are joined together by phosphodiester linkages that are covalently bonded from phosphate group of one nucleotide to 3'-OH group of another nucleotide's sugar. Continuous linkages of nucleotide units constitute the sugar-phosphate backbone with 5' to 3' directionality (Fig. 1.1B). In two DNA strands, purine bases [adenine (A) or guanine (G)] from one strand are connected to pyrimidine bases [thymine (T) or cytosine (C), respectively] of another strand by Watson Crick hydrogen bonds, resulting in a double helix DNA (Fig. 1.1C).

A DNA double helix has three canonical forms: A, B or Z-DNA, depending on surrounding environmental conditions and DNA sequences ⁴. B-DNA with right-handed helices is the most predominant form of DNA double helix in nature (Fig. 1.2A). The B-form DNA has 10 base pairs for each helical turn. The helical diameter, that is a distance between two phosphorous atoms of a base pair is around 2 nm. The B-form DNA has C2'-endo ribose sugar, with wide and deep major grooves. A-DNA is another form of right-handed helices found in dehydrated condition (Fig. 1.2B). The A-form diameter (2.6 nm) is wider than that of B-form (2.0 nm) along with 11 base pairs per complete turn and it adopts C3'-endo ribose sugar conformation, which results in narrow and deep major grooves ⁵. Beyond the A and B forms of DNA, Z-DNA is a rare form of DNA double helix

which is left-handed (Fig. 1.2C). Sequences with alternating purines and pyrimidines can favor the formation of Z-DNA. Electrostatic repulsion between two phosphate backbones is minimized in high salt environment, which leads to small helical diameter (around 1.8 nm) and flattening of major groove. The features of these structures are summarized in Table 1.1 ⁶.

Figure 1.1 Schematic of a helical DNA.

(A) DNA double helix schematic with strand directionality (5' to 3') indicated by a black arrow. (B) A chain of polynucleotide connected by the phosphodiester linkage. (C) Pairing of purine (adenine and guanine) and pyrimidine (thymine and cytosine, respectively) bases through hydrogen bonds (dotted lines). Figure is adapted and edited from ⁶.

Figure 1.2 Double helical DNA conformation

(A) right handed B-DNA, (B) right handed A-DNA and (C) left handed Z-DNA, represented in ribbon view (left), top view (middle), and space-filling representation (right). The figure is adapted from ⁷.

	Type of DNA		
	A	B	Z
Handed helical direction	Right	Right	Left
Base pair per turn (bp)	11.0	10.0	12.0
Helical diameter (nm)	~2.6	~2.0	~1.8
Type of sugar pucker	C3'-endo	C2'-endo	C2'-endo (pyrimidine)
			C3'-endo (purine)
Characteristic of major groove	Narrow and deep	Wide and deep	Flat
Characteristic of minor groove	Wide and shallow	Narrow and deep	Narrow and deep

Table 1.1 Structural characteristic of various DNA conformation

1.1.2. G-quadruplex DNA

Beside the various double helical DNA conformations described above, oligonucleotides rich in guanines can potentially adopt a non-canonical structure called G-quadruplex (also termed as G4-DNA, G4 or G-DNA) ⁸⁻⁹. Unlike the double stranded DNA,

G-quadruplex has four DNA backbone strands, and is composed of a G-tetrad core (also called as tetrad, or G-quartet) connected by loops. The G4 core is composed of at least two G-tetrads¹⁰, with three and four layer G4 most commonly reported by either X-ray crystallography or NMR methods¹¹⁻¹². As the G4 core exhibits different strand directionality and loop types, this results in G4 to be structurally diverse. Other factors contributing to G4 polymorphism includes the DNA sequence length, DNA concentration and ion types in solution.

A G-tetrad is composed of four guanine bases self-assembled in a square planar arrangement and stabilized by cationic ions. The G-tetrad is held in a plane by Hoogsteen hydrogen bonds between imino proton N1-H1 and amino proton N2-H2 of a guanine with acceptors O6 and N7 of a neighboring guanine, respectively (Fig. 1.3). Cations such as K^+ , Na^+ in the central cavity coordinate with O6 carbonyl group of each guanine to stabilize the G-tetrad (Fig. 1.3A)¹³.

Figure 1.3 Schematic of a G-tetrad

(A) Arrangement of guanines on a plane, (B) and (C) schematic representation of a G-tetrad.

Loops are linkers from nitrogenous bases of one G-tetrad to other tetrads. There are three different types of loops commonly found in G4-DNA. Edgewise (or lateral) loop connects two adjacent backbones of opposing directionality (Fig. 1.4A)¹⁴, double-chain-reversal (or propeller) loop links two neighbouring with the same strand orientation (Fig. 1.4B)¹⁵ and diagonal loop connects two opposite strands (Fig. 1.4C)¹⁶⁻¹⁷. A V-shaped loop first introduced by Patel's group is an uncommon loop which occurs when one backbone strand is broken (Fig. 1.4D)¹⁸. In addition, loops of a G4 may also be formed from bulges¹⁹, or multiple duplex stems²⁰.

Figure 1.4 Loop arrangement.

(A) Edgewise loop, (B) double-chain-reversal loop, (C) diagonal loop, and (D) V-shaped loop. Arrows represent strand directionality from 5'-end to 3'-end.

G4 scaffold is constituted by four individual phosphate-sugar backbone strands. These strands originated from one, two or four molecules. G4 stoichiometry also affect the number of loops participating in the G4 structure. For examples, tetramer G4 is formed by four molecules without any loop connectivity (Fig. 1.5A), two G-rich molecules constitute a dimer G4 with two edgewise loops (Fig. 1.5B), or one molecule makes up a monomer G4 with three propeller loops (Fig. 1.5C).

Figure 1.5 G4 stoichiometry.

(A) Tetramer, (B) dimer and (C) monomer.

Sugar – phosphate backbone DNA strand directionality results in different G4 structures: parallel, hybrid or anti-parallel. The strand directionality is defined from 5'-end to 3'-end of DNA backbone. Parallel G4 (4+0) contains four strands of the same directionality (either upward or downward) (Fig. 1.6A). G4 with hybrid structure (3+1) has three unidirectional strands, with one strand pointing in the opposite directionality (Fig. 1.6B). Anti-parallel G4 (2+2) comprises two strands (adjacent or opposite) orientated with opposition direction from the other two strands (Fig. 1.6C and D).

Figure 1.6 Directionality of DNA phosphate backbone

(A) (4+0) Parallel, (B) (3+1) hybrid, (C & D) (2+2) anti-parallel. Red arrows illustrates opposite directionality to others.

Nitrogenous base covalently bonds to C1' of deoxyribose sugar creates an N-glycosidic bond. Rotation of guanine within a G4 may adopt different glycosidic conformation, *syn*, or *anti* orientation (Fig. 1.7A and B, respectively) ¹⁶. Guanosine adopts *syn* conformation when the nitrogenous base rotates toward the sugar, while *anti*-conformation has base facing out of the sugar. The *anti*-guanine can be formed by either C2' or C3'-endo ribose sugar, whereas *syn*-guanine favors C2'-endo sugar conformation.

Figure 1.7 Glycosidic conformation of guanosine

(A) *syn*-guanine and (B) *anti*-guanine.

The stacking and rotation of guanines on each tetrad along with two torsion angles of glycosidic bonds create significantly different groove dimensions within the G4 structure. They are classified into three types based on the distance from phosphorous of one guanine to neighboring one within the same G-tetrad (Fig. 1.8). Wide, medium and narrow groove spans 19 Å, 16 Å and 12 Å, respectively. The existence of various grooves contributes to the structural diversity of G4 conformation.

Figure 1.8 Schematic representation of different types of groove dimension (wide, medium and narrow).

Phosphorous is indicated by yellow circles for clarity. (The figure is taken from A.T. Phan, unpublished).

1.1.3. Biological relevance of G-quadruplex

Guanine-rich sequences have been found to be prevalent in parts of the human genome, appearing mostly in oncogenes, telomeres and some key growth regulatory genes²¹⁻²⁵. Up to 376,000 putative G4 forming sequences (PQS) were predicted in promoter regions by computing bioinformatics with the algorithms form of $G_{\geq 3}N_{1-7}G_{\geq 3}N_{1-7}G_{\geq 3}N_{1-7}G_{\geq 3}$, where N is adenine, guanine, cytosine or thymine²⁶⁻²⁸. Herein, a putative G4 sequence is composed of 4 stretches, with each stretch containing at least three guanines. Within the human genome, putative G4 has been found with higher abundance at the telomeres (Fig. 1.9B)²⁹, gene promoters²⁷, intron, exon (Fig. 1.9A)³⁰, and at human replication origins (Fig. 1.9C)³¹. Additionally, putative G4 were also reported with high incidence in the immunoglobulin switch regions³²⁻³⁴ and the 5' untranslated regions (5'-UTR) of mRNA

in the cytoplasm (Fig. 1.8D) ³⁵⁻³⁷. The conserved G-rich sequences in these regions suggest a key regulatory function of G4 formation in cells.

Figure 1.9 Possible locations of putative G4 in cells ³⁸.

(A and C) DNA-G4 involves to transcription and replication processes, respectively. (B) Putative G4 appears in 3' overhang of telomeres. (D) Possible RNA-G4 formation in mRNA molecule effects to translation pathway.

a. G4 formation within the Telomeres

Telomeres are composed of 1000 to 2000 tandem repeat of the sequence TTAGGG, ^{29, 39} located at the end of a human chromosome to serve as a cap for protecting chromosome from DNA degradation or unwanted repair ⁴⁰⁻⁴¹. In successive cycles of cell division, telomeres are gradually shortened due to incomplete replication of the template strand. Normal human cell induces senescence or apoptosis process when telomere length becomes critically short. However, in tumor cells, telomere length is protected from shortening by the action of telomerase ⁴² which acts to extend the length of the telomere and causes cells to be immortalized ⁴³. G4 formation at the telomeres has the potential to inhibit telomerase activity (Fig. 1.10) ⁴⁴⁻⁴⁷ as the bulky G4 structure sterically hinders the telomerase. As such, stabilization of G4 structure in telomeres as an anti-cancer therapeutic strategy is increasingly explored ⁴⁶⁻⁴⁷. The increasing availability of high-resolution structure of short telomeric G4 formed by one, two, three or four TTAGGG repeats (Fig. 1.11) also helps in the application of G4 as an anti-cancer therapeutic ¹⁶.

Figure 1.10 Schematic representation of the impact of G-quadruplex formation on a tumor cell. The figure is adapted from ⁴⁸.

Figure 1.11 Structural schematic of human telomeric DNA G-quadruplex from one, two, three or four TTAGGG repeat ¹⁶.

(A) A tetrameric parallel G-quadruplex formed by a single TTAGGG repeat in K^+ solution ⁴⁹. Two repeats of TTAGGG may assemble as dimeric G-quadruplexes of parallel (B) or anti-parallel (C) conformation in K^+ solution ^{12, 50}. Asymmetric dimeric (3+1) G-quadruplex in Na^+ solution was observed in (D) two strands of three repeat sequences and (E) association of a single repeat strand and three repeats one ⁵¹. (F-J) Formation of monomeric G-quadruplex from four repeat sequence adopt (F, J) anti-parallel ^{10, 29}, (G) parallel ¹², (H-I) (3+1) structure ⁵².

b. Impact of G4 formation in cellular processes

Within the human genome, DNA may be transiently assembled as G4 during biological processes of DNA replication, transcription or DNA repair, as a result of the breaking of Watson Crick hydrogen bonds of double helix DNA⁵³⁻⁵⁴. In promoter regions, formation of G4-DNA regulates the transcription of some genes such as C-MYC²², C-KIT⁵⁵⁻⁵⁷, BCL-2²⁵, HIF-1 α ⁵⁸, K-RAS²⁴. These genes encode for key regulatory factors of tumor cells. As such, controlling the formation of G4 in these regions can be potentially applied for chemotherapeutics treatment (Fig. 1.12)⁵⁹⁻⁶². In contrast, resolving G4 on these sites can cause transcriptional activation of these genes⁶³⁻⁶⁴. At least one PQS was found in more than 40% promoters of the human genome²⁷. The role of G4 in mediating transcriptional process was further confirmed by the occurrence of G4 in promoter and UTRs of important metabolic enzymes, receptors or regulatory proteins⁶⁵.

Figure 1.12 Schematic of putative G4 at promoter regions.

Formation of G4 in this region is possible to alter transcription of target genes. The figure was adapted from⁶⁶.

Single strand RNA molecules are believed to easily adopt G4 structure⁶⁷ and are more thermodynamically stable than DNA G4s^{35, 68-70}. As RNA PQS are prevalent at 5'-UTRs, with around 3000 positions suggested by bioinformatics studies³⁵. G4 can also serve as a regulatory function in post-transcription through the formation of RNA G4 close to the translation start sites. Several studies have demonstrated the successful down-regulation of some important clinical genes such as the neuroblastoma RAS oncogene encoded p21 protein through the stabilization of RNA G4 structures on the 5'-UTRs^{35, 61}.

c. *G-quadruplex binding proteins*

The role of putative G-quadruplex structures in a variety of cellular processes also involves the specifically interaction of G-quadruplex binding proteins. These proteins can be classified into three groups based on their biological functions (Table 1.2). The first group includes proteins that recognize and bind G-quadruplex to increase its stability and regulate some cellular processes. The second group contains some proteins which promote the formation of G-quadruplex from its single stranded form as well as stabilize its structure. The third group includes protein enzymes that resolve structure of G-quadruplex in an ATP-hydrolysis dependent manner (helicases).

Table 1.2 Proteins that bind to G-quadruplex

Type of G-quadruplex interactive proteins	Proteins	G-quadruplex location / name	Proposed function	References
Binding and stabilizing G-quadruplex	MutS α	Immuno-globulin switch (S) regions	tightly binds to transcribed S regions, promotes DNA synapsis and recombination	71
	MyoD	Promoters of muscle-gene	up-regulate the muscle-gene transcriptions	72
	NPM1 (Nucleo-phosmin)	c-MYC promoter	electrostatic interactive binding of G-quadruplex inactivates NPM1's nucleolar localization	73
	Wild-type p53	Nuclease hypersensitive element (NHE) III1 region of MYC promoter	regulates the transcription of MYC genes	74-75
Promoting the formation of G-quadruplex	Nucleolin	c-MYC promoter	promotes and increases the stability of G-quadruplex to reduce activity of c-Myc promoter.	76
	Topo1	Telomeres and promoter regions	promotes the formation of G-quadruplex leads to inhibit Topo 1 activity in double strand DNA cleaving	77 78
	EWS	Telomeres	binds to G-rich ssDNA, promotes G-quadruplex formation at the RGG domain of the C-terminal in EWS	79
Unwinding G-quadruplex conformation	FANCI	Helicase	binds and unfolds G-quadruplex structure at the 5'-single strand DNA tail to reduce the apoptosis and DNA damage in breast cancer cells	80

RecQ	Helicase	unfolds G-quadruplex DNA structures to increase the genomic stability in Bloom's syndrome	81 82
RHAU	Helicase / RNA G-quadruplex	resolves G-quadruplex conformation in mRNA to control the transcription and translation process	83

1.2. *AGRO100*

1.2.1. Nucleic acid aptamers

Aptamers are relatively short single stranded DNA or RNA molecules often selected from a library of random nucleic acid by a method of Systematic Evolution of Ligands by EXponential enrichment (SELEX). Aptamers have a unique three-dimensional structure which recognizes and binds to molecular targets such as peptides, proteins, small molecules, cells, virus or bacteria (Fig. 1.13) ⁸⁴. Aptamers specifically and selectively interact with their targets through hydrogen bonds, van der Waals, or via hydrophobic or electrostatic interactions ⁸⁵. Due to the high affinity of aptamers to targets from micromolar (μM) to picomolar (pM) range, its non-immunogenicity and stability in a range of temperature and pH environment, aptamers are increasingly applied in a variety of applications such as therapeutic treatments ⁸⁶, diagnostic ⁸⁷, drug delivery ⁸⁸ or biosensor devices ⁸⁹⁻⁹⁰. Additionally, its small size facilitates structural control and chemical modification ^{85, 91}. The use of aptamers in therapeutic treatment is gaining popularity for the treatment of some important diseases such as diabetes ⁹², inflammation ⁹³, and thrombosis ⁹⁴.

Figure 1.13 Schematic diagram of a functional aptamer forming aptamer-target complex. The figure was adapted from ⁹⁵.

1.2.2. *AGRO100* as first anti-cancer oligo aptamer in clinical trial

AGRO100 is a G-rich DNA sequence containing 26 nucleotides d(GGTGGTGGTGGTTGTGGTGGTGGTGG). *AGRO100*, also known as *AS1411*, is the first anti-cancer aptamer that reached Phase II clinical trials for acute myeloid leukemia and renal cell carcinoma ⁹⁶. The first version of this aptamer is the 29 nucleotides long *GRO29A* d(5'-TTTGGTGGTGGTGGTTGTGGTGGTGGTGG-3'), which is modified with a 3'-aminoalkyl group ⁹⁷. This aptamer was shown to form G4-DNA, binding specifically to multifunctional phosphoprotein nucleolin and inhibiting the proliferative activity of a range of cancer cell lines *in vitro*. However, *GRO29A-OH*, is *GRO29A* without a 3'-aminoalkyl modification, exhibits the same activity and nuclease resistance as *GRO29A* version ⁹⁸. Finally, *AGRO100*, a 26-bases oligonucleotide, is a truncated version of *GRO29A* without the 5'-TTT and nucleotide modifications is the current version used in the clinical trial ⁹⁹.

1.2.3. *AGRO100* as anti-cancer agent

The aptamer *AGRO100* was evaluated for its anti-cancer property through a range of more than 80 cancer cell lines. The concentration of testing drug required to inhibit 50% cancer cell growth (GI₅₀) was lower than 10 μ M, while normal human cell lines were unaffected ¹⁰⁰⁻¹⁰². *AGRO100* was then evaluated for its bioactivity in animal models, showing a non-toxic effect at a dose of 10 mg/kg/day for both rats and dogs ¹⁰³⁻¹⁰⁴. *AGRO100* underwent Phase I clinical trial in 2003 as a first anti-cancer aptamer tested in human ¹⁰⁵, and the study was successfully completed on 17 metastatic or advanced cancer patients with non-toxic response in humans ¹⁰⁶. The Phase II clinical trial was then started in 2007 for 35 patients who had acute myeloid leukemia and renal cell carcinoma ⁹⁶. One patient exhibited a dramatic and durable response. The moderate side adverse event were observed in 34% of patients. However, the ongoing clinical trial of *AGRO100* (or *AS1411*) was terminated in 2011 due to the failure of unrelated drug candidate in pivotal Phase 3 trials ¹⁰⁷. As *AGRO100* has an excellent safety profile, this aptamer is still being developed to improve its bioactivity for cancer treatment.

Proposed mechanism of AGRO100 mediating cancer treatment

The precise mechanism of *AGRO100* for anti-proliferative activity and cytotoxicity to a wide type range of cancer cells is still unclear. Formation of G4 was believed to give biological activities when comparing *AGRO100* to other non-G4 forming G-rich sequences¹⁰⁸. It is most likely that nucleolin is a molecular target of *AGRO100* due to its association with multiple cancer types. Nucleolin (NCL) is a multifunctional protein found abundantly in the nucleolus¹⁰⁹⁻¹¹¹. Nucleolin is also over-expressed on a cell surface and cytoplasm in some cancer cell types¹¹²⁻¹¹⁵ and involves to modulate cell proliferation¹¹⁶, cell cycle regulation¹¹⁷ and apoptosis¹¹⁸. Thus, nucleolin is considered as a target for cancer cells in therapeutic treatment.

Nucleolin was initially speculated as a cell surface receptor, and the binding of *AGRO100* to nucleolin on plasma membrane was thought to mediate DNA internalization⁹⁷. However, further research has found that *AGRO100* enters cells via macropinocytosis (a type of fluid phase endocytosis) and specifically targets and binds to cytoplasmic nucleolin, where the *AGRO100*–nucleolin complex is thought to inhibit various growth factors signaling activity to result in cell death¹¹⁹. Bates' group has proposed several mechanisms in which *AGRO100* selectively accumulates in cancer cells to cause cell death (Fig. 1.14 and 15, respectively)^{107, 119}.

Figure 1.14 Proposed accumulation process of *ASI411* (*AGRO100*) in cancer cells.

ASI411 enters both normal and cancer cells efficiently at the beginning. *ASI411* binds to cytoplasmic nucleolin and induces further macropinocytosis in cancer cell but not in normal cells. *ASI411* finally selectively accumulates while is cleared from normal cells that has less nucleolin. The figure is adapted from ¹⁰⁷.

Figure 1.15 Proposed mechanism of *ASI411* (*AGRO100*) induces cancer cell death.

(Left) In cancer cells, nucleolin (NCL) blocks the activity of Rac1 (GTPase enzyme which mediates macropinocytosis and methuosis) leading to cell growth and metastasis. **(Right)** In the presence of *ASI411*, aptamers bind to cytoplasmic nucleolins and inhibit recognition of NCL to Rac1 and lead to active Rac1. Hyperactivation of Rac1 induces methuosis causing cell death. The figure is adapted from ¹¹⁹.

1.2.4. Polymorphism of *AGRO100* structure

Although *AGRO100* has outstanding effect in cancer treatment *in vitro*, the high-resolution structure of this 26-mer oligonucleotide is still unknown. The structure of *AGRO100* is challenging as the G-rich sequence is known to adopt multiple structures in solution¹²⁰. At least eight monomeric structural conformations of *AGRO100* have been detected by size exclusion chromatography (Fig. 1.16 left)¹²¹. Circular dichroism (CD) spectra of these isolated fractions predominantly exhibited the CD signature of a parallel G4 conformation, characterized by a positive trough at 260 nm and negative ones at 240 nm and near 290 nm.¹²¹

Figure 1.16 Analysis *AGRO100* conformation in potassium solution.

(Left) Size exclusion chromatography separated multiple fractions and (Right) CD spectra of isolated fractions of *AGRO100* solution. The result was adapted from¹²¹.

To reduce the structure polymorphism of *AGRO100*, Do and co-workers modified the *AGRO100* sequence via the addition of thymine residues at both 5' and 3' ends of *AGRO100* sequence to prevent formation of higher order structures or aggregation¹²², and reducing number of guanine residues in sequence from 17 to 16 by substituted one G to T to favour a single form of G4. In this manner, one of the *AGRO100* variant, *AT11* with G to T substitution at position 11, d(TGGTGGTGGTTGTTGTGGTGGTGGTGGT), was resolved as a single G-quadruplex structure in solution via NMR spectroscopy¹²³. CD spectra of *AT11* (Fig. 1.17-B1) confirmed the formation of a right-handed parallel structure, which matches the predominant forms of *AGRO100* in potassium solution.

Figure 1.17 Analysis of modified *AGRO100* sequence.

CD spectra (**A1 and B1**) and representative structure of left-handed (**A2**) and right-handed (**B2**) G4. The results were adapted from ¹²⁴ and ¹²³.

Another *AGRO100* variant modified at position 27, called *AT27* d(TGGTGGTGGTGGTTGTGGTGGTGGTGGTT), was later shown to be the first known left-handed G4 structure by both X-ray crystallography and NMR spectroscopy ¹²⁴. CD spectra of *AT27* (Fig. 1.17-A1), with maxima at 240 nm and minima at around 280 nm, was shown to be similar to the unusual fraction 2 (Fig. 1.16 right, green curve) isolated from *AGRO100*.

Both right-handed G4 *AT11* and left-handed G4 *AT27* structures comprise of two stacked G4 blocks wherein each block contains two G-tetrad layers connected through a central linker T16 of *AT11* and T13-T14 of *AT27*. However, two subunits of *AT11* exhibited 3' to 5' stacking mode while *AT27* exhibited 5' to 5' stacking. The strand progression of *AT11* followed the right-handed manner while that of *AT27* was left-handed (Fig. 1.17-A2 & B2).

1.3. FUNCTIONAL POLYPEPTIDES FOR MATERIAL COATING

1.3.1. Elastin polypeptides

As discussed above, DNA carries genetic information of living organism. DNA dictates the function of protein via intermediate biopolymer RNA. A protein consists of one or many polypeptide chains which are constituted from more than 20 types of amino acids via peptide bonds. There are different types of proteins such as structural proteins, enzyme, or messenger proteins that made up a living organism.

Elastin is a type of extracellular matrix (ECM) protein, found commonly in connective tissues, such as skin, lung, heart and blood vessels ¹²⁵. Elastin provides elasticity and resilience to tissues, allowing them to stretch and deform ¹²⁶. Elastin also binds fibrillin, a large glycoprotein, to form a network of elastic fibers in the ECM. Elastin and elastin-like proteins (ELPs) have been shown to promote cell proliferation and cell adhesion ¹²⁷. Due to their mechanical properties, elastin is widely used in drug delivery and tissue engineering in combination with growth factors for wound dressings or dermal substitute applications ¹²⁸.

1.3.2. Recombinant functional elastin protein as a novel coating material

A functional fusion protein GPG-AG3 which contains elastin-like domains and silver-binding motifs for silver nanoparticles mineralization has been reported ¹²⁹. The artificial protein construction was presented in Figure 1.18. The elastin-like domain comprised of two parts, Proline-rich domain (denoted as P) in the centre, and two Glycine-rich domains (denoted as G). Both P and G domains were originated from elastin precursor – tropoelastin ¹³⁰. Another short 12 amino acids AG3 domain which was able to nucleate silver particles was conjugated at C-terminal elastin ¹³¹. The artificial elastin GPG-AG3 was not only inherited elastin feature to undergo self-assembly and form protein aggregates but also can induce silver nanoparticle (AgNPs) formation (Fig. 1.19A). The AgNPs-elastin protein shows successful inhibition of gram-negative *E.coli* growth on 2x YT agar plate (Fig. 1.19B). The work has provided a novel biocompatible anti-bacterial material that is potential for tissue engineering and regenerative application ¹²⁹.

Figure 1.18 Sequence of recombinant artificial elastin protein GPG-AG3 ¹²⁹.

Figure 1.19 Recombinant elastin like protein GPG-AG3.

(A) TEM image of AgNPs (black dots) on elastin template (transparent aggregates). (B) Anti-bacterial efficiency of silver-coated GPG-AG3 film ¹²⁹.

1.3.3. Antibacterial activity of silver nanoparticles (AgNPs)

Silver is one of the most studied metal due to its excellent electrical conductivity ¹³², optical properties ¹³³ and wide-spectrum antimicrobial effects ¹³⁴. For several decades, silver had been demonstrated to have antimicrobial properties against gram-negative and gram-positive bacteria, as well as viruses, protozoa, fungi and antibiotic resistant strains ¹³⁵. As a result, silver has been widely incorporated in a wide variety of applications, including biosensors, coatings for biomedical implants, wound dressings and coatings in food packaging ¹³⁶. However, the mechanisms by which silver interacts with bacteria are still being elucidated. One hypothesis is widely accepted that is disruption of bacteria cell membrane occurred when the cell cytoplasm interacts with silver ions. Others have shown that interaction of silver ions with the ribosome halts the enzymes and proteins expression essential to ATP production ¹³⁷.

Silver nanoparticles (AgNPs) are widely explored as antibacterial agents due to their ability to penetrate the bacteria cell wall more effectively. Currently, AgNPs are synthesized via the chemical reduction of silver ion precursors in the presence of additives.

These additives include polymers and surfactants, which are essential for the initial nucleation, stabilization and growth of AgNPs. Recently, peptide-based strategies have also been explored for the synthesis of silver nanoparticles due to their intrinsic solubility in aqueous media and increased biocompatibility¹³⁸.

1.3.4. Bio-coating material in biomedical devices

Compatible biomaterials are on the high demand for coating biomedical devices such as implants, biosensors, drug delivery or tissue engineering. However, the mechanism of interaction between organisms and materials remains unknown, which leads to poor systematic design of bio-coating materials¹³⁹⁻¹⁴⁰. Currently, construction of biofunctional interface develops notably in scientific research and engineering, to have a better understanding of these aspects. General conditions of coating material are a capability to tolerate organism, communication in both ways between devices and tissue, or biocompatibility of surfaces to the environment¹⁴¹.

Coating material selection depends on some factors such as mechanical properties, topography, biocompatibility, etc.¹⁴². However, some functional surfaces such as silane on silica substrate or fatty acid on metal oxide material varied biological responses of materials to the specific environment¹⁴³. Hence, the combination of coating material and surface functionalization is ideal in case of retaining material properties while enhancing chemical specification on a material surface. From that, we can control the reaction of material in surrounding environment as well as tune functional surface to various purposes¹⁴⁴.

Nowadays, researchers attempt to design bioactive surfaces on a material to form biocontact properties and regenerate tissues in biomedical application¹⁴⁵. For example, adhesive receptors or carbohydrates of growth factors and enzymes were grafted on a biomimetic material to enhance a bioinert environment¹⁴⁶. For antibacterial material on the surface of medical devices such as heart stents, valves, catheters, antimicrobial peptides which are short peptides, exhibiting fungicidal and bactericidal, were employed¹⁴⁷. Alternatively, nanocomposites such as silver nanoparticles are also developed to be effective antimicrobial materials¹⁴⁸.

1.4. RESEARCH OBJECTIVES

The overall goal of this Ph.D. thesis is to investigate the role of functional polynucleotides and polypeptides in application of medical treatment and medical device, respectively. As such, the work can be divided into two main parts.

a. AGRO100 aptamer as an anti-cancer agent

We resolved a novel G4 structure which is a modification from *AGRO100* sequence. The structure allows for better understanding of the oligonucleotide aptamer *AGRO100* and potentially enables development of drugs in clinical treatment and delivery system. **(Chapter 3)**

Following the structural reveal of this modified anti-cancer aptamer, the bioactivity has been evaluated in human lung carcinoma cells (A549). The work contributes to disclose the unknown mechanism of *AGRO100* bioactivity. **(Chapter 4)**

b. Recombinant elastin protein as an anti-bacterial coating material

We engineered the recombinant elastin protein to be an ideal platform for small size AgNPs nucleation. We expect this antibacterial material can be readily and safely applied to any surface, potentially of any size, to serve for the coating on biomedical devices. **(Chapter 5)**

2. CHAPTER 2 – METHODOLOGY

2.1. DNA preparation

In this project, DNA oligonucleotides were in-house synthesized or purchased from IDT Singapore where the standard desalting purification method was selected. The ordered oligos were dialyzed against water to remove unwanted chemicals and then lyophilized to obtain the clean DNA powder. The dried forms were diluted in potassium buffer and stored at -30 °C for further analysis.

2.1.1. In-house DNA synthesis

DNA oligonucleotides were synthesized on an Applied Biosystems 394 DNA synthesizer (USA). The synthesis is started from 3'-end to 5'-end of oligos. The system operates based on the automated solid-phase method¹⁴⁹⁻¹⁵⁰ in which nucleoside phosphoramidites (Cambridge Isotope Laboratories, MA, USA)¹⁵¹ (Fig. 2.1) are utilized.

Figure 2.1 Schematic diagram of nucleoside phosphoramidite block.

DMT group is located at 5'-end of sugar to protect the nucleoside from unwanted side reactions.

In general, first phosphoramidite nucleoside which is bound to a solid support undergoes detritylation to remove DMT protecting group, which results in the exposure of 5'-hydroxyl group. The phosphoramidite is then ready for coupling to the second phosphoramidite (Fig. 2.2, Step 1). The failure products in the coupling step are capped at 5'-hydroxyl group by acetyl group to prevent it from the successive synthesis (Fig. 2.2, Step 2), while the successful coupling products are oxidized by iodine to form a stable pentavalent phosphate triester (Fig. 2.2, Step 3). Next, the stabilized products are detritylated to continue to the next cycle until the desired sequence is fully synthesized (Fig. 2.2, Step 4). The synthesized products are treated with concentrated ammonium hydroxide at 55 °C overnight (minimum 12 hours) to cleave the linkage between the

oligonucleotide and solid support as well as to remove the protecting groups on the phosphates.

Figure 2.2 Schematic representation of a cycle of oligonucleotide synthesis ¹⁵²

The oligonucleotides are then ready to go through the purification process which is based on the hydrophobic stationary phase by using Poly-Pak™ II cartridges. The purified samples are then dialyzed against 20 mM KCl solution. The final dry product was obtained by lyophilisation.

2.1.2. Synthesis of DNA with the 3'-fluorescent dye

3'-(6-FAM)-CPG (Glen Research, Sterling, VA) which formed by a 6-carboxyfluorescein (6-FAM) bound to a controlled pore glass (CPG) was used as a starting point of FAM-DNA synthesis (Fig. 2.3). DMT protective group of 3'-(6-FAM)-CPG was cleaved by detritylation to expose the hydroxyl group. The first phosphoramidite was then coupled to 3'-(6-FAM) and continued the cycle of oligonucleotide synthesis (Fig. 2.2). The synthesized products were treated with concentrated ammonium hydroxide (32%) at 55 °C in 16 hours to cleave FAM-DNA from CPG. The FAM-oligonucleotides were then purified by reverse-phase HPLC using an XBridge BEH C18 OBD Prep column (130 Å, 5 µm, 10 mm x 250 mm, Waters). The eluted samples were again undergone the purification process based on the hydrophobic stationary phase by using Poly-Pak™ II cartridges to remove DMT label. The final FAM-oligonucleotide products were characterized by MALDI-TOF (JMS-S3000, JEOL) and examined the obtained purity by denaturing PAGE (Polyacrylamide Gel Electrophoresis) (Fig. S1 and S2 in Appendices section). The FAM-products were then dialyzed against water, lyophilized and stored at -80 °C prior to use.

Figure 2.3 Schematic represents synthesis of DNA with 3'-fluorescent dye

2.1.3. Synthesis of site-specifically labelled deoxyoligonucleotide containing ¹⁵N labelled guanosine or ¹³C, ¹⁵N labelled thymidines

The site-specifically labelled oligonucleotides were synthesized on the Applied Biosystems 394 DNA synthesizer (USA). 2% labelled 2'-deoxyguanosine phosphoramidite (¹⁵N5, 98%, NLM-6826-PK, Cambridge Isotope Laboratories) and 4% labelled thymidine phosphoramidite (¹³C10, 98%; ¹⁵N2, 98%, CNLM-6824-PK, Cambridge Isotope Laboratories) were prepared in guanosine phosphoramidite and thymidine phosphoramidite bottles, respectively. The labelled guanosine or thymidine bottle was inserted at position 5 in the instrument. The site specifically labelled position was replaced as 5 in the DNA sequence which was edited in the system. The labelled DNA

synthesis was operated based on the automated solid-phase method and followed by the purification process which is described in part 2.1.1.

2.2. Ultraviolet (UV) Spectroscopy

In this work, a JASCO V-650 UV spectroscopy was used to quantify DNA concentrations and register the melting experiments. To date, UV spectroscopy is a common approach to determine the concentration of DNA in solution. The principle is based on the absorbance of the base aromatic rings from nucleic acid at around 260 nm¹⁵³. The absorbance at 260 nm is taken when ultraviolet light is radiated on the DNA solution.

2.2.1. DNA concentration measurement

The samples were prepared by dilution of concentrated DNA samples (dilution ratio of either 1:50 or 1:100) to a volume of 500 µL in a quartz cuvette. DNA was then heated until 95 °C to unfold any structure that may exist before executing UV reading. The UV results were an average of three scans for each experiment. Concentrations of samples were calculated from Beer-Lambert Law equation¹⁵⁴

$$A = \epsilon cl$$

where,

A is the absorbance value at 260 nm

ϵ is oligonucleotide molar extinction coefficient, the data can be obtained from <http://sg.idtdna.com/calc/analyzer>

c is oligonucleotide strand concentration

l is quartz cuvette path length (1 cm or 0.2 cm was used in this project)

2.2.2. DNA melting temperature measurement

Melting temperature (T_m) of DNA is defined as the temperature point at which 50% of DNA molecules are dissociated into unfolded state. In this work, UV melting experiments were performed over a wide range of temperatures from 15 to 90 °C. In the case of G4 structure, the absorbance of DNA at 295 nm, used as a measurement of structure folding/unfolding, was recorded as a function of temperature¹⁵⁵. DNA samples at 5 µM concentration were prepared in the respective buffers. Each sample was equilibrated in 10 minutes at 90°C and then cooled down to 15°C at a rate of 0.1°C/min. Data were recorded

with 0.5°C increment. Melting points were obtained from the resulting melting curve, normalized by baseline correction ¹⁵⁶.

2.3. Circular Dichroism (CD) Spectroscopy

CD spectroscopy is an excellent technique for determining the basic secondary structure of biomolecules ¹⁵⁷⁻¹⁵⁹. It is based on the absorbance difference of the left and the right-handed polarized light that is irradiated into chiral samples. Different G4 topologies are characterized by CD signature profiles that are usually performed from 220 nm to 320 nm ¹⁶⁰⁻¹⁶¹. Generally, the parallel strand G4 exhibits a maximum peak around 260 nm and a negative peak near 240 nm (Fig. 2.4, red curve). While the antiparallel G4 conformation shows a positive trough at near 295 nm (Fig. 2.4, blue and green curve).

Figure 2.4 CD spectra of different DNA conformation in buffer containing ~ 100 mM K⁺, pH 7.0.

[The example was taken from *Dao Nguyen Thuan's Ph.D thesis*, NTU, 2013].

In this work, JASCO-815 spectropolarimeter (Japan) was employed to obtain CD spectra of DNA samples. 500 μ L solution of 5 μ M DNA were prepared in the respective buffer and then placed in a 1-cm path length quartz cuvette. The data were acquired from 220 nm to 320 nm with 100 nm/min scan speed, 1nm step, and 1 nm bandwidth. The average of three scans was taken for every experiment.

CD melting – Melting temperature (T_m) of the biomolecules can also be obtained by CD spectroscopy. The process is similar as UV melting's procedure (section 2.2).

2.4. Nuclear Magnetic Resonance (NMR) Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is a powerful technique to determine the structure of biomolecules ¹⁶². The phenomenon of NMR is when nuclei absorb and behave specifically under electromagnetic radiation at a specific resonance frequency. Magnetically active nuclei with a spin $\frac{1}{2}$ (such as ^1H , ^{13}C or ^{15}N) are able to experience this phenomenon and therefore are used in NMR spectroscopy. The magnetization of the nuclear spins that aligns in a direction of an applied external magnetic field (z axis) (Fig. 2.5a) is rotating around the external field when a radio frequency pulse is applied perpendicular to the field (x axis) (Fig. 2.5b). Net magnetization precesses about the external magnetic field with a frequency that is exactly equal to the energy difference between two states of the spin are called Larmor frequency $\omega_0 = \gamma B_0$, where γ is the specific gyromagnetic ratio for each nucleus type, and B_0 is the external field. This precession induces a current along the detection coil on XY plane, and gives a free induction signal as a function of time (Fig. 2.5b). The induction signal is gradually decreased over time when the nuclear spin system reverts to the equilibrium state (relaxation), designated as a free induction decay (FID). NMR signal is a frequency domain result of a Fourier Transform of the FID (time domain) (Fig. 2.5c&d).

Figure 2.5 NMR spectroscopy fundamental diagram.

Electron clouds that surround a nucleus generates a local magnetic field which opposes the applied external magnetic field. It leads to a decrease (shielding) or increase (de-shielding) in the apparent magnetic field and hence shifts in NMR frequency. As a result, the resonance frequency of the sample will change if different field strength is applied and thus introduce incomparable results between different machines. A chemical shift (δ) scale is applied to facilitate this matter by the equation (Fig. 2.5e)

$$\delta = \frac{\nu - \nu_{ref}}{\nu_{ref}}$$

Where

ν is the sample resonance frequency

ν_{ref} is the resonance frequency of a reference compound where δ is 0. Such reference compounds are tetramethylsilane (TMS) or 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS).

2.4.1. Nucleic acid chemical shift

The chemical shift equation displayed above exhibits the independence of an applied magnetic field strength and thus is universal for each atom which experiences the magnetic field in the same environment. Chemical shift of a proton ^1H in nucleic acid is expressed in figure 2.6. The unit is represented as a part per million (ppm) due to the multiplication to 10^6 of an infinitesimal chemical shift. Characterization of a single nucleus proton, especially amino protons H1 and H3 of guanine and thymine, respectively, assists the assignment processes in DNA structure determination.

2.4.2. Two-Dimensional NMR

In order to investigate the detailed structure conformation of DNA, two-dimensional (2D) NMR is often employed to achieve the correlation between nearby protons. The process generates the signals with two time variables, t_1 and t_2 . The final NMR spectrum yields from double Fourier transform of these signals (Fig. 2.7). Figure 2.7 is a 2D NMR spectrum diagram that shows self-peaks (black dots) and symmetrical cross-peaks (white dots) of two ^1H protons A and B.

Figure 2.6 Characterized chemical shift of various protons ^1H from purines and pyrimidines of DNA.

A, T, G, C correspond to adenine, thymine, guanine and cytosine, respectively. Protons are labelled to their respective numbers in the bases. (Figure was taken from *Lim Kah Wai's Ph.D thesis*, NTU, 2013)

Figure 2.7 2D NMR spectrum schematic diagram.

Two ^1H protons A and B create self-peaks (black dots) and symmetrical cross-peaks (white dots).

There are two types of 2D NMR experiments in terms of the mechanism in observing the correlation between two magnetic nuclei, the first one uses through-bond correlation, and the second uses through-space interaction. Through-bond correlation is based on J-coupling which is mediated through the chemical bond connecting two spins. Homonuclear or heteronuclear describes the coupling partners of the same atoms (such as ^1H - ^1H) or different atoms types (such as ^1H - ^{13}C), respectively. This 2D NMR experiment gives information within a molecule connectivity, such as heteronuclear single-quantum

correlation (HSQC), total correlation spectroscopy (TOCSY) or heteronuclear multiple-bond correlation (HMBC).

In contrast, through-space NMR experiments give information of two close nuclei that is depended on dipole-dipole interaction between them. The interaction gives rise to the Nuclear Overhauser Effect (NOE). NOE magnitude is inversely proportional to the distance between two magnetic nuclei governed by the relationship, $\text{NOE} \propto 1/r^6$, where r is the scalar distance between two nuclei. The nuclear Overhauser effect spectroscopy (NOESY) shows cross-peak of two nuclei up to ~ 5 Å in space. More intense cross-peak means shorter scalar distance between the two nuclei.

In this work, all NMR experiments were performed on Bruker ADVANCE 600-MHz at 25°C unless state otherwise. DNA samples of 2D NMR experiments were prepared at 2 – 3 mM concentration, while that of 1D experiments were at 0.1 – 0.5 mM. Due to the high concentration of water (~ 50 mM) compared to that of the DNA samples, NMR signal of water could overwhelm the signals of interest. Hence, jump and return (JR) water suppression pulse sequence method¹⁶³ was utilized to suppress water signals. DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) was used in the sample buffer as a reference to calibrate the chemical shift where DSS resonates at 0 ppm. The oligonucleotides were typically dissolved in buffer containing 70 mM KCl, 20 mM KPi, 20 mM DSS, pH 7.2 unless otherwise state.

2.5. NMR structural calculation

The processes of *AT21S* structural calculation is described in detail below.

2.5.1. NOE Distance restraints

Resonance signal of exchangeable (or labile) protons ($^1\text{H1}$ and $^1\text{H3}$ of guanine and thymine, respectively) were obtained from NOESY experiment with a mixing time of 200 ms. The peaks intensity were classified as very strong, strong, medium, weak and very weak with the distance restraints of 2.5 ± 0.7 , 3.2 ± 0.8 , 4.2 ± 1.2 , 5.0 ± 1.4 and 6.0 ± 1.8 Å, respectively. Similarly, resonance signal of non-exchangeable protons ($^1\text{H8}$ and $^1\text{H6}$ of guanine and thymine, respectively) were also achieved from NOESY spectrum when sample was treated in 99.99% D_2O . The peaks intensities were classified as very strong,

strong, medium, weak and very weak with the distance restraints of 2.4 ± 0.6 , 3.0 ± 0.75 , 3.8 ± 0.95 , 4.8 ± 1.2 and 6.0 ± 1.5 Å, respectively. Herein, the strong and medium intensities were obtained from NOESY experiment with 100 ms mixing time, while the weak peaks were extracted from NOESY experiment with 300 ms mixing time.

2.5.2. Planarity restraints

G-tetrad planarity restraints for *AT21S* structure were assigned for G2·G14·G8·G19 and G3·G6·G9·G13. Triad planarity restraints are for G20·T15·T17 and base pair planarity restraints is for G16·T21.

2.5.3. Hydrogen-bond distance restraints

Cyclic hydrogen bonds from Hoogsteen faces between guanines on a G-tetrad were assigned to H21–N7, N2–N7, H1–O6 and N1–O6 with the distance restraints of 2.0 ± 0.2 , 2.9 ± 0.3 , 2.0 ± 0.2 and 2.9 ± 0.3 Å, respectively. While hydrogen bonds of G·T base pair were assigned to H3(T)–O6(G), N3(T)–O6(G), H1(G)–O4(T), N1(G)–O4(T) with the distance restraints of 2.0 ± 0.2 , 2.9 ± 0.3 , 2.0 ± 0.2 and 2.9 ± 0.3 Å, respectively. Hydrogen bond of T·T was assigned on H3–O2 and N3–O2 with the distance restraints of 2.0 ± 0.2 , 2.9 ± 0.3 Å, respectively.

2.5.4. Dihedral restraints

Syn-guanine (G₂, G₈, G₁₃, G₁₉) and *anti*-guanine (G₃, G₆, G₉, G₁₄, G₁₆, G₂₀ and G₂₂) residues were determined based on glycosidic χ torsion angle restraints of $(60 \pm 30)^\circ$ and $(240 \pm 70)^\circ$, respectively.

2.5.5. Structural calculation

XPLOR-NIH program¹⁶⁴⁻¹⁶⁵ was used to compute the structure of oligonucleotides, while PyMOL¹⁶⁶ is used to display the structure. NOE distance restraints (both exchangeable and non-exchangeable protons), planarity restraints, hydrogen-bond distance restraints and dihedral restraints were all set up to initiate the structure calculation. 100 structures were generated by two steps (i) distance geometry-simulated annealing and (ii) distance-restrained molecular dynamics refinement. 10 lowest energy structures were finally refined and viewed by PyMOL program.

2.6. Gel electrophoresis

2.6.1. Native gel electrophoresis

Migration of secondary structure of oligonucleotides is viewed on a native PAGE (Polyacrylamide Gel Electrophoresis). In this work, the native polyacrylamide gel was made of 20% polyacrylamide, 100 mM KCl in 1X TBE buffer (89 mM Tris-borate, 2 mM EDTA, pH 8.3). Running buffer is 1X TBE supplemented with 10 mM KCl. The gel was visualized by UV shadowing.

2.6.2. Denaturing gel electrophoresis

The denatured DNA (primary structure) was viewed on the denaturing gel to achieve the relative size of DNA. Polyacrylamide gels were prepared from 7 M Urea, 20% polyacrylamide in 1X TBE buffer. DNA samples were heated at 95 °C for 5 minutes right before loading into gels. Running buffer is 1X TBE.

2.7. DNA degradation

Oligodeoxynucleotides used in this experiment have been conjugated with fluorescein (FAM dye) at 3'-end. 2 µM DNA-FAM was incubated in the complete media containing DMEM (Dulbecco's modified eagle medium, Gibco) supplemented with 10% FBS (Fetal bovine serum, Gibco) and 1% Penicillin-Streptomycin (Sigma) at 37 °C. At a specific incubation time, 1 µL of reaction was stopped by dissolving in 9 µL stopping solution containing 95% formamide (Sigma), 0.025% SDS (Merck) and stored at -30 °C for the denaturing gel electrophoresis.

2.8. Cell culture and cell viability assay

Human non-small-cell lung cancer cells (A549) and human fibroblast normal cell (WI38) used in this work were obtained from ATCC. Initially, cells were grown in complete media containing DMEM supplemented with 10% FBS and 1% Penicillin-Streptomycin at 37°C, 5% CO₂ in humidified condition. The cell media was changed every 2 – 3 days till the cell density reached a confluency around 90%. Cells were subcultured in 96-well-plate for the cell viability assay. A549 and WI38 were seeded at density of 1000 and 2000 cells per well, respectively, overnight. The media was then replaced with fresh

complete media containing the specific concentration of the interested oligonucleotides and the cells continued to grow at 37°C, 5% CO₂ in humidified condition overtime. MTT Cell proliferation assay kit from ATCC (USA) was employed to evaluate the viability of cells on different types of oligonucleotides. The assay kit includes MTT reagent (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) and stopping solution. At a specific incubation time, 10 µL MTT reagent was added into each reaction and incubated at 37°C for 4 hours. 100 µL stopping solution was added to stop the reaction and to dissolve the purple formazan crystal and again incubated at 37°C overnight. The resulting color was absorbed at 570 nm with reference at 650 nm by utilizing a microplate reader TECAN Infinite 200 Pro.

3. CHAPTER 3 – ANTI-PARALLEL FOLDING TOPOLOGY OF MODIFIED G- QUADRUPLEX *AGRO100* [#]

[#] Results from this chapter are being prepared for publication in “Truong, T. H. A¹, Fernaldo, R. W. ¹, and Phan, A. T., *The formation of unprecedented G-quadruplex knot*, to be submitted, (2018)”.

¹T.T.H.A and F.R.W. contributed equally to this work.

3.1. Introduction

AGRO100 (also known as *AS1411*) is an anti-cancer aptamer that has been tested in human and has reached Phase II clinical trials for acute myeloid leukemia and renal cell carcinoma ⁹⁶. *AGRO100* was proposed to bind selectively to cytoplasmic nucleolin, which induces some signalling pathway that causes anti-proliferation of cancer cells ¹⁰⁷. Despite its outstanding biological characteristic, the actual mechanism is still unclear to date. Additionally, the undefined structures of *AGRO100* also attribute to difficulties in drug design and therapeutic treatment.

AGRO100 26-mer sequence is rich in guanines with the coexistence of at least 8 different G4 folding topologies in potassium solution as reported by Dailey *et al.* ¹²¹. In this work, 5 fractions in *AGRO100* solution were isolated by size-exclusion chromatography. Four of them showed the same CD spectra with maxima at 260 nm, that characterized a parallel G4 structure. And the other one has inverse CD spectrum which characterized a left handed G4 structure, as previously discovered by our group ¹²⁴. Recently, our group has also reported the structure of a right-handed two propeller-type subunits G4 structure derived from *AGRO100* ¹²³. Its CD profile is similar to most of the fractions from *AGRO100*, which might imply that the major structural forms of *AGRO100* in solution is right-handed G4.

In this chapter, we continue to explore a novel G4 structure derived from *AGRO100*. We found that one particular G4 structure is composed of 2 G-tetrads and a stabilizing triad stacked on top. This novel model can serve as a platform for the design of novel drug delivery system and cancer treatment.

3.2. Single substitution from G to T forms a unique structure of *AT21*

To reduce the multiple structure formation of *AGRO100*, thymine residues were added at both 5' and 3' ends of *AGRO100* sequence, preventing formations of higher order structures or aggregation ¹²². Additionally, the number of guanine residues in the sequence was also reduced from 17 to 16 by substituting one G to T to favor a single form of G4.

Interestingly, we found a novel G4 folding when guanine was substituted at position 21 (designated as *AT21*) (Fig. 3.1A).

The CD profile of *AT21* showed a positive maximum at 290 nm and a negative minimum at 260 nm, suggesting the formation of an anti-parallel G4 structure¹⁶⁷. It is different from the spectrum of *AGRO100* and *AT11*, in which their maxima at 260 nm suggested parallel structure formation (Fig. 3.1B), as well as from *AT27* which showed the inverse spectra of right handed parallel structure, indicating the formation of parallel left-handed G4. This CD result of *AT21* was unlike any other CD profile of separated fractions of *AGRO100* in potassium solution from Dailey *et. al.* (Fig. 1.15 right)¹²¹.

Figure 3.1 (A) Sequences and (B) CD spectra of modified *AGRO100*.

The 4 μ M oligos were dissolved in a solution containing 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25 °C. The bolded letters indicate the modified positions from *AGRO100* sequence.

3.3. Proposed G4 scaffold of *AT21* oligonucleotide

3.3.1. NMR spectra

NMR spectrum of *AGRO100* sequence in phosphate buffer containing 100 mM K^+ displayed multiple overlapped peaks resonating at 10.0 to 12.0 ppm (Fig. 3.2A, black spectrum). Guanine imino protons involved in G-tetrad usually resonate in this region, suggesting the G4 formation in *AGRO100* solution. The badly-resolved peaks are due to existence of multiple conformations, which caused difficulties in structural studies. On the other hand, NMR spectrum of *AT21* in phosphate buffer containing 100 mM K^+ showed 12 well-resolved imino peaks at 9.5 – 12.4 ppm (Fig. 3.2A, red spectrum), suggesting the

formation of a single major G4 structure. The clear and distinct NMR peaks make it feasible for structural interpretation.

Figure 3.2 NMR spectra of *AT21*

(A) 1D imino proton NMR of *AGRO100* (black) and *AT21* (red) in solution containing 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25 °C. (B) ^1H — ^{15}N HSQC of *AT21*. G and T were denoted for guanine and thymine imino protons, respectively. DNA was prepared at 2 mM concentration.

Imino proton peaks arise from both guanine (H1) and thymine (H3) in the *AT21* sequence. In order to differentiate these protons, [^1H — ^{15}N]-HSQC experiment was done, using the coupling between imino protons and their magnetically active imino nitrogen (^{15}N) at natural abundance. The HSQC profile of *AT21* showed that out of the 12 imino peaks, 9 were coming from guanine residues, and 3 from thymine residues (Fig. 3.2B). This result was based on the difference in chemical shift of the imino nitrogens of guanines (N1) and thymines (N3) (Fig. 3.2B, F1 axis). In a G-tetrad, each guanine imino proton forms a hydrogen bond to a nearby oxygen acceptor from the adjacent guanine (N1-H1...O6). This type of hydrogen bond results in the imino proton peak to appear in the 9.5 to 12.5 ppm region of the NMR spectrum. Thus, the fact that *AT21* contains 9 guanine imino proton peaks in this region suggests that there are 2 G-tetrad layers in the structure. The 3 thymine imino proton peaks in the same region also suggest hydrogen bond formations from each thymine imino proton to an oxygen, resulted from an unusual base pairing. In contrast,

common Watson-Crick base pair of thymine (involving N3-H3 $\cdots$ N) has a characteristic imino proton peak at 12 to 14 ppm (Chapter 2, 2.3).

3.3.2. Spectral assignment of exchangeable and non-exchangeable protons

Unambiguous proton assignment is the initial step of NMR structure determination of *AT21*. Protons that are covalently bonded with a labelled isotope (^{15}N or ^{13}C) can be differentiated from other protons by using a filtered experiment, where the intensity of the peak is proportional to the concentration of the neighbouring isotope (^{15}N or ^{13}C)¹⁶⁸. Thus, guanine imino proton assignments were obtained by using a 2% labelled ^{15}N guanosine in one guanine position at a time (Fig. 3.3). Similarly, 4% ^{13}C , ^{15}N thymidines were site-specifically labelled in each thymine position to achieve the assignments of thymine imino protons (Fig. 3.3).

The assignment of imino protons verified guanosine involvement in G-tetrads. Meanwhile, information of aromatic protons tracks the correlation of these protons with the surrounding environment. There are some methods to achieve the assignment of aromatic protons such as through-bond correlations between imino and aromatic protons¹⁶⁹, and/or substitution of active protons with deuteriums at specific positions¹⁷⁰. In this work, non-exchangeable protons (H8) of guanines were assigned via filter long range ^{15}N 7- ^1H 8 coupling correlation, in which guanine residue of *AT21* had been enriched in 2% ^{15}N , one at a time (Fig. 3.4)¹⁶⁸. As for thymine aromatic protons, their assignments (H6) were achieved via one bond ^{13}C 6- ^1H 6 coupling correlation, in which the thymine residues of *AT21* had been enriched in 4% ^{13}C , ^{15}N , one at a time (Fig. 3.4).

Figure 3.3 *AT21* Imino protons assignment.

NMR assignment of imino protons from *AT21* sequence through site-specific 2% ^{15}N and 4% ^{13}C , ^{15}N -labeling of guanine and thymine, respectively. Imino protons locations of guanine and thymine were colored in yellow. Concentrations of the site-specifically labelled oligonucleotides were range from 0.5 to 1 mM in solution containing 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25 °C.

Figure 3.4 NMR assignment of aromatic protons

NMR assignment of aromatic protons through long-range coupling to site-specific 2% ^{15}N and one bond coupling to 4% ^{13}C , ^{15}N -labeling of guanine and thymine, respectively, in AT21 structure. Aromatic proton locations of guanine and thymine were colored in yellow.

Concentrations of the site-specifically labelled oligonucleotides were range from 0.5 to 1 mM in solution containing 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25 °C.

3.3.3. NMR spectrum in D₂O solvent

In the strategy of determining G4 folding topology, checking the exchange rates of imino protons in the G4 core provides important information for determining their positions in the G4 structure. To obtain this, oligo *AT21* was lyophilized after the G4 structure is formed in H₂O solvent containing 100 mM K⁺. The lyophilized G4 was then dissolved in the solvent containing 99.99% D₂O. The solution was then monitored by 1D NMR proton after 60 minutes. NMR spectrum of DNA in D₂O solvent (Fig. 3.5, 60' spectrum) showed that guanine imino protons of G₂, G₁₁, G₁₂, G₁₈, and G₂₃ were relatively long-lived, while the rest of the peaks had diminished in comparison with the ones in H₂O solvent (Fig. 3.5, ref. spectrum). The presence of these long-lived peaks was caused by the slow exchange of ¹H protons with ²H deuterium that are present in the solvent. Therefore, it implies that the imino protons of G₂, G₁₁, G₁₂, G₁₈, and G₂₃ were well-protected by the surrounding environment. In the case of a usual G4 structure containing more than 3 G-tetrad layers, the protected guanine protons usually come from middle G-tetrad which is sandwiched between the top and bottom tetrads. However, in the case of *AT21*, which only contain 2 G-tetrads, imino protons of G₂, G₁₁, G₁₂, G₁₈, and G₂₃ might be sandwiched between a G-tetrad and a bundle of long loops.

Figure 3.5 D₂O solvent exchange experiment for imino protons of *AT21*.

NMR imino proton spectrum of 2 mM *AT21* in H₂O solvent (**ref.**) and in D₂O solvent after 60 minutes (**60'**). D₂O solvent was added in *AT21* dry powder which was dissolved in 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25 °C.

3.3.4. Alignment of G-tetrads

Following the resulting assignment of imino and aromatic protons, the interaction between nearby protons in *AT21* structure were examined by two-dimensional (2D) NMR spectrum. The spatial distance between an imino proton H1 of one guanine and an aromatic proton H8 of another adjacent guanine within a G-tetrad is around 4.7 Å. Two protons can give an NOE cross-peak if they are separated by less than a certain distance (usually up to 7-8 Å)¹⁷¹. Moreover, the NOE interaction can also be detected through a mediated transfer, where the connectivity pathway is mediated by amino protons N2 resulting in intensity enhancement. In a G-tetrad, there are typically four NOE cross-peaks indicating the cyclic connectivity of the four guanines (Fig. 3.6A, NOE connectivity pattern). The NOE inter-connectivities of *AT21* sequence (in H₂O buffer containing 100 mM K⁺ at 25 °C) were observed in a NOESY spectrum (200 ms mixing time) (Fig. 3.6B). In this spectrum, 4 NOEs between the imino protons (H1) of G₂, G₁₈, G₁₁, G₂₃ and the aromatic protons of (H8) G₁₈, G₁₁, G₂₃, and G₂ respectively have established the alignment of a G-tetrad G₂·G₁₈·G₁₁·G₂₃ (Fig. 3.6B, red boxes). Another G-tetrad alignment was G₃·G₉·G₁₂·G₁₇, but only 3 NOEs between the imino protons (H1) of G₃, G₉, and G₁₂ to the aromatic protons (H8) of G₉, G₁₂, and G₁₇ respectively were detected (Fig. 3.6B, blue boxes). There was a missing NOE between the imino proton of G₁₇ to the aromatic proton G₃ due to the inability to observe imino proton peak of G₁₇. The broadening of some protons due to its motions or those of the neighbouring residues has been reported previously.

3.3.5. Folding topology of *AT21* G-quadruplex

Taking together all the results, the initial G4 folding topology of *AT21* is proposed to be a core of 2 G-tetrads held by 2 long edgewise loops (Fig. 3.6D). The first segment G₂G₃ spans 2 tetrads resulting one G4 backbone followed by a first long 5-bases edgewise loop T₄G₅G₆T₇G₈ (denoted as loop A). The G₉T₁₀G₁₁ segment is making a V-shape loop in which G₉ of the bottom G-tetrad is the starting point of the loop and G₁₁ of the upper G-tetrad is located on adjacent G4 backbone. The G₁₁G₁₂ segment is making the third G4 backbone followed by a second long 4-bases edgewise loop T₁₃T₁₄G₁₅T₁₆ (denoted as loop B). The last G4 backbone is made by G₁₇G₁₈ segment. The undefined loop T₁₉G₂₀T₂₁T₂₂ brings G₂₃ into upper G-tetrad resulting in a discontinued G4 backbone of G₉G₂₃.

The unobservable G₁₇ imino proton as well as some unassigned aromatic protons posed significant challenges for structure determination of *AT21*. This may come from the dynamic movement of two long edgewise loops in the solvent. Guanine imino protons of G₂, G₁₁, and G₁₈ which are from upper G-tetrad were well-protected in D₂O exchange experiment (Fig. 3.5). This suggests that there might be an unusual folding formation on the top of the upper G-tetrad which, together with the lower G-tetrad, sandwiched the upper tetrad and therefore shielding it from surrounding solvent (D₂O). More conclusive proof and elucidation of this fold has come from the analysis of sequences with modified loops (see below).

Figure 3.6 NMR spectrum assignment of AT21.

(A) Characteristic cyclic NOE connectivity in G-tetrad. Arrows indicate inter NOE connectivity from H1 to H8. (B) NOESY spectrum (mixing time 200 ms) of 2 mM AT21 in solution containing 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25 °C. The NOE connectivity within a G-tetrad was framed and labeled with the same color (red or blue). (C) Alignment of G-tetrad. (D) Schematic diagram showing partial folding topology of G4 AT21. Guanines are colored in blue except G₁₇ is colored grey representing the predicted position inside the tetrad. Arrows represent DNA strand direction from 5' to 3'-end of AT21 sequence.

3.4. Characteristic of G4 AT21

3.4.1. Effect of K⁺ concentrations

In G4 structure, counterions are not only supporting the formation but also stabilizing the structure of G4 by coordinating with O6 atoms of guanines in cavity of G4

core¹³. There are two ways for counterions to affect the G4 structure. One is the specific binding, where ions such as K^+ or Na^+ coordinate to inner O6 of guanine with a release of water molecule. The specific binding gives rise to the structure conformational transition of G-rich sequences. Another is the non-specific binding, where counterions interact with the electrostatic fields of negative charges along the phosphodiester DNA backbones. The interaction results in counterion condensation around DNA polyanions¹⁶⁷.

Figure 3.7 G4 AT21 in different KCl concentrations.

(A) CD spectra and (B) Melting temperature profiles of AT21 in various KCl concentrations and 20 mM potassium phosphate buffer (pH 7.2) at 25°C. (C) Melting temperatures T_m of AT21 in various potassium ions concentrations. The stated K^+ concentration was a total amount of K^+ counterions from KCl and KPi buffer. (D) Dependence of T_m versus various concentrations of potassium cations. DNA were prepared at 4 μM concentration.

In this work, we tested the stability of G4 AT21 in various potassium ions concentration (Fig. 3.7). CD spectra of AT21 in increasing KCl concentration showed

similar profiles with a maximum point at 290 nm and a minimum point at around 260 nm (Fig. 3.7A). These features are characteristics of anti-parallel G4 structure. The result showed clearly that low potassium ion concentration (20 mM KCl) is sufficient to drive the G4 formation of *AT21* sequence.

In addition, melting temperatures (T_m) were examined in various potassium chloride concentration to investigate the stability of the structure (Fig. 3.7B). T_m is a temperature of the transition point at which 50% of folded and 50% of unfolded structures coexist. The result showed that the melting curves were shifting from low to high temperatures with increasing KCl concentration. From these curves, melting temperatures (T_m) were extracted (Fig. 3.7C) and plotted versus the change of potassium ion concentration (Fig. 3.7D). The plot exhibited a slow change of T_m as a function of potassium ion concentration. The CD profile suggested that 50 mM K^+ (is a total of 20 mM K^+ from KCl and around 30 mM K^+ from potassium buffer) is sufficient to coordinate with all the guanine O6 atoms in G4 cavity. The increase of overall T_m maybe the result of dissociation of K^+ accumulation around the polyanions phosphodiester backbone of the DNA.

3.4.2. Effect of different types of counterions on *AT21* structure

The coordinations of cations to O6 atoms of guanines significantly contribute to G4 stability. Both monovalent and divalent cations are able to make the coordination. Venczel's group suggested that the order of G4 structure in stability was based on types of monovalent cations as $K^+ > Rb^+ > Na^+ > Li^+ = Cs^+$ and divalent ions as $Sr^{2+} > Ba^{2+} > Ca^{2+} > Mg^{2+}$ ¹⁷². Therein, the majority of G4 formation studies were focused on K^+ and Na^+ ions since they are physiologically relevant. However, other cations also affect the formation of G4. Hence, we also examined the effects of different types of monovalent cations in G4 structure formation (Fig. 3.8). The oligonucleotides *AT21* were dissolved in buffer containing around 100 mM cations of K^+ , Na^+ , Li^+ or NH_4^+ at pH 7.2. The samples were then recorded using NMR and CD. The NMR spectra showed that *AT21* in media containing K^+ and NH_4^+ ions exhibited imino proton peaks at 10 – 12 ppm region which indicate the presence of G4 (Fig. 3.8A, K^+ and NH_4^+ spectra). In contrast no peak was visualized in the case of media containing Na^+ and Li^+ ions (Fig. 3.8A, Na^+ and Li^+ spectra). Chemical shifts of imino protons were similar for both cases of K^+ and NH_4^+ ions. In these

spectra, a group of around 9 protons located downfield at 11 - 12.2 ppm region and other 3 protons located upfield at 9.5 – 10.2 ppm region. Additionally, CD profiles of *AT21* in media containing K^+ and NH_4^+ showed the similar spectra with a positive trough at 290 nm and a negative trough at around 260 nm indicating anti-parallel G4 structures. Taken together, both K^+ and NH_4^+ are able to induce the anti-parallel G4 formation of *AT21* sequence. It is reasonable due to the ionic radius of K^+ and NH_4^+ ions are almost the same (1.33 Å and 1.48 Å, respectively), whereas those of Na^+ and Li^+ ions are smaller (0.95 Å and 0.6 Å, respectively) ¹⁷³. The smaller ionic radius of Na^+ and Li^+ requires more ions to coordinate guanine O6 atoms in G4 cavity. In addition, the Na^+ and Li^+ ions might destabilize the G4 formation just due to the nature of *AT21* sequence.

Figure 3.8 Effect of counterions on the formation of *AT21* structure.

(A) NMR imino proton spectra and (B) CD spectra profiles of *AT21* in various types of cations. *AT21* was prepared at 2 mM concentration.

3.4.3. Effect of pH on *AT21* structure

We tested the stability of G4 *AT21* in some acidic conditions (Fig. 3.9). In previous experiments, *AT21* oligonucleotide was dissolved in a medium containing 100 mM K^+ with pH 7.2 to favor the formation of G4. Based on the NMR as well as CD signals, we have confirmed that *AT21* forms G4 at pH 7.2. The pH of the medium was then reduced by adding drops of HCl to achieve the desired pH values (6.0, 5.2 or 4.1). Four NMR spectra were recorded for *AT21* at pH 7.2, 6.0, 5.2 and 4.1. Interestingly, all of the 12 well-resolved imino proton peaks in pH 7.2 sample were still there in the samples of pH 6.0, 5.2 and 4.1. This suggests that there was no depurination occurred under the acidic conditions. However, more distinct peaks emerged in the region of 10.5 – 11.5 ppm for lower pH samples if compared with the hump observed on pH 7.2 sample. It is possible that acidic

conditions induce the formation of minor forms of *AT21*. The results confirmed the stability of *AT21* structure in acidic conditions even at low pH (pH 4.1). It should be kept in mind that pH values of some organelles (such as early endosome, Golgi apparatus or lysosome) in cells are lower than the physiological pH 7.2 due to secretion of acid in metabolism processes ¹⁷⁴. Additionally, pH values of tumor cells are observed to be quite low compared to those of normal cells due to excess of lactic acid secretion and ATP hydrolysis activity, which are required to provide adequate nutritional needs for invasion processes ¹⁷⁵. Thus, the stability of this modified aptamer *AT21* in acidic conditions might be significantly important for therapeutic application.

Figure 3.9 NMR spectra of G4 *AT21* in different pH environment.

2 mM *AT21* was dissolved in 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25 °C. pH of the media was adjusted by adding HCl 1 M solution. Red stars represent new peaks.

3.4.4. Effect of temperature on *AT21* structure

The changes of *AT21* structure from various temperatures in media containing 70 mM KCl and 20 mM potassium phosphate (pH 7.2) were also observed by NMR spectroscopy (Fig. 3.10). 1D NMR imino proton spectra showed that additional peaks appeared as temperature were increased (from 25 °C to 40 °C) (Fig. 3.10). New set of peaks became obvious at higher temperature of 40 °C, which was above the melting temperature (T_m) of *AT21* (35 °C) (Fig. 3.7C). At the melting temperature, 50% of the folded structures

are transient to the unfolded forms. Thus, the decreased intensity of the folded peaks were expected as the temperature increased. However, the emergence of new set of peaks meant that there was one or more minor forms that were favored in higher temperature as opposed to only an unfolded form.

In addition, the major peaks of *AT2I* still unchanged when the temperature was decreased (from 25 °C to 5 °C) respond to its stable structure (Fig. 3.10). Some new peaks appeared obviously may be originated from some imino protons which not having hydrogen bonds. The phenomenon can be explained that the exchange rate and dynamic movement of these imino protons were reduced in low temperature condition and then facilitated their detection in NMR spectroscopy. The stability at low temperature of *AT2I*, a modified aptamer, makes it an ideal candidate to be designed and served as a specific probe to detect and purify some target proteins in cell lysate or protein display.

Figure 3.10 Variant temperature of *AT2I* solution.

2 mM of *AT2I* was dissolved in solvent containing 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25 °C.

3.5. Loops modification of *AT21*

The folding topology of *AT21* (Fig. 3.6C) was proposed above with two G-tetrad planes in monomeric G4. The G4 core is held by two long edgewise loops. Resonance of G₁₇ imino proton was undetected in 1D NMR imino spectra (Fig. 3.3). The dynamic movement of two long loops might cause peak broadening thus rendering it undetectable, which made it difficult further structure determination work. Thus, we hypothesized that modifying both edgewise loops may reduce their effect on G4 core, allowing further elucidation of the *AT21* structure.

In the proposed G4 structure of *AT21*, we denoted the sequence T₄G₅G₆T₇G₈ as loop **A**, and T₁₃T₁₄G₁₅T₁₆ as loop **B** (Fig. 3.6D). Both loops were modified by substituting G to T and/or removing G residues. In most cases, A and C were mostly not selected to modify the loop to avoid formation of Watson Crick base pairing with T and G in the sequence of *AT21*, respectively. Loop modifications are listed in table 3.1.

No.	Label	Sequence (5' - 3')	Description
1	AT21-D(A)-5T	TGGTTTTTGTGGTTGTGGTGTGGTGGT	Change loop A to 5T
2	AT21-D(B)-4T	TGGTGGTGGTGGTTTTGGTGTGGTGGT	Change loop B to 4T
3	AT21-D(AB)-all T	TGGTTTTTGTGGTTTTGGTGTGGTGGT	Change both loop A and B to all T
4	AT21-D(AB)-3T	TGGTTTGTGGTTTGGTGTGGTGGT	Change both loop A and B to 3T
5	AT21-D(AB)-2T	TGGTTGTGGTTGGTGTGGTGGT	Change both loop A and B to 2T
6	AT21-(DA-3T)-(DB-2T)	TGGTTTGTGGTTGGTGTGGTGGT	Change loop A to 3T and B to 2T
7	AT21-(DA-2T)-(DB-3T)	TGGTTGTGGTTTGGTGTGGTGGT	Change loop A to 2T and B to 3T
8	AT21-(DA-1T)-(DB-3T)	TGGTGTGGTTTTGGTGTGGTGGT	Change loop A to 1T and B to 3T
9	AT21-(DA-TA)-(DB-3T)	TGGTAGTGGTTTTGGTGTGGTGGT	Change loop A to TA and B to 3T

Table 3.1 Description of the modified loops in *AT21* structure.

The purpose of the experiment was to reduce the effect of the bottom loops, allowing to detect the G₁₇ imino proton of *AT21*. Thus, the modified sequences were dissolved in 70 mM KCl and 20 mM potassium phosphate (pH 7.2) at 25 °C and then qualitatively analyzed by NMR and CD. The 8th case AT21-(DA-1T)-(DB-3T) was the only sequence which exhibited significantly different spectrum compared with *AT21* (Fig. 3.11A, 8th spectrum). Thus this sequence was no longer discussed. We observed that NMR spectra of the rest of the modified sequences were similar to that of the original *AT21*. The spectra were separated into two groups, one containing 3 peaks located upfield (9.2 – 10.5

ppm), and another containing multiple peaks located downfield (11.2 – 12.5 ppm) (Fig. 3.11A). This results suggested that these modified sequences were able to form G4 and their foldings were similar to *AT21* structure. In addition, NMR spectra of these modified sequences showed 13 peaks at imino proton regions (Fig. 3.11A), except the 5th case AT21-D(AB)-2T where it showed more than 13 peaks (Fig. 3.11A, 5th spectrum). The expected additional peak was located in the downfield (11.2 – 12.5 ppm) region which is a characteristic for imino protons in G4 core. Furthermore, CD spectra of these structures exhibited the same features of anti-parallel G4 *AT21* in which a maximum at 290 nm and a minimum at around 260 nm were recorded (Fig. 3.11B). Taken together, we confirmed that these modified sequences behaved similarly to *AT21*. We decided to choose the 7th case AT21-(DA-2T)-(DB-3T) and denoted the sequence as *AT21S* to further explore the folding topology and structure of *AT21*. The decision was made based on the number of imino protons appeared and the clean spectra to facilitate further characterization.

3.6. *AT21S* as a replacement of *AT21*

3.6.1. NMR spectra and assignment of exchangeable protons

AT21S is the modified sequence of *AT21* in which the first T₄G₅G₆T₇G₈ and the second T₁₃T₁₄G₁₅T₁₆ edgewise loops were shortened to TT and TTT sequences, respectively (Fig. 3.12A). The 1D NMR spectrum profile of *AT21S* in H₂O solvent containing 100 mM K⁺ at 25 °C showed 13 peaks corresponding to 10 guanines and 3 thymines imino protons, deduced based on the [¹H - ¹⁵N]-HSQC experiment of natural abundance ¹⁵N-¹H correlation (Fig. 3.12B). The number of detected thymine imino protons of *AT21S* was the same as *AT21*, while there was one additional guanine imino protons in *AT21S* spectrum as expected. 2D NOESY profile (200 ms mixing time) of *AT21S* in H₂O solvent containing 100 mM K⁺ at pH 7.2 was superimposed on the spectrum of *AT21* (Fig. 3.12C) for comparison. The high similar pattern of the NOESY profiles of both sequences indicated the same NOE relations. Thus, assignment of *AT21S* imino protons in 1D NMR spectrum can be inferred from that of *AT21*. The 9 guanine imino protons of *AT21S* were then numbered according to the 9 relative positions of these protons in *AT21* (Fig. 3.12D). The last guanine imino proton of *AT21S* was numbered as G₁₃ which was translated from the undetected G₁₇ imino proton in *AT21* (Fig. 3.12D, red number). Imino proton peaks of G₂, G₃, G₆, G₈, G₉, G₁₃, G₁₄ and G₁₉ of *AT21S* were located in the downfield region (11.5 – 12.4 ppm) suggesting their involvement in 2 G-tetrads of G4. The other 2 guanine imino

proton peaks of G₁₆ and G₂₀ were located upfield indicating hydrogen bond formations of these guanine imino protons with thymine oxygen acceptors. The 3 thymine imino protons T₁₅, T₁₇ and T₁₉ resonated at 9.5 to 12.5 ppm suggesting hydrogen bond formations of mismatched base pairs.

Figure 3.11 (A) NMR spectra and (B) CD profiles of loop modifications of *AT21* structure.

0.2 mM of each modified oligonucleotide was dissolved in 70 mM KCl and 20 mM potassium phosphate (pH 7.2) at 25 °C. The red dashed box represents for the final selected sequence for further characterization.

Figure 3.12 Comparison of AT21 and AT21S.

(A) DNA sequence of AT21 (black) and AT21S (red). (B) $[^1\text{H} - ^{15}\text{N}]$ HSQC of the ^{15}N - ^1H correlation of AT21S. (C) Superimpose NOESY spectra (mixing time 200 ms) of AT21 (black) and AT21S (red). (D) 1D NMR spectra of AT21 (black) and AT21S (red) with their assignments. 2mM oligos were dissolved in solution containing 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25°C.

3.6.2. Assignment of non-exchangeable protons

The loop modification also gave a cleaner NMR spectrum of *AT21S* aromatic protons if compared to that of *AT21* (Fig. 3.13A). Well-resolved spectrum facilitates the assignment of aromatic protons that is critical for the structural calculation.

Guanine aromatic protons H8 of *AT21S* in H₂O buffer containing 100 mM K⁺ were assigned by long range through-bond correlations between imino and aromatic protons via ¹³C5 at natural abundance in JR-HMBC experiments (Fig. 3.13B) ¹⁶⁹. Other guanine H8 and thymine H6 aromatic protons were assigned by tracing out the sequential NOE connectivity from H8/6-H1'(n-1) intraresidue to H1'(n-1)-H8/6(n) inter-residue from NOESY spectrum (mixing time 300 ms) of *AT21S* in ²H₂O buffer containing 100 mM K⁺ (Fig. 3.13C). There were some discontinuous tracts that might be caused by the loop geometry ¹⁷¹. Taken together, the assignment of non-exchangeable protons of *AT21S* was fully achieved (Fig. 3.13C).

3.7. Folding topology of *AT21S*

3.7.1. Alignment of G-tetrads, triad and base pair

Following the assignments of imino and aromatic protons, cyclic NOE H1-H8 inter-connectivities within G-tetrads (Fig. 3.14A2, NOE connectivity pattern) were adequately established. The result was extracted from NOESY spectrum (200 ms mixing time) of *AT21S* in H₂O buffer containing 100 mM K⁺ at 25 °C (Fig. 3.14A1). Thus, the arrangement of 2 G-tetrads in G4 core was established to be G₂·G₁₄·G₈·G₁₉ and G₃·G₆·G₉·G₁₃ (Fig. 3.14A2), with the guanine glycosidic conformation arrangement of *syn-anti-syn-syn* and *anti-syn-anti-anti*, respectively. Classification of guanosine glycosidic conformation was based on the intra-residue NOE intensity of H8-H1' that were extracted from NOESY spectrum (mixing time 300 ms) (Fig. 3.13C). *Syn*-guanosines have a very strong intensity of this NOE because the guanine base rotates facing to sugar resulting in the short distance between H8 and H1' (~2.5 Å). On the other hand, guanosines that adopt *anti*- glycosidic conformation have a longer distance between H8 and H1' (~3.5 Å) resulting in the medium NOE intensities. From these facts, it was deduced that G₂, G₈, G₁₃ and G₁₉ adopt *syn*-conformation in G4 core, while G₃, G₆, G₉ and G₁₄ adopt *anti*-conformation in G4 core.

Figure 3.13 NMR assignment of G4 AT21S.

(A) 1D NMR spectra of AT21 (black) and AT21S (red). (B) and (C) are 2D NMR spectra. (B) Intra-correlation of guanine H1 and H8 protons via through-bond connectivity in JR-HMBC experiment. The inset shows long-range J-coupling of guanine protons H1 and H8. (C) NOESY spectrum (mixing time 300 ms) shows NOE correlation of H8/6-H1'(n-1) intraresidue and H1'(n-1)-H8/6(n) inter-residues. The continuous tracing lines were colored in red, purple and green. Tract interruptions are shown by different colors. The residue numbers are labeled

for intra-connectivity. X represents for weak inter-crosspeak. 2mM oligos were dissolved in solution containing 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25°C.

We observed that NOE of exchangeable protons in NOESY spectrum (mixing time 200 ms) presented cross-peaks of G₂₀·T₁₅ and T₁₅·T₁₇ (Fig. 3.14B1) indicating a possible formation of a triad G₂₀·T₁₅·T₁₇ (Fig. 3.14B2). Within the triad, atoms H3 and O4 of thymine T₁₅ are hydrogen bonded to O6 and H1 of guanine G₂₀, respectively. Additionally, acceptor O6 of T₁₅ also creates a hydrogen bond with H3 of T₁₇ (Fig. 3.14B2). Other than that, another mismatched G·T wobble base pair was observed in G₁₆·T₂₁ where O6 and H1 of G₁₆ create hydrogen bond to imino H3 and O4 of T₂₁, respectively (Fig. 3.14B3).

3.7.2. Folding topology of *AT21S*

Taken all the facts into account, the G4 folding topology of *AT21S* was established (Fig. 3.16C). The G4 scaffold contains 2 G-tetrads G₂·G₁₄·G₈·G₁₉ and G₃·G₆·G₉·G₁₃ connected by 2 edgewise loops and a V-shaped loop. The triad G₂₀·T₁₅·T₁₇ stacks over on the upper G-tetrad of G4 scaffold, and the base pair G₁₆·T₂₁ stacked onto the triad. In detail, the first segment G₂G₃ spans 2 tetrads resulting one G4 backbone followed by a first edgewise loop T₄T₅. The G₆T₇G₈ segment is making a V-shape loop in which G₆ of the bottom G-tetrad is the starting point of the loop and G₈ of the upper G-tetrad is located on adjacent G4 backbone. The G₈G₉ segment is making the third G4 backbone followed by a second edgewise loop T₁₀T₁₁T₁₂. The G₁₃G₁₄T₁₅G₁₆ segment continues to be the last G4 backbone (G₁₃G₁₄) and spans to the triad and base pair layers. T₁₇T₁₈G₁₉ segment brings G₁₉ into upper G-tetrad resulting a discontinued G4 backbone G₆G₉. Lastly, G₂₀T₂₁ segment spans to the triad and base pair layers to complete *AT21S* topology. We observed that guanosines of the 3 continuous backbones of G4 core followed the glycosidic conformation of 5'-*syn-anti*-3'. Whereas the discontinued backbone (G₆G₉) has an inverse manner 5'-*anti-syn*-3' due to the interruption of the strand.

In this conformation, the upper G-tetrad is sandwiched between the lower G-tetrad and the triad. As a result, imino protons of the upper G-tetrad (G₂, G₈, G₁₄ and G₉) were well protected from exchanging with deuterium in D₂O solvent (Fig. 3.15).

Figure 3.14 Folding topology of *AT21S*.

(A1) NOESY spectra (mixing time 200 ms) shows (A2) a cyclic connectivity of guanine H1-H8 within a G-tetrad. (B1) NOESY spectra (mixing time 200 ms) shows NOE of exchangeable protons. Hydrogen bond alignment for (B2) the triad G·T·T and (B3) the G·T base pair. Red arrows show NOE correlation. (C) Schematic of *AT21S* folding topology. *Syn* and *anti* guanines are colored magenta and cyan, respectively. Thymine is colored in orange.

Figure 3.15 D₂O solvent exchange of *AT21S* oligonucleotide.

Deionized water in 2 mM DNA sample that contained 70 mM KCl and 20 mM potassium phosphate buffer (pH 7.2) was replaced with solvent containing 99.9% D₂O.

3.8. Structure of *AT21S*

The high-resolution structure of the *AT21S* G-quadruplex in solvent containing 100 mM K⁺, 25 °C was calculated (Chapter 2, 2.6) based on NMR restraints that are summarized in Table 3.2. From the calculation, superimposition of 10 lowest-energy refined structures showed a good convergence (Fig. 3.16A). The antiparallel G4 contains 2 G-tetrads, 1 triad, 1 base pair as well as 2 edgewise loops and 1 V-shape loop (Fig. 3.16). The base pair G₁₆·T₂₁ is located on top of the triad T₁₅·T₁₇·G₂₀. The base pair plane tilts down to the triad plane at the side of C7 (T₂₁) and N2 (G₁₆). The triad T₁₅·T₁₇·G₂₀ stacks on the upper G-tetrad G₂·G₁₄·G₈·G₁₉. Base stackings between the planes are shown in Figure 3.16 C-E; hydrogen bonds alignments on each plane are shown in Figure 3.17.

We observed that the thymine bases of both edgewise loop T₄T₅, T₁₀T₁₁T₁₂ and V-shape loop T₇ point toward the bottom G-tetrad. The thymine T₁₈ stacks on top of the base pair G₁₆·T₂₁. Both T₁ at 5'-end and G₂₂G₂₃T₂₄ at 3'-end swings freely in the structure (Fig. 3.16A and B).

A. NMR restraints		
Distance restraints	Exchangeable	Non-exchangeable
Intraresidue	275	4
Interresidue	115	82
Other restraints		
Hydrogen bond		42
Dihedral angle		10
B. Structure statistics		
NOE violations		
Number (>0.2 Å)	0.300 ± 0.458	
Deviations from the ideal covalent geometry		
Bond lengths (Å)	0.003 ± 0.000	
Bond angles (deg)	0.695 ± 0.006	
Impropers (deg)	0.366 ± 0.006	
Pairwise all heavy atom rmsd values (Å)		
All heavy atoms	0.726 ± 0.162	
G-tetrad core	0.483 ± 0.123	

Table 3.2 NMR restraints and structure statistic.

Figure 3.16 Structure of modified aptamer AT21S in potassium solution.

(A) Superimposition of 10 lowest energy structures. (B) Ribbon schematic view of a lowest-energy structure. (C, D and E) Base stacking patterns of (C) base pair G₁₆·T₂₁ and triad T₁₅·T₁₇·G₂₀, (D) triad T₁₅·T₁₇·G₂₀ and upper G-tetrad G₂·G₁₄·G₈·G₁₉, (E) 2 G-tetrads G₂·G₁₄·G₈·G₁₉ and G₃·G₆·G₉·G₁₃. The residues are labelled according to their base types and

positions in sequence. Thymine, *syn*-guanines and *anti*-guanines are coloured in orange, violet and aquamarine, respectively.

Figure 3.17 Alignment of hydrogen bonds in a representative AT21S structure.

(A) Base pair G16-T21, (B) triad T15-T17-G20, (C) G-tetrads G₂-G₁₄-G₈-G₁₉ and (D) G-tetrad G₃-G₆-G₉-G₁₃. The black dash lines indicate hydrogen bonds. The residues are labelled according to their base types and positions in sequence. Thymine, *syn*-guanines and *anti*-guanines are coloured in orange, violet and aquamarine, respectively.

3.9. Stability of AT21S

3.9.1. CD and melting spectra

AT21S CD profile was exhibited similar to that of AT21 indicating the formation of antiparallel G4 structure (Fig. 3.18A). Melting temperature (T_m) of AT21S was 40 °C which was 5 °C higher than AT21 suggesting that the shorter loops of AT21S stabilized the

structure (Fig. 3.18B). The result of T_m is consistent with the conclusion from Mergny's group in which the T_m is decreasing with increasing loop length ¹⁷⁶.

Figure 3.18 CD spectra and melting temperature of AT21 and AT21S

(B) (A) CD spectra and (B) melting temperature (T_m) of 4 μ M AT21 (black) and 4 μ M AT21S (red) in solution containing 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25°C.

3.9.2. Modified 5'-end and 3'-end of AT21 and AT21S

The robustness of the AT21 and AT21S scaffolds were also examined by modifying both ends of the sequences. According to the proposed folding topology of AT21, G₂₆G₂₇T₂₈ at 3'-end does not contribute to the core structure. Thus the modification was focused on removing these long tail. List of sequence modifications of AT21 was displayed on Table 3.3. NMR imino proton spectra of these modified sequences were recorded and depicted in Figure 3.19. All spectra were similar with the reference AT21, with the main characteristic of the presence of 12 imino protons (Fig. 3.19). Some additional peaks appeared in samples 2, 4 and 5 suggesting the formation of one or more minor forms. Furthermore, removing T₁ at 5'-end of AT21S (Fig. 3.20, DT1) also has no effect on the structure, as depicted on the NMR spectrum in Fig. 3.20. Taken together, the result suggested that modification of both tails of the sequences were allowed, without any effect on the G4 structures. This opens more possibility for modification of the aptamer for therapeutic application.

No.	Label	Sequence (5' - 3')	Description
1	AT21	TGG TGG TGG TGG TTG TGG TGT TGG TGG T	
2	AT21-D(26-28)	TGG TGG TGG TGG TTG TGG TGT TGG T	Remove G ₂₆ G ₂₇ T ₂₈
3	AT21-D(27-28)	TGG TGG TGG TGG TTG TGG TGT TGG TG	Remove G ₂₇ T ₂₈
4	AT21-D(26-28)-5T	TGG TGG TGG TGG TTG TGG TGT TGG TTT TTT	Replace G ₂₆ G ₂₇ T ₂₈ to 5 T
5	AT21-5'-5T	TT TTT TGG TGG TGG TGG TTG TGG TGT TGG TGG T	Add 5 T at 5'-end

Table 3.3 List of modification of sequence *AT21* oligonucleotide.

Figure 3.19 NMR spectra of modified *AT21* oligonucleotide.

The oligos were dissolved in 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25°C. Red dots indicate original imino protons peaks in *AT21* sequence. DNA concentrations were 0.2 mM.

Figure 3.20 NMR spectra of modified *AT21S* oligonucleotide. The 0.2 mM oligos were dissolved in 70 mM KCl and 20 mM potassium phosphate (pH 7.2), 25 °C. Red dots indicate original imino protons peaks in *AT21S* sequence. *AT21S* sequence is d(TGGTTGTGGTTTGGTGTGTTGGTGGT) and *DT1* is d(GGTTGTGGTTTGGTGTGTTGGTGGT).

3.10. Conclusion

A novel G4 conformation derived based on the *AGRO100* aptamer *AT21* was discovered. The 28-mer sequence *AT21* as well as its shorter version 24-mer *AT21S* favors the monomeric antiparallel G4. The G4 core contains only two G-tetrads sandwiched between a triad and 2 successive loops. The stability of the structure in acidic condition and in sequence modification makes the G4 scaffold be a robust new scaffold in drug design for therapeutic application.

4. CHAPTER 4 - ACTIVITY OF *AGRO100* VARIANTS ON HUMAN LUNG CANCER CELL A549 [#]

[#]Some results from this chapter regarding the *AT11* oligonucleotide were published in “Do, N. Q., Chung, W. J., Truong, T. H. A., Heddi, B., & Phan, A. T., *G-quadruplex structure of an anti-proliferative DNA sequence*. Nucleic Acids Research, 2017, Volume 45, Issue 12, 7 July 2017, Pages 7487–7493”.

4.1. Introduction

AGRO 100 is a well-known aptamer with potent anti-proliferative activity against cancer cells that have reached Phase 2 clinical trial. Two mechanisms were proposed for the anticancer activity of *AGRO100*: (1) G4 structures of *AGRO 100* bind nucleolins and result in cell apoptosis¹⁰², or (2) G-containing degradation products of *AGRO100* causes cell cytotoxicity¹⁷⁷. However, the well-established mechanism is still under investigation. Thus, an exploration of the *AGRO100* structure-activity relationship may provide further insights into the anticancer mechanism of this oligo and may be an important step towards drug design and therapeutic treatment.

Structures of two monomeric G4 structures derived from *AGRO100* have been solved; they are a right-handed and a left-handed G4 containing 4 G-tetrads, which are designated as *AT11* and *AT27*, respectively¹²³⁻¹²⁴. In addition, another sequence (termed *AT21*) derived from *AGRO100* that exhibits a novel structure, in which the main G4 core contains only 2 G-tetrads has been explored (**Chapter 3**). These modified sequences adopt different unique G4 conformations, but their biological properties haven't been explored yet.

In this chapter, we examined the anti-proliferative effect of these modified sequences on non-small-cell lung cancer cells A549, in which *AGRO100* significantly induced the cell apoptosis¹⁷⁸⁻¹⁷⁹.

4.2. Effect of *AGRO100* variants on the proliferation of tumor cells

The deoxy-oligonucleotides that were used in this work were bought or chemically synthesized according to the list in table 4.1. In this table, *AGRO100*, *AT11*, *AT27*, *AT21*, and *AT21S* are the G4 sequences derived from the anti-cancer aptamer *AGRO100*. Another intramolecular G4 is *T95-2T*. *AGRO-comp* and *AT21S-comp* are the complementary strands of *AGRO100* and *AT21S*, respectively. *Poly-T*, *CRO* and *non-CRO* are the non-G4 structures which were used as the oligo controls (Table 4.1).

No.	Label	Sequence (5' - 3')	Description
1	<i>AGRO100</i>	GGT GGT GGT GGT TGT GGT GGT GGT GG	Aptamer
2	<i>AT11</i>	TGG TGG TGG TTG TTG TGG TGG TGG TGG T	<i>AGRO100</i> variant
3	<i>AT27</i>	TGG TGG TGG TGG TTG TGG TGG TGG TGT T	<i>AGRO100</i> variant
4	<i>AT21</i>	TGG TGG TGG TGG TTG TGG TGT TGG TGG T	<i>AGRO100</i> variant
5	<i>AT21S</i>	TGG TTG TGG TTT GGT GTT GGT GGT	<i>AT21</i> variant
6	<i>T95-2T</i>	TT GGG T GGG T GGG T	Monomeric G4
7	<i>AGRO-comp</i>	CCA CCA CCA CCA CAA CCA CCA CCA CC	Complementary of <i>AGRO100</i>
8	<i>AT21S-comp</i>	ACC ACC AAC ACC AAA CCA CAA CCA	Complementary of <i>AT21S</i>
9	<i>Poly T</i>	TTT TTT TTT TTT TTT TTT TTT TTT TT	Poly T sequence
10	<i>Non-CRO</i>	CTT CTT CTT CTT TCT CTT CTT CTT CT	Non i-motif sequence
11	<i>CRO</i>	CCT CCT CCT CCT TCT CCT CCT CCT CC	Potential i-motif sequence

Table 4.1 List of oligonucleotides used in bioactivity experiments.

A549 cells were sub-cultured in complete media for a day before they were treated by the media containing various concentrations of the oligonucleotides (the detail was stated in Section 2-2.8). The cell proliferative assay MTT was employed to determine the relative abundance of the cells. The absorbance of living cells in this assay was then normalized with the absorbance of the untreated cells (UTC).

In figure 4.1, the proliferation of non-small-cell lung cancer cells A549 was significantly inhibited by *AGRO100*, *AT11*, *AT21*, and *AT27* at the concentrations of 5, 10 to 20 μ M. In contrast, at these doses the control oligonucleotide *CRO* has no effect on the cancer cells' proliferation (Fig. 4.1A). On the other hand, the normal human lung fibroblast WI38 exhibited the healthy proliferation in the media containing these oligonucleotides with the same tested concentrations as for the cancer cells (Fig. 4.1B). It is to be noted that the absorbance of the culture media without cells was around 0.2 (data not shown). We calculated the IC_{50} values of these oligos to be around 2.5 μ M which is consistent with the reported value¹⁰³. The IC_{50} is a value of the applied drug's concentration at which 50% of the tested cells' growth are inhibited. The results imply that the anticancer activity of *AGRO100* variants (*AT11*, *AT21*, *AT27*) is similar to that of the original aptamer.

Figure 4.1 Anti-proliferative activity of the modified *AGRO100* aptamers on (A) A549 and (B) WI38 cells in 5 days of incubation.

In addition, the shorter version of the 28-mer *AT21*, the 24-mer *AT21S* also inhibited the tumor cell proliferation with similar doses as *AGRO100* and *AT21* (Fig. 4.2A). This result again verified the anti-cancer property of the *AGRO100* variants. *CRO* was used as an oligo control when compared with the anti-cancer aptamer *AGRO100*⁹⁹. However, *CRO* contains repeats of (CCT)_n which has potential to adopt the specific i-motif structure. Thus, other oligo controls *Poly-T* and *non-CRO* were used to test their anti-cancer properties. As expected, cancer cells A549 grew well in the presence of these structures (Fig. 4.2B). In addition, another G-rich oligo *T95-2T* that adopts a stable parallel intramolecular G4¹⁸⁰ was also tested. Cancer cells A549 growth was inhibited to around 40% to 60% by 10 to 20 μM of *T95-2T*, respectively. This implied that the bio-activity of G4 *T95-2T* is lower than that of the G4 *AGRO100* aptamer and its analogs.

Effect of *AGRO100* aptamer and *AT21S* were also observed overtime (Fig. 4.2 C and D, respectively). The bioactivity of *AGRO100* and *AT21S* were similar on lung cancer cell A549. The number of living cells was reduced gradually overtime indicating the slow response of the cancer cells to the aptamer and its variant. The highest cell toxicity was observed after 5 days of incubation. The cancer cell's behavior in 10 μM oligos were similar to that of 20 μM suggesting that 10 μM of the oligonucleotides caused a maximum cell growth inhibition. We observed that 1 μM of DNA also slowly caused 40% of cell death after 5 days. A slight increase of alive cell numbers for 1 to 5 μM *AGRO100* and 1 to 20 μM of *AT21S* after 6 days of incubation may come from the depletion of the oligonucleotides that entered the cell cytoplasm (Fig. 4.2C and D). The result is consistent

with a report from Bates' group where the prolonged exposure of *AGRO100* in cancer cells caused the cytostasis that inhibits the process of cell growth and proliferation¹⁰⁰.

Figure 4.2 Anti-proliferative activity of *AGRO100* variants and controls.

(A) *AT21S* compared to *AGRO100*, (B) intramolecular G4 and non-G4 structure to lung cancer cell A549 proliferation after 5 days of incubation. (C and D) Cell proliferation on different days of *AGRO100* (C) and *AT21S* (D) on A549.

As discussed above, *AGRO100* aptamer adopts multiple conformations, while its derivations *AT11*, *AT21*, *AT21S*, and *AT27* are identified as a single form of G4 in the potassium solution. Although these versions adopt different G4 structure formation (parallel right-handed four G-tetrad *AT11*, anti-parallel two G-tetrad *AT21* or *AT21S*, parallel left-handed 4-G-tetrad *AT27*, respectively), these oligos showed the excellent anti-tumor proliferation without harming the healthy cells.

4.3. Effect of non-G4 *AGRO100* and *AT21S* on cell viability

We also tested the anti-proliferative effect of the same sequences *AGRO100* and *AT21S* when not folded in a G4 conformation. The DNA was prevented from forming G4 by incorporating their complementary strands to favor a helical duplex DNA conformation. The formation of DNA duplex from *AGRO100* and its complementary was confirmed by

the native PAGE electrophoresis (Fig. 4.3A, lane 1-3). In the native gel, *AGRO100* (lane 1) showed multiple bands indicating many structural forms, which are consistent with the published data ¹²¹. Lane 3 exhibited one band indicating the formation of a DNA duplex from *AGRO100* and its complementary strand. A similar instance was expected on *AT21S* duplex (Fig. 4.3A, lane 4-6). The resulting duplexes of *AGRO100* and *AT21S* were denoted as *AGdx* and *ATdx*, respectively, and were tested for their anti-cancer effect.

Figure 4.3 Effect of the non-G4 *AGRO100* and its variants.

(A) Native PAGE of *AGRO100* and *AT21S* in potassium solution. Sample concentration of each lane was similar. Lane 1 is *AGRO100* which separated into many bands that correspond to different conformations. Thus *AGRO100* signal is weak compared to a unique conformation of other samples. Antiproliferative activities of duplex (B) *AGRO100* and (C) *AT21S* on A549 after 5 days of incubation.

From the result, these duplexes did not significantly inhibit the cancer cells growth (Fig. 4.3B and C). Even though 20 μM of *AGdx* and *ATdx* also exhibited anti-proliferative effect (around 20%), but its effect was too little compared with that of the G4 structures of *AGRO100* and *AT21S* (more than 80% at 10 μM). The resulting data might suggest the relation of *AGRO100*'s G4 structure in the anti-proliferation of cancer cells.

4.4. Effect of guanine-containing degradation products on cells proliferation

4.4.1. Nuclease resistance of G-rich oligonucleotides

Recently, Zhang *et. al.* has reported that the anti-cancer activity of *AGRO100* was caused by their nature of G-rich sequence rather than their G4 structure¹⁷⁷. In this report, they provided the evidence that the cancer cell cytotoxicity was caused by the degradation products of some guanine rich oligonucleotides rather than their intact sequences. They used *AGRO100* and other G-rich sequences that have low nuclear resistances for the experiments. However, these G-rich sequences do not resemble the *AGRO100* structure.

Here, we examined the degradation of *AGRO100* and its analogs in cell culture media containing 10% fetal bovine serum (FBS) to further verify this hypothesis. *AGRO100* and *AT21S* were labeled with a fluorescent-FAM dye at 3'-end of the oligos. The fluorescent-labeled oligos were then incubated in 10% serum media at 37°C. The nuclease resistance of oligos was evaluated by using denaturing gel electrophoresis method (Fig. 4.4A-E). The non-degraded oligos were measured quantitatively by ImageJ based on the fluorescence band intensity on the denaturing gels (Fig. 4.4F). The observed results implied that *AGRO100* and *AT21S* were quickly degraded within 24 hours in 10% serum media (50% and 80% molecules, respectively). Further incubation (until 168 hours) led to around 80% and 95% degradation of *AGRO100* and *AT21S*, respectively. In contrast, *AGRO100* and *AT21S* from duplex strands *AGdx* and *ATdx*, were stable in the serum media even after 168 hours, where the amount of degraded products were negligible (Fig. 4.4C, D and F). Additionally, *T95-2T* also exhibited a high stability with more than 90% of the oligos are still intact in the serum media for 120 hours incubation (Fig. 4.4E and F).

The experiment results contribute to interpreting the outcomes of the anti-proliferative activity. *AGRO100* and *AT21S* that are characterized by a rapid degradation in serum within 24 hours superbly inhibited the cancer cells A549 proliferation. In contrast, the low digested oligos *AGdx*, *ATdx* and *T95-2T* were less effective on the cell proliferation. Our results agree with the results from Zhang *et. al.*¹⁷⁷ which imply the significance of guanine-degraded products to the anti-cancer cells mechanism.

Figure 4.4 Nuclease resistance of G-rich oligonucleotides.

(A) *AGRO100*, (B) *AT21S*, (C) *AGdx*, (D) *ATdx* and (E) *T95-2T* in DMEM medium containing 10% serum. (F) Degradation graph extracted from the data of denaturing gel electrophoresis.

4.4.2. Effect of guanine – based nucleotides and nucleosides

Nucleotides and nucleosides of guanine have been reported to cause cytotoxicity to some cancer cells¹⁸¹⁻¹⁸². However, the affecting threshold was varied from various cell lines and the mechanism is still unclear. Thus, we tested guanine-based nucleotides and nucleosides on small lung cancer cells A549 and normal fibroblast cells WI38 to validate their properties (Fig. 4.5). The result showed that 100 μ M of guanine, guanosine, guanosine triphosphate (GTP) and deoxyguanosine triphosphate (dGTP) inhibited around 70% of cancer cells proliferation (Fig. 4.5A), but does not cause cytotoxicity to normal cell WI38 (Fig. 4.5B). Moreover, other types of deoxynucleotide dATP, dCTP and dTTP did not affect both cancer cell nor normal cell proliferations (Fig. 4.5A and B). The data verified the cytotoxicity of guanine-based products on A549 cancer cell line. Herein, dGTP exhibited significantly an anti-cancer activity in which 20 μ M dGTP caused almost 50% cell inhibition. The cancer cell proliferation was also observed to decrease gradually overtime (Fig. 4.5C). In this experiment, the media reached maximum toxicity after 5 days

of incubation in 100 μM dGTP and had a slight increase in cell numbers after 6 days due to depletion of dGTP. The result was consistent with the time course experiments of *AGRO100* and *AT21S* (Fig. 4.2 C and D, respectively), where 10 μM of *AGRO100* and *AT21S* can be theoretically degraded to be 170 μM and 120 μM dG, respectively. The high concentration of dG in *AGRO100* caused a continuous anti-proliferative effect to cancer cells after 6 days incubation (Fig. 4.2C). Therefore, the cancer cell antiproliferation of *AGRO100* and variants may be inferred from the formation of their guanine-degraded products.

Figure 4.5 Antiproliferative effect of nucleobases

Proliferation effect of nucleobases on (A) cancer cell A549 and (B) normal fibroblast cell WI38. (C) Cell proliferation on different days of dGTP on A549.

4.4.3. Effect of guanine-degraded products of *AGRO100* and *AT21S*

The guanine based nucleotides and the quickly-degraded guanine rich sequences were observed to have the anti-cancer proliferation effect. To further confirm the result, the antiproliferative effect of the degraded products from *AGRO100* and *AT21S* in cancer cells A549 was also tested. Firstly, the oligonucleotides were incubated in 10% serum for 24

hours at 37°C to allow the degradation (denoted as post-degraded oligos). The culture media containing new oligos (denoted as pre-degraded oligos) and another media containing post-degraded oligos were utilized as the culture media for cancer cells A549, which were then incubated in 37°C, 5% CO₂ for 1 day. After that, the media was replaced with new culture media without DNA to stop the oligo degradation process that could affect the cell behavior. Lastly, the cells were further incubated for 4 days in 37°C, 5% CO₂. The cell proliferation and DNA internalization were then evaluated by MTT assay and flow cytometry, respectively (Fig. 4.6). The experiments showed that more than 5 µM of the post-degraded *AGRO100* (Fig. 4.6A) and *AT21S* (Fig. 4.6B) inhibited 30% of the cancer cell proliferation, while that of the pre-degraded *AGRO100* (Fig. 4.6A) and *AT21S* (Fig. 4.6B) only caused 20% of anti-cancer proliferation. Interestingly, both post-degraded *AGRO100* and *AT21* were entered cells less than the pre-degraded products (Fig. 4.6C and D, respectively). It is to be noted that the flow cytometry data were recorded after 1 day of cell incubation in the media containing DNA.

The result showed that the cytotoxicity came from the guanine-degraded products, however, the cancer cell uptake was higher for the intact products than the degraded ones. A possible explanation to that is that the intact *AGRO100* and *AT21S* adopts the compact G4 structure that could fit easily to the vesicles of endocytosis to enter the cells, while the unstructured forms of the degraded DNA could not. Another possible explanation is that the G4 structures of *AGRO100* and *AT21S* recognized and bound specifically to nucleolins or other proteins which were considered as a membrane receptor to induce DNA internalization¹⁰⁷. However, the more amount of the intact aptamers in cell's cytoplasm does not correspond to a higher anticancer effect. Further experiments should be performed to interpret this matter.

Figure 4.6 Antiproliferative assays and internalization of the degraded DNA products. Anti-proliferative assay (A and B) and internalization (C and D) of degraded products *AGRO100* and *AT21S*, respectively, on small lung cancer cells A549. The internalization of FAM-conjugated DNA into cells was determined in the black gate. UTC indicates the untreated cells.

4.5. Conclusion

In summary, we have showed the significant anti-proliferative effect of *AGRO100* variants (*AT11*, *AT21*, *AT21S* and *AT27*) exclusively to cancer cells. We also showed that the cytotoxicity of these G4 sequences was caused by the degradation products which were formed in the cell serum medium. Further systematic studies on the effect of the digested G-rich products may contribute significantly for understanding the bioactivity mechanism of *AGRO100* aptamer. However, the G4 structure of *AGRO100* and its variants might contribute to the aptamer localization into cell cytoplasm suggesting the refined structures could be a platform for drug design in therapeutic treatment.

5. CHAPTER 5 - FIBROUS ANTIBACTERIAL COATINGS FROM SELF-ASSEMBLED SILVER-BINDING ELASTINS [#]

[#] Results of this chapter were published in “Yan, L., Anh, T. T.¹, Shang-You, T., & Fong, E., *Fibrous antibacterial coatings from self-assembled silver-binding elastins*. RSC Advances 5.107 (2015): 88027-88031.”

¹ [All the results in this work was done by Anh, T. T. except the results from figures 5.4f, 5.6, 5.7 and 5.9. The work was done from Aug-2013 to Dec-2014 by Anh, T. T. before Anh, T. T. left Dr. Eileen Fong Wen Mei’s lab to join Prof. Phan Anh Tuan’s lab in Jan-2015.]

The experimental method of this chapter was given in the appendices section.

5.1. Introduction

Antibacterial surfaces play an important role in disrupting the adhesion and growth of harmful microbes. In particular, silver and nanosilver has been applied to a variety of materials and surfaces, and have been used with great success to resist bacteria adhesion¹⁸³. While the mechanism by which silver kills cells is still under debate, it has been widely accepted that silver ions disrupt the bacteria cell membrane, leading to cell death¹⁸⁴⁻¹⁸⁵. Nonetheless, the well-established antimicrobial properties of silver have led to the widespread development of silver-based materials for use in biomedical¹⁸⁶, textiles¹⁸⁷, food and packaging industries¹⁸⁸.

Silver, particularly nano-sized silver particles have been shown to have more potent antimicrobial properties due to their large surface areas. Since, nanosilver has been immobilized onto surfaces¹²⁹, incorporated as coatings¹⁸⁹, or directly impregnated into polymers and fabrics¹³⁰. Recently, immobilization of silver nanoparticles onto electrospun polymeric nanofibers has been reported to yield materials with potent antibacterial properties, further exploiting the high surface areas and porosities of the nanofibrous architectures¹⁹⁰⁻¹⁹².

Conventionally, nano-silver is synthesized using chemical approaches involving the reduction of silver precursors¹⁹³. Alternative approaches to mineralize silver nanoparticles from soluble precursors such as silver nitrate, have also been explored. For example, Kaplan and coworkers developed silk fusion proteins that incorporated silver-binding domains, and showed that films made from such fusion proteins have the ability to mineralize silver¹³⁵. Recently, Philip *et al.*, also designed mussel adhesive fusion proteins that were able to synthesize silver nanoparticles from soluble precursors after 6 days¹⁹⁴. Likewise, we previously reported successful preparation of fusion proteins containing elastin-like domains and silver-binding motifs (named GPG-AG3)¹²⁹. We also showed that aggregates or thin films made out of GPG-AG3 proteins biomineralized silver nanoparticles with dimensions ranging from 20 nm to several microns in diameters.

In this work, we demonstrate a straightforward and green approach to prepare antibacterial coatings on polypropylene (PP) surfaces using GPG-AG3. PP is a polymer used in a wide variety of applications; yet untreated PP suffers from poor wettability and printability due to its hydrophobic characteristics¹⁹⁵⁻¹⁹⁶. Here, we exploit the self-assembling characteristics of the GPG domains to create fibrous templates for silver nucleation. The self-assembled silver-

coated protein fibers were subsequently deposited directly onto untreated PP films, and allowed to evaporate in air, leaving behind a thick fibrous silver mat (Fig. 5.1).

Figure 5.1 Schematic illustrations of the steps involved in the formation of silver nanoparticle (AgNPs) covered protein nanofibers on PP films.

The entire process is environmental friendly; both synthesis and coating processes were performed in physiological buffers and temperatures, eliminating the use of toxic organic solvents, otherwise required in methods such as electrospinning^{192, 197}. The coating process is simple, does not require specialized equipment and can be applied to any hydrophobic engineering plastics. It is also possible to mass produce the silver-coated proteins elsewhere and brought on-site to prepare antibacterial coatings on the required surfaces.

5.2. Formation of silver nanoparticle (AgNPs) on elastin nanofibers platform

Here, trifluoroethanol (TFE) was added to 20 μ M GPG-AG3 protein solution (final TFE concentration of 30%) to trigger the formation of protein fibers. The self-assembled GPG-AG3 fibers were subsequently exposed to silver nitrate for 3 days at room temperature and dialyzed. Figure 5.2a and b show AFM images of the self-assembled GPG-AG3 fibers. Each fiber was found to be more than 10 μ m in length with an average diameter of around 50 nm. A magnified image of the fibers revealed a bead-like morphology, where each protein fibril consisted of protein “beads” joined end-to-end; each “bead” is about 20–50 nm in diameter (Fig. 5.2b). In the presence of TFE, the hydrogen bonding between water and protein is disrupted, and this drives the irreversible formation of beta-turn or beta-sheet secondary structures to result

in the fibrous morphology¹⁹⁸. The protein fibers were found to remain stable when stored in water at 4 °C for 1 week, with no observable changes in morphologies observed from TEM images (Fig. 5.3).

Transmission electron microscopy (TEM) confirmed the presence of GPG-AG3 protein fibers, where each fibril was indeed coated with silver nanoparticles with diameters of 10–50 nm (Fig. 2c and d). The selected area electron diffraction (inset in Figure 2d) indicated that the silver nanoparticles were crystalline in nature, and have [111] face-centered-cubic lattice structures.

Figure 5.2 Characterization of elastin fibers and silver-coated fibers.

(a and b) AFM images of self-assembled GPG-AG3 protein fibers. (c and d) TEM images of silver-coated GPG-AG3 protein fibers. Inset in (d) shows the selected area electron diffraction (SAED) patterns of the silver nanoparticles.

Figure 5.3 TEM images of Ag-protein fibers after storage in water in 4 °C for 1 week.

5.3. Deposition of AgNPs-covered protein fibers onto PP film

Solutions containing varying concentrations of silver-coated GPG-AG3 fibers were directly deposited onto 8 mm circular untreated PP films, and allowed to dry in air. Dried PP films were analysed using FESEM and UV-Vis. Figure 5.4a shows a representative FESEM image of a PP film treated with 10 μM of proteins. A magnified image of the coating revealed that a dense network of silver-coated protein fibers was indeed present on the surface of the film (Fig. 5.4b). Energy-dispersive X-ray spectroscopy (EDX) spectrum further confirmed the presence of elemental silver (Fig. 5.4c). Other elements such as C, O, Cu, Si, K and Ca were due to the presence of protein matter and silicon wafer sample holder.

The silver content on the PP films could be varied by varying GPG-AG3 protein concentrations. At a protein concentration of 20 μM , a complete coverage of the film surface was achieved ($\sim 95.8\%$) (Fig. 5.5). This observation was also consistent with FTIR spectra of the PP films (Fig. 5.4d) as well as the darkening coloration in the PP films (Fig. 5.4e). Peaks corresponding to amide I (wavelengths 1627 cm^{-1} and 1534 cm^{-1}) and amide II peaks (wavelength 3285 cm^{-1}) could be detected in all of the protein-coated films except for when the protein concentration was 1 μM . This could be due to the low surface coverage ($\sim 5\%$) at a protein concentration of 1 μM (Fig. 5.5).

Figure 5.4 Characterization of silver-fibers-coated Polypropylene (PP).

(a) SEM image of protein-coated PP films (10 μ M). (b) Magnified image of the area enclosed by the red box of sample shown in (a). (c) Energy dispersive X-ray (EDX) spectrum obtained from (b), confirming the presence of elemental silver. (d) UV-Vis spectra of PP films coated with 20 μ M of GPG-AG3 protein fibers. Characteristic peaks at 440 nm confirmed the presence of silver. a.u. represents arbitrary units. (e) FTIR spectra of PP coated with varying concentrations of silver-coated protein nanofibers. Untreated PP was used as negative control. (f) Appearance of PP films coated with varying concentrations of silver-protein solutions.

Figure 5.5 Percent surface coverage of PP films when treated with varying concentrations of silver-bound GPG-AG3.

5.4. Antibacterial effect of AgNPs-coated fiber on PP films

5.4.1. Effect of AgNPs-coated fiber PP film on agar plates containing *E. coli* and *S. aureus*

We showed that silver-coated GPG-AG3 protein fibers could be readily deposited onto the surfaces of untreated PP *via* a simple evaporation step. We next evaluated the antibacterial properties of the protein-coated PP films using both gram-negative *Escherichia coli* (*E. coli*) and gram-positive *Staphylococcus aureus* (*S. aureus*). First, PP films containing varying concentrations of silver-coated protein fibers were placed in contact with bacteria (at 10^7 CFU) pre-coated onto 2× YT agar plates. Agar plates were incubated overnight at 37 °C and the widths of the inhibition zones were determined. Figure 5.6 shows images of agar plates incubated with various protein-coated PP films for *E. coli* and *S. aureus* respectively. Magnified images of the inhibition zones could be found in Figure 5.7. Black arrows indicate the edges of the bacterial inhibition zones in each sample. Table 5.1 summarizes the measured widths of the inhibition zones for all the samples analyzed. A maximal width of inhibition zone (~1 mm) was achieved at 15 μM for *E. coli* where further increase in protein concentrations did not increase the widths of the inhibition zones. In comparison, an inhibition zone with widths of 0.9 mm was obtained for *S. aureus*. As expected, untreated PP film controls had no effect on bacteria growth. The formation of inhibition zones was likely a result of released Ag^+ ions¹⁹⁹ from the silver

nanoparticles found on the surfaces of the GPG-AG3 protein fibers; the antibacterial properties of Ag^+ has been reported by others^{185, 199-202}. We noted higher amounts of silver-coated GPG-AG3 protein fibers were needed to kill *S. aureus*, as compared to *E. coli*. This could be due to the thicker cell walls of *S. aureus*, which are harder for silver ions to penetrate and disrupt¹⁸⁵.

Figure 5.6 Effect of AgNPs-coated fiber PP film on agar plates containing *E. coli* and *S. aureus*.

Images of agar plates with PP films treated with varying amounts of silver-coated protein nanofibers for *E. coli* (left panel) and *S. aureus* (right panel). Magnified images of individual samples are provided in Figure 5.7.

Figure 5.7 Magnified images of the bacterial inhibition zones for PP films

Magnified images of the bacterial inhibition zones for PP films containing varying concentrations of silver-coated GPG-AG3 fibers for *E. coli* and *S. aureus*. Black arrows mark the edges of the inhibition zones.

Protein Concentration (μM)	Width of inhibition zone (mm)	
	<i>E. coli</i>	<i>S. aureus</i>
1	N.D.	N.D.
5	0.59 ± 0.08	0.46 ± 0.14
10	0.64 ± 0.10	0.82 ± 0.10
15	1.11 ± 0.12	0.87 ± 0.18
20	1.16 ± 0.13	0.93 ± 0.18

Table 5.1 Widths of inhibition zones for *E. coli* and *S. aureus*

5.4.2. Effect of AgNPs-coated fiber PP film on media containing *E. coli* and *S. aureus*

Growth kinetics of bacterial strains were used to assess the relative rate and extent of bactericidal activities of the protein-coated PP films. Bacteria were cultured in 2× YT media in the presence of protein-coated PP films, and agitated at 250 rpm at 37 °C. Bacteria growth was monitored by measuring the optical density at 600 nm (OD 600 nm) overtime. Figure 5.8a and 5.8b show the time courses of bacteria growth in the presence of PP films coated with varying concentrations of silver-coated GPG-AG3 protein fibers. Films coated with at least 15 μM of proteins were found to be effective in inhibiting the growth of both *E. coli* and *S. aureus*. In addition, we also found that silver nanoparticles presented together with GPG-AG3 fibers had enhanced antibacterial properties compared to silver nanoparticles alone (Fig. 5.8c and d). This could be due to a higher concentration of Ag^+ ions resulting from the large surface areas provided by the fibrous network.

Figure 5.8 Effect of AgNPs-coated fiber PP film on media containing *E. coli* and *S. aureus*

(a and b) Growth curves of *E. coli* and *S. aureus* in the presence of PP films coated with varying concentrations of silver-coated protein nanofibers. (c and d) Growth curves of *E. coli* and *S. aureus* in the presence of coated PP film (20 μM , black curve), silver-coated GPG-AG3 (20 μM , red curve), silver nanoparticles (20 μM , blue curve) and blank PP film control (green curve).

5.4.3. Stability of AgNPs-coated fiber PP film in ddH₂O, 2 $\times$ YT and PBS

Finally, the stability of the protein coatings deposited onto PP films was examined. PP films coated with 20 μM of GPG-AG3 fibers were placed in 50 mL conical tubes containing 30 mL of ddH₂O, 2 $\times$ YT or PBS, and shaken at 60 rpm for 24 h at 25 $^{\circ}\text{C}$. Washed samples were subjected to FTIR and UV-Vis analysis to determine the remaining protein and silver contents after washing (Fig. 5.9a and b). There were no observable changes in both amide I and II peaks for the films washed in ddH₂O and PBS, compared to the unwashed sample (Fig. 5.9a). Likewise, we also did not detect significant decrease in silver contents for films washed in ddH₂O and PBS (Fig. 5.9b). However, we noted a slight decrease in the intensities of both amide I and II peaks for samples washed in 2 $\times$ YT (Fig. 5.9a). However, there was no significant drop in the silver content in films washed in 2 $\times$ YT (Fig. 5.9b). Nonetheless, all washed films exhibited comparable antibacterial properties compared to the unwashed samples (Fig. 5.9c).

Figure 5.9 Stability of AgNPs-coated fiber PP film

(a) FTIR spectra of silver-protein coated PP films (20 μ M) before and after washing in water, PBS and 2x YT for 24h at 25°C. (b) Absorbance values @440nm of the unwashed and washed samples determined using UV-VIS. a.u. represent arbitrary units. Error bars are S.D. (c) Agar disks showing the presence of inhibition zones of washed samples for *E. coli* and *S. aureus* respectively.

5.4.4. AgNPs-coated fiber PP film in solvent containing 2% SDS

We also found that the protein GPG-AG3 fibers were physically adsorbed onto the PP surfaces, since no proteins could be detected after washing in 2% SDS at 70 °C for 1 h. The SDS wash test is used to determine the type of interactions between proteins and surface²⁰³⁻²⁰⁴. FTIR analysis show dramatic reduction in the intensities of both amide I and II peaks for films subjected to SDS washing (Fig. 5.10). Given the high concentration of proteins in the solution, it is likely that the final coating consisted of multiple protein layers. While several layers of

proteins on top of the coating could be removed by rigorous washing in ddH₂O, 2× YT and PBS, it is likely that there remain sufficient layers of silver-coated proteins on the PP surfaces to confer antibacterial properties.

Figure 5.10 FTIR spectra of protein-coated PP films subjected to 2% SDS washing

FTIR spectra of protein-coated PP films subjected to 2% SDS washing at 70 °C for 1h (blue curve). Intensities of amide I and II peaks were dramatically reduced after washing, confirming that the proteins were physically adsorbed onto PP.

5.4.5. Direct bactericidal effect of AgNPs-coated fiber PP film on *E. coli* and *S. aureus*

Finally, we investigated if the coatings themselves indeed have the bactericidal abilities. Bacteria (10^5 CFU) were cultured directly on protein-coated PP films for 2 h, and the surface was washed in 1 mL of 2× YT to harvest the bacteria. 100 µl of the culture was directly plated onto 2× YT agar plates and incubated overnight at 37 °C. The next day, the colonies found on the agar plates were counted to obtain the CFU. Figure 5.11 shows representative images of the agar plates for uncoated PP films and protein-coated PP films. The average CFU for protein-coated PP films was 10^2 , 100-fold less than the value obtained for uncoated PP film control (10^4). Hence, our protein coatings are indeed able to kill bacteria upon contact, confirming their antibacterial characteristics.

Figure 5.11 Representative images of agar plates used for CFU counting for uncoated PP film control (left) and protein-coated PP film (20 μ M; right).

5.5. Conclusion

In summary, a simple, low-cost approach to fabricate antibacterial coatings on PP surfaces was demonstrated. We showed that silver-binding fusion proteins (GPG-AG3) readily self-assembled into fibrous templates for the nucleation of silver under physiological conditions. We showed that silver-coated protein coatings could be generated using a single evaporation step to confer antibacterial properties to PP surfaces. Protein-coated PP films inhibited the growth of both gram-negative *E. coli* and gram-positive *S. aureus*, demonstrating a scalable and green strategy to prepare antibacterial coatings. Future work to strengthen the adhesion of the proteins to the underlying substrates is necessary to prolong the antibacterial effects of the coatings.

6. CHAPTER 6 – CONCLUSION AND FUTURE WORK

CONCLUSION

Two main classes of natural biopolymers are polynucleotides and polypeptides. These molecules contribute to the diversity of living organisms. This thesis is focused on G-quadruplex nucleic acids of polynucleotides and elastin proteins of polypeptides.

G-quadruplex is a four-stranded structure of a G-rich sequence containing 2 or more stacked G-tetrads which is constituted from 4 guanines in a cyclic formation connected by Hoogsteen hydrogen bonds. Various G-quadruplex structures are classified by molecularity, numbers of G-tetrads, glycosidic conformation, strand orientations, connecting loops and groove widths.

AGRO100, the first anti-cancer aptamer tested in humans, exhibits G-quadruplex structural polymorphism which hinders the understanding of its mechanism and application. Thus, exploration of their structural conformation is important. Structure of a novel G-quadruplex of *AT21* which is derived from the *AGRO100* sequence was resolved. *AT21* adopts a monomeric antiparallel G-quadruplex containing only two G-tetrads sandwiched between a triad and 2 successive loops. The stability of the structure in acidic condition and with sequence modification showed that this new G4 scaffold is robust for therapeutic application. **(Chapter 3)**

Significant bioactivity of *AGRO100* variants (*AT11*, *AT21*, *AT21S* and *AT27*) to cancer cells A549, but not to normal cell WI38 was observed. IC₅₀ of the variants are 2.5 μ M, similar to that of their original sequence *AGRO100*. We observed that guanine based degraded products also played a role in the anticancer effect of these oligos. **(Chapter 4)**

In the context of polypeptides, we showed that silver-binding fusion proteins (GPG-AG3) readily self-assembled into fibrous templates for the nucleation of silver with the size of 10 to 50 nm in diameter under physiological conditions. The silver-coated protein coatings could be generated using a single evaporation step to confer antibacterial properties to PP surfaces. Protein-coated PP films inhibited the growth of both gram-negative *E. coli* and gram-positive *S. aureus*, demonstrating a scalable and green strategy to prepare antibacterial coatings. **(Chapter 5)**

FUTURE WORK

The low potency of *AGRO100* in Phase II clinical trial could come from the drug being rapidly cleared from human body ¹⁰⁷. Thus, much research has been directed toward applying this agent into a drug cancer-selective delivery system to improve its accumulation in cells and result in high potency.

Nanoparticles or compounds that conjugates the aptamer *AGRO100* are supposed to increase the drug accumulation in cancer cells and subsequently improve target treatment. The conjugates were developed from gold nanospheres ²⁰⁵, gold nanostars ²⁰⁶⁻²⁰⁷, PEG-PLGA ²⁰⁸, silica nanoprobe ²⁰⁹, quantum dots ²¹⁰, DNA nanostructure such as DNA tetrahedra structures ¹⁷⁹. Bates' group has conjugated around 40 nucleotides *AGRO100* onto a gold nanosphere with 5 nm diameter ²⁰⁵. The conjugates exhibited a significant selective accumulation of aptamers in breast cancer cells compared to aptamers without conjugation. The effect of cell anti-proliferation was highly inhibited and followed by cell death. The outcome of these researches make *AGRO100* conjugates highly potential for nanomedicine in therapeutic treatment. Thus, *AGRO100* variants with the same bioactivity of *AGRO100* are as potent for conjugation onto the nanoparticle. Furthermore, their refined structures facilitate the drug design and are very potent for improving their anti-proliferative activity on various cancer cell lines.

In our current work on elastin polypeptide, we have successfully demonstrated that GPG-AG3 proteins were able to self-assemble into long nano-fibers and were able to serve as a template for AgNPs synthesis. Silver fibers absorbed on PP showed antibacterial capacity on both gram negative *E. coli* and gram positive *S. aureus*. However, the concern is the long term toxicity of silver proteins-coated PP against these bacteria in harsh environment (2% SDS), since the protein coating on PP gradually lost overtime. Hence, we propose another method other than the absorption method to obtain a covalent attachment of protein on PP surface. Macdonald *et al.* has developed one-step method in dry condition to obtain the covalent bonding between functional protein and material surface ²¹¹. The success of this method does not require addition of crosslinker or chemical reaction which may be harmful for the application of the biomaterial on the medical device. The work will help to strengthen the adhesion of the proteins to the underlying substrates, which is

necessary to prolong the antibacterial effects of the coatings. Eventually, the silver-coated proteins can be employed in medical devices, to enable large scale manufacturing as an effective long-term antibacterial material.

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APPENDICES

1. EXPERIMENTAL DETAILS OF CHAPTER 5

Materials and methods

Bacteria strains *Escherichia coli* (*E. coli*, DH5 α) and *Staphylococcus aureus* (*S. aureus*, ATCC #25923) were obtained from Life Technologies and American Type Culture Collection respectively. All chemicals were purchased from Merck Millipore, except 2,2,2-Trifluoroethanol (TFE) and silver nitrate (AgNO₃) which were purchased from Sigma-Aldrich. Silicon wafers were purchased from Lotech Scientific Supply (Singapore). Polypropylene (PP) films were purchased commercially from Landmark Plastics (Singapore).

Self-assembly of GPG-AG3 protein nanofibers

Expression and purification of GPG-AG3 proteins was performed as previously reported¹²⁹. Lyophilized GPG-AG3 proteins were weighed and dissolved in deionized water (dH₂O) at 4 °C. Subsequently, TFE was added to protein solution to induce the formation of nanofibers. The protein solution (final concentration of 20 μ M) containing 30% TFE was allowed to incubate at 60 °C for 3 days before being finally cooled to room temperature. Concentration of protein was decided by NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc, Wilmington, DE, USA).

Synthesis of silver nanoparticles using GPG-AG3

Silver nitrate (AgNO₃, 100 mM) was added to the different concentrations of protein nanofibers solution prepared previously. The mixture was shaken on a rocker for 1 h. Subsequently, 1 mM NaCl was then added to solution and further incubated for 24 h at room temperature to provide electrostatic stability to the nucleation of silver nanoparticle on AG3 peptide. The mixture was then dialyzed in dH₂O to remove the excess of AgNO₃ and NaCl. The final solution was stored at 4 °C for further use.

Adsorption of silver-coated GPG-AG3 nanofibers on polypropylene (PP) films

Untreated PP films were cut into 8mm circular disks using an 8mm biopsy punch. 200 μ L of silver-coated GPG-AG3 solutions were dropped directly onto the PP films and allowed

to evaporate in air overnight. Varying concentrations of silver-coated GPG-AG3 solutions (i.e., 1, 5, 10, 15 and 20 μ M) were used to prepare coatings of varying silver content on identical PP films. The coated samples were then rinsed in ddH₂O to remove any unbound proteins and dried in nitrogen gas.

Atomic force microscopy (AFM)

GPG-AG3 protein nanofibers were subjected to atomic force microscopy (AFM) to examine the morphology of the self-assembled GPG-AG3 protein nanofibers. 1 μ L of the fiber-formed protein solution was dropped onto a piece of untreated silicon wafer and allowed to dry in air. Imaging was performed on the dried sample using atomic force microscopy (Veeco, Dimension V), with accompanying Nanoscope 7.3 software, in tapping mode at a scan rate 0.7 Hz with a Si cantilever at 25°C.

Field Emission Scanning Microscopy (FESEM) and Transmission Electron Microscopy (TEM)

Protein-coated PP samples were visualized using Field Emission Scanning Microscopy (JOEL, FESEM, 6340F), at an accelerating voltage of 5 kV and an emission current of 12 mA. Images were acquired at random positions of the films (i.e., near the edge and at the center of the films). The thresholds of the acquired SEM images were adjusted to 50% of the maximum intensities, before converting to binary images. The white areas (i.e., areas covered by proteins) were expressed as percentages of the total areas to yield the percentage coverages of protein on the PP films. For transmission electron microscopy (TEM), an emission current 105 mA and an accelerating voltage of 200 kV were used to visualize the samples.

UV-VIS and FTIR

The presence of silver nanoparticles on GPG-AG3 protein fibers was confirmed using UV-Vis Spectrometer 2501PC. The spectrum was collected over a wavelength range of 300 – 900 nm with a resolution of 0.5 nm using water as reference. FT-IR spectra were recorded on a Fourier transform infrared spectrometer (PerkinElmer) with a DGTS detector.

Antibacterial assays

The antibacterial properties of the PP films containing 1, 5, 10, 15 and 20 μM of silver-coated proteins were evaluated using gram-negative *Escherichia coli* (*E. coli*) and gram-positive *Staphylococcus aureus* (*S. aureus*). Approximately 107 colony forming units (CFU) of bacteria were spread on 2x YT (Yeast Extract Tryptone) agar plates. Coated PP films were placed in direct contact with the bacteria and incubated overnight at 37 °C. The “inhibition zone” was defined as the bacteria-free zone. The widths of the inhibition zone (i.e., distances between the bacteria lawns and the PP films) were measured manually using ImageJ. For each sample, at least 15 measurements were taken and the means $\pm$ SD were reported.

The growths of both bacteria strains in liquid media in the presence of protein-treated PP films were evaluated. Bacteria cultures were grown to log phase (i.e., optical density OD_{600nm} 0.6 – 0.8) and subsequently re-innoculated at 1:100 dilution into 4 mL of 2x YT media. Cultures were immediately supplemented with PP films containing varying amounts of silver-coated proteins and agitated at 250 rpm at 37°C. Bacteria growth was monitored by measuring the optical density at 600 nm (OD_{600nm}) at regular time intervals for up to 24 h.

Evaluation of stability of protein coatings

Protein-coated PP films were subjected to rigorous washing to determine the stability of the coatings. Briefly, samples were immersed in 10 ml of ddH₂O, PBS or 2x YT and agitated at 225rpm for 24h at 25 °C. Washed samples were air-dried before being subjected to FTIR and UV-VIS analysis to determine the residual protein and silver content.

Statistical analysis

For all experimental data, results are represented as means $\pm$ SEM of 3 independent experiments. The statistical significance of differences was determined using ONE-WAY ANOVA. Differences were taken to be statistically significant at $P \leq 0.05$.

2. Synthesis of 3'-6FAM-oligonucleotide

Figure S1. AGRO100-6FAM-3' purification and characterization. (A) Elution spectrum of DNA purification by using reverse phase HPLC. Black arrow represents for the eluted fraction which was then removed DMT labeled and characterized by MALDI-TOP. (B) MALDI-TOP spectrum of AGRO100-6FAM-3' with expected MW of 9146 (sequence: 5'-GGTGGTGGTGGTTGTGGT GGTGGTGGT-6FAM-3'). (C) Denaturing PAGE of AGRO100-6FAM-3'. From the result of MALDI-TOP spectrum and denaturing PAGE, the obtained purity of labelled – DNA is more than 95 %.

Figure S2. AT21S-6FAM-3' purification and characterization. (A) Elution spectrum of DNA purification by using reverse phase HPLC. Black arrow represents for the eluted fraction which was then removed DMT labeled and characterized by MALDI-TOP. (B) MALDI-TOP spectrum of AGRO100-6FAM-3' with expected MW of 8108.3 (sequence: 5'-TGGTTGTGGTTTGGTGTGGTGGT-6FAM-3'). (C) Denaturing PAGE of AT21S-6FAM-3'. Black arrow represents for the eluted fraction which was removed DMT labeled and characterized by MALDI-TOP. From the result of MALDI-TOP spectrum and denaturing PAGE, the obtained purity of labelled – DNA is more than 95 %.

LIST OF PUBLICATIONS

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