Probing the effects of non-base-triple-forming nucleotides on triplex formation

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**Probing the effects of non-base-triple-forming nucleotides
on triplex formation**

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on triplex formation**

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ABSTRACT

Triplex is emerging as a prevalent structural element found in DNA, RNA and DNA-RNA structures for diverse biological functions. Better understanding of the factors affecting nucleic acid triplex structures may help develop an accurate method for the search for novel functional triplexes present in nature, and develop highly stable artificial triplex structures for biological applications. In this thesis, we characterized by UV-absorbance-detected thermal melting experiment how a triplex structure stability may be affected by the residues adjacent to the triplex. Triplexes with varied strand compositions (DNA-DNA₂, RNA-DNA₂, and RNA-RNA₂) were studied. We found that the non-base-triple-forming Watson-Crick base pair of the duplexes adjacent to the 5' ends of the triplex-forming oligonucleotide (TFO) can strengthen the DNA-DNA₂ and RNA-DNA₂ triplex formation. A 2-aminopurine residue was attached to the 5' and 3' ends, respectively, to monitor triplex formation. The 2-aminopurine fluorescence intensity is enhanced upon binding to a duplex to form a triplex, suggesting that the 2-aminopurine base becomes less stacked upon triplex formation. 2-Aminopurine fluorescence titration was used to measure the K_D values of the triplex forming reactions and the effects of pH, Mg²⁺ and spermine on triplex stability.

LIST OF ABBREVIATIONS

TFOs	Triplex-Forming Oligonucleotides
rHP	RNA hairpin
dHP	DNA hairpin
T_m	melting temperature
EDTA	Ethylenediaminetetraacetic acid
MES	2-(N-morpholino)ethanesulfonic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
ssRNA	single-stranded RNA
dsRNA	double-stranded RNA
2AP	2-aminopurine
K_D	equilibrium dissociation constant

Chapter 1. Introduction

1.1 DNA and RNA

1.1.1 The primary structure of DNA and RNA

Deoxyribonucleic acid (DNA) is a molecule that carries most of the genetic information in the growth, development, functioning and reproduction of human beings and other living creatures and viruses (1). In 1953, James Watson and Francis Crick discovered that the molecular structure of DNA is a double helix structure by using X-ray diffraction data and modeling (Figure 1.1). There are two types of DNA/RNA bases: the purines, guanine (G) and adenine (A), and the pyrimidines, thymine (T) (or uracil for RNA) and cytosine (C) (Figures 1.2 and 1.3) (2). In a DNA double helix, the bases form complementary Watson-Crick base pairs (guanine-cytosine and adenine-thymine, see Figure 1.4). On the outside, there is a negatively charged sugar-phosphate backbone making the DNA molecule hydrophilic, while the important atoms of bases are protected in the hydrophobic interior.

The primary structure of a DNA is a linear sequence of nucleotides, such as: 5'-TCTCTCTCTTTC-3'. DNA monomers are linked by phosphodiester bonds between the 5' and 3' oxygen atoms. Each polynucleotide has a 5' end and a 3' end. DNA is a

polar molecule due to the fact that the chemical properties of the two ends are different. Normally, a single or multiple phosphate groups appear at the 5' end while a hydroxyl group exists in the 3' end for enzymatically synthesized DNAs. Chemically synthesized DNA may have no phosphate on either end. Generally, there is no branch in a DNA strand. So we can use the sequence of a DNA to describe the covalent structure of a DNA molecule.

Figure 1.1 Double helical structure of a DNA (PDB: 2n0l).

Figure 1.2 Chemical structures of four nucleotides with unmodified purine and pyrimidine bases in DNA.

Figure 1.3 Chemical structures of four nucleotides with unmodified purine and pyrimidine bases in RNA.

1.1.2 The secondary structure of DNA and RNA

The secondary structure of DNA is the base-pairing interactions in one molecule or a set of molecules (3). The secondary structures of DNA and RNA are different: most natural DNA are fully base paired double helices, while natural RNA are single strands and often have complicated base-pairing interactions because they have extra hydroxyl

groups in the ribose sugars which can form hydrogen bonds (Figures 1.2 and 1.3). In 1952, Austrian chemist Erwin Chargaff found that DNA from any cell of all organisms should have the same number of pyrimidine and purine bases. What's more, the number of cytosine is equal to guanine while the amount of adenine is the same with thymine, which is called Chargaff's rules (Table 1.1) (4,5).

In 1953, James Watson and Francis Crick described the double helix structure of DNA, using X-ray diffraction and the mathematical modeling. In the Watson-Crick model, duplex DNA is a right-handed helical structure (Figures 1.1, 1.4, and 1.5), which is formed by two complementary single stranded DNAs in an antiparallel orientation. The sugar and phosphate group can form a hydrophilic backbone on the outside of the duplex DNA which contain the large hydrophobic interior bases inside (Figures 1.1 and 1.4). The interior bases carry the genetic information (2).

Table 1.1 Erwin Chargaff's 1952 data, containing the numbers of bases of DNA from some organisms (6).

Organism	A%	G%	C%	T%	A/T	G/C	GC%	AT%
φX174	24.0	23.3	21.5	31.2	0.77	1.08	44.8	55.2
Maize	26.8	22.8	23.2	27.2	0.99	0.98	46.1	54.0
Octopus	33.2	17.6	17.6	31.6	1.05	1.00	35.2	64.8
Chicken	28.0	22.0	21.6	28.4	0.99	1.02	43.7	56.4
Rat	28.6	21.4	20.5	28.4	1.01	1.00	42.9	57.0
Human	29.3	20.7	20.0	30.0	0.98	1.04	40.7	59.3
Grasshopper	29.3	20.5	20.7	29.3	1.00	0.99	41.2	58.6
Sea Urchin	32.8	17.7	17.3	32.1	1.02	1.02	35.0	64.9
Wheat	27.3	22.7	22.8	27.1	1.01	1.00	45.5	54.4
Yeast	31.3	18.7	17.1	32.9	0.95	1.09	35.8	64.4
E.coli	24.7	26.0	25.7	23.6	1.05	1.01	51.7	48.3

Figure 1.4 Chemical structure of DNA duplex. Dotted lines are hydrogen bonds. The figure was reproduced from Wikipedia: <https://en.wikipedia.org/wiki/DNA>.

1.1.3 The tertiary structure of DNA and RNA

The tertiary structure of DNA/RNA refers to the three-dimensional structure. DNA and RNA require precise 3D tertiary structures for the biological functions. There are many

motifs for DNA/RNA tertiary structure, such as double helix (7), quadruplexes (8), coaxial stacking (9), tetraloop-receptor interactions (10), major and minor groove triplexes (11), A-minor motif (11), and ribose zipper (12).

1.1.3.1 Double helix

The double helix is the main tertiary structure of natural DNA and RNA. There are three DNA conformations that have been found in nature: A-DNA, B-DNA, and Z-DNA (see Figure 1.6). B-DNA is the most common DNA in cells (2,7), which has a width of 23.7 Å and a length of 34 Å per 10 base-pairs of sequence. One turn of B-DNA has 10.4-10.5 base pairs in the solution. There are more base pairs per turn in the A-DNA. Compared with B-DNA, the minor groove of A-DNA is wide and shallow while the major groove is deep and narrow. Watson-Crick based paired RNA duplexes are in A form.

Z-DNA is much different from A-DNA and B-DNA due to the fact that it is left-handed. Z-DNA was first found by Robert Wells et al in the studies of a repeating polymer of inosine-cytosine (13). He found a "reverse" circular dichroism spectrum for these DNAs, and interpreted that this means that the strands wrapped around one another in a left-handed fashion. The relationship between Z-DNA and B-DNA was investigated by Pohl and Jovin, who found that the ultraviolet circular dichroism of poly (dG-dC) was almost inverted in sodium chloride solution (14). Then Thamann et al used the Z-

DNA crystals and Raman spectra of these solutions to prove that this was the result of a conversion from B-DNA to Z-DNA (15). There may be biological significance of Z-DNA such as providing torsional strain relief during DNA transcription (16,17). A comparison of regions with a high sequence-dependent, predicted tendency to form Z-DNA in human chromosome 22 with a set of known gene transcription sites indicated there is a connection (18). The toxic effect of ethidium bromide (EtBr) on trypanosomas is because of a change from their kinetoplastid DNA to Z-DNA (19). This transformation is caused by the insertion of EtBr and subsequent loosening of DNA structure resulting in the unraveling of the DNA supercoils. Z-DNA formation can cause the inhibition of DNA replication (19).

Table 1.2 Structural parameters of the three DNA duplexes (20,21)

Geometry attribute	A-DNA	B-DNA	Z-DNA
Helix Sense	Right-handed	Right-handed	Left-handed
Repeating unit	1 bp	1 bp	2 bp
Rotation/bp	32.7°	34.3°	60° /2
Bp/turn	11	10.5	12
Inclination of bp to axis	+19°	-1.2°	-9°
Rise/bp along axis	0.23 nm	0.33 nm	0.38 nm
Pitch/ turn of helix	2.82 nm	3.32 nm	4.56 nm
Mean propeller twist	+18°	+16°	0°
Glycosyl angle	anti	anti	C: anti G: syn
Sugar pucker	C3'-endo	C2'-endo	C: C2'-endo G: C2'-exo
Diameter	2.3 nm	2.0 nm	1.8 nm

Figure 1.5 Right-handed and left-handed duplexes. The figure was reproduced from <https://s3.amazonaws.com/classconnection/411/flashcards/6689411/png/righthand-14B3C099C3F025F5759.png>

Figure 1.6 Molecular models of A-DNA, B-DNA and Z-DNA. The A-form and B-form structures are right handed. The Z-form structure is left handed. The figure was

reproduced from <https://zh.wikipedia.org/wiki/Z-DNA>

1.1.3.2 G-quadruplexes

G-quadruplexes (G4-DNA) are structures which have a guanine-rich sequence. Four guanine bases can bind together by Hoogsteen hydrogen bonds to form a square planar structure (Figure 1.7). These guanine tetrads can stack on top of each other to form a G-quadruplex. Basically, a quadruplex structure can be stabilized by a cation, such as potassium, which is located in the central channel between each pair of tetrad (22). G-quadruplexes can be formed in DNA, RNA, and PNA bi-molecularly, intra-molecularly, and tetra-molecularly (23). The way of quadruplex folds depends on the length of the sequences. Short sequences with only one contiguous run of three or more guanine bases, need four individual strands to form a quadruplex. This kind of quadruplex is called tetramolecular quadruplex, meaning that it needs four separate strands to form the structure. Long sequences with two runs of three or more guanine bases, where the guanine regions are separated by one or more bases, only need two such sequences to form a quadruplex. These structures, formed between two separate G-rich strands, are called bimolecular quadruplexes. Sequences containing four runs of guanine bases can form stable quadruplex structures by themselves; these are intramolecular quadruplexes (24).

Figure 1.7 Molecular model of G-quadruplex structure. The figure was adapted from <https://en.wikipedia.org/wiki/G-quadruplex>

Previous research suggested that quadruplexes are important for immunoglobulin heavy chain switching (25). It has been reported that quadruplexes are involved in transcriptional regulation, and are related to programmed recombination of immunoglobulin heavy genes and the pilin antigenic variation system of the pathogenic *Neisseria* (26). G-quadruplex formation can be stabilized or destabilized by the binding of ligand, small molecules or proteins. There are a lot of naturally occurring proteins

that have been reported to be able to selectively bind to G-quadruplexes, such as the helicases involved in Bloom's and Werner's syndromes and the *Saccharomyces cerevisiae* protein RAP1 (27). A rule has been widely used in algorithms to predict the sequences that can form G-quadruplexes. A typical G-quadruplex-forming sequence has the pattern of $d(G_3+N_{1-7} G_3+N_{1-7} G_3+N_{1-7} G_3+)$, where N could be any base (28).

1.1.3.3 Tetraloop-receptor interactions

Tetraloop-receptor interaction is formed by a tetraloop and a receptor of an RNA duplex by hydrogen bonds and base stacking interactions; this interaction can stabilize the tertiary structure of the RNA molecule (29). Generally, a tetraloop contains four nucleotides and they can be cataloged into three families base on the sequence: CUYG, UNCG and GNRA tetraloops, where N can be any nucleotide, Y is any pyrimidine, and R is purine nucleotide (29,30). The GNRA tetraloop is the most common tetraloop found in tetraloop-receptor interactions. The first and fourth nucleotides of the tetraloop can form a base pair to stabilize the stem-loop, and the composition of this base pair and the composition of the loop bases determine the stability of the tetraloop (31). Due to the covariation of the sequences of the tetraloop and the receptor, different isoform of the tetraloop and its receptor could have the same type of tertiary contact (32).

1.1.3.4 A-minor motif (A common type of minor-groove base triple structures found in RNA)

A-minor motif is an important component of RNA structure which is formed by the interaction of an unpaired nucleotide with the minor groove of an RNA duplex. The unpaired nucleotide could be adenine, cytosine, guanosine and uridine, but adenine is the most common one. In the A-minor motif, the N1-C2-N3 edge of the inserting base can form hydrogen bonds with the 2'-OH's and the bases of the duplex (Figures 1.8 and 1.9). Normally, the host duplex is a G-C base pair. Based on the position of the inserting base relative to the two 2'-OH's of the Watson-Crick base pair, A-minor motifs can be separated into four classes (11). In the type 0 A-minor motif, the N3 of the adenine is outside the O2' of the far strand of the receptor helix which is the rarest form of the motif. In the type I A-minor motif (Figure 1.8), the inserted base has to be an A. The O2' and N3 atoms of the A are inside the receptor helix minor groove, which is the most common A-minor motif type. In the type II A-minor motif (Figure 1.9), the inserted base for this must be an A, too. The O2' of the A is outside the close strand O2' of the helix while the N3 of the A located in the minor groove. In the type III A-minor motif structure, the O2' and N3 of the residue stay outside the strand O2' of the receptor helix (33). Generally, A-minor motif interactions can stabilize the RNA duplexes' helices and loops, such as in the core of group II introns (34).

The A-minor motif is a very important RNA structural motifs in the ribosome, which

can help the binding of the cognate tRNA to the small subunit (35). In the anticodon recognition, the ribosome will insert an adenine base into the minor groove to discriminate the correct and incorrect codon-anticodon base pairs. If it is an incorrect pair, the helical geometry will be distorted which means the A-minor interaction can't stabilize the binding, subsequently, the incorrect tRNA will dissociate (36).

Figure 1.8 The structure of type I A-minor interaction (37). The figure was reproduced from https://en.wikipedia.org/wiki/Nucleic_acid_tertiary_structure#A-minor_motif

Figure 1.9 The structure of type II A-minor interaction (37). The figure was reproduced from https://en.wikipedia.org/wiki/Nucleic_acid_tertiary_structure#A-minor_motif

1.1.3.5 Ribose zipper

A ribose zipper is an RNA tertiary structural element that is formed by bifurcated hydrogen bonds between 2'-OH groups on the ribose sugars of different strands (38). In 1991, Bevilacqua and Turner found the ribose-ribose interactions are present in the structure of Group I intron of *Tetrahymena thermophila* (39). In 1996, Cate et al. crystallized the P4-P6 domain of the Group I intron from *T. thermophila* and coined the term “ribose zipper” (38). Ribose zipper elements have the following common

structural characteristics. Generally, ribose zippers formed by shared hydrogen bonds between the 2'-OH of one nucleotide and the 2'-OH and pyrimidine O2 or purine N3 of the another nucleotide (Figure 1.10) (38,40). By inserting one molecule's backbone into another molecule's minor groove, ribose zippers bring two backbone strands closer via interdigitated 2'-OH interactions (41,42). Most of the ribose zippers' two residues are in anti-parallel chains (43). Ribose zipper elements have been found in rRNA structures (43-47), ribozymes (48-52), tRNA (53), riboswitches (54,55), RNA/ligand interactions (56), and other RNA molecules (57).

Figure 1.10 The structure of a ribose Zipper (38). The figure was reproduced from https://en.wikipedia.org/wiki/Nucleic_acid_tertiary_structure#Ribose_zipper

1.1.3.6 Major and minor groove triplexes

A-minor motif is a minor-groove triple and is a common RNA structure, which is not present in DNA, because the 2'-OH groups of the ribose sugar are important to form the interactions of the A-minor motif interactions in the minor groove (see section 1.1.3.4). The minor groove allows for extensive hydrogen bonding, and hydrophobic surface burial, optimal van der Waals contacts, and can form a highly energetically favorable interaction (11). The A-minor motif triplexes are important elements in the large RNAs such as the ribosome, the group I intron (58), the group II intron (59) because the minor groove triples can pack a free helix and loop.

Pyrimidine motif major-groove triplex has been found in many DNA and RNA structures, which include Watson-Crick and Hoogsteen interactions (Figure 1.11). The red strand shown in Figure 1.11 is a polypurine strand, the blue one is a polypyrimidine strand and the yellow strand is the third polypyrimidine strand in the major groove of the duplex (60).

Figure 1.11 An example of triplex. The figure was reproduced from (60).

1.1.3.7 RNA functions

There are many types of RNA, such as transfer RNA (tRNA), messenger RNA (mRNA), ribosomal RNA (rRNA), transfer-messenger RNA (tmRNA) and small interfering RNA (siRNA). siRNA is a class of double-stranded RNA with 20-25 base pairs. siRNA can operate with the RNA interference (RNAi) pathway, where it can interfere with the expression of specific genes with complementary nucleotide sequences by degrading mRNA. Messenger RNA (mRNA) is a common RNA transcript from DNA based on the central dogma of molecular biology. The DNA serves as a template, with the help of transcription enzymes, for the synthesis of the precursor mRNA (pre-mRNA) in eukaryotes. The pre-mRNA will be processed through three main modifications: 5'

capping, 3' polyadenylation, and RNA splicing. Then it will become mature mRNA. With the help of ribosome and tRNA, the mRNA can be translated to proteins. Transfer RNA contains about 80 nucleotides which can transfer an amino acid to a polypeptide chain for the translation of the mRNA. Ribosomal RNA is a part of the ribosomes. Eukaryotic ribosomes consist of four rRNA molecules: 5S, 5.8S, 18S and 28S rRNA. The ribosomes can bind to mRNA and help protein synthesis. Most of the RNAs found in eukaryotic cells are rRNA. Transfer-messenger RNA can be found in plastids and bacteria (61). It can tag proteins encoded by mRNAs and prevents the ribosomes from stalling.

1.1.4 Triplex-forming oligonucleotide

A triplex-forming oligonucleotide (TFO) is a major groove ligand that can form Hoogsteen hydrogen bonds with nucleic acid duplexes or hairpins (Figure 1.11). TFOs are very useful in many biological processes such as controlling the gene expression, catalyzing some biological reactions, and communicating responses to cellular signals (62-65). Many DNA and RNA duplex regions are important binding sites for enzymes (66-72). Therefore, we want to have a better understanding of how the sequences and other conditions such as pH and salt affect the stability of RNA-RNA triplex and RNA-DNA triplex.

Cytosine N3 atom needs to be protonated to form two Hoogsteen hydrogen bonds with the G-C base pair (Figure 1.11), which means the lower pH is favored for the triplex structure (73-82). Thus, we designed different TFOs with RNA and DNA hairpins to form triplexes in different pH buffers, and we use UV-absorbance-detected thermal melting and 2-aminopurine fluorescence titration to measure the stabilities of the triplexes.

Cations such as spermine (Figure 1.12) and Mg^{2+} have been reported to be able to strongly stabilize triplex structures (80,83-88). Spermine has four amine groups which are protonated at physiological pH to give four positive charges. Spermine is present in many living cells and plays an important role in many cellular processes such as influencing on the cell cycle, relating the transcription and translation, modulation of kinase activities and effect on the stability of nucleic acid (89-91). In the pyrimidine·purine·pyrimidine (Y·R·Y) triplex (Figure 1.11), the third strand contains many thymines or uracils and protonated cytosines, and binds to the duplex with a parallel orientation relative to purine strand. The multivalent cations, such as magnesium and spermine can enhance the stability of the triplex structures (83,92-98). To have a better understanding of how the Mg^{2+} and spermine affect the stability of triplex, we have used UV-absorbance-detected thermal melting and 2-aminopurine fluorescence titration to measure the stabilities of the triplexes in the presence and absence of Mg^{2+} and spermine.

Natural triplexes are important for RNAs in forming complex three-dimensional architectures and many biological functions (42,99,100). For instance, triplex form in telomerase RNAs (101-106), group I introns (107-109), group II introns (34), long noncoding RNAs (110-112), -1 ribosomal frameshift-inducing mRNA pseudoknots (113-118) and metabolite-sensing riboswitches (51,119-125).

Figure 1.12 The structure of spermine.

1.2 UV-absorbance-detected thermal melting

Ultraviolet-visible spectroscopy (UV-Vis) is absorption spectroscopy in the ultraviolet and visible spectral region. The absorption in the visible range determines the color of the samples. Molecules undertake electronic transitions in this region of the electromagnetic spectrum. This technique is different from fluorescence spectroscopy because the fluorescence spectroscopy detects signals from the excited state to the ground state, but the absorption spectroscopy dealing with transitions from the ground state to the excited state.

UV-Vis spectroscopy is often used for the concentration determination of RNA and

DNA. In a melting experiment, a triplex is often disrupted in two steps by increasing the temperature (126-130). If the binding of TFO to the hairpin is tight, the two transitions may merge into one. If the reaction is in equilibrium, we can get the thermodynamic parameters, such as hairpin melting and duplex melting. In most cases, the triplex formation reaction is not in equilibrium due to the fact that the triplex forming and disruption are too slow compared to the typical temperature ramp rate (0.2 °C /min) (65).

1.3 2-Aminopurine

2-Aminopurine (2AP, see Figure 2.1), an analog of adenine, is a common fluorescent molecular marker used in nucleic acid research (131-133). The natural unmodified RNA/DNA bases (adenine, guanine, cytosine, thymine and uracil) do not display any significant fluorescence, while 2AP has significant fluorescent properties. The absorbance maximum wavelength is around 303 nm and the emission maximum wavelength is 370 nm (132-134). 2AP can be incorporated into oligonucleotides by chemical synthesis. Incorporation of 2AP residues can help us to characterize the structure, energetics, and dynamics of DNA/RNA structures by monitoring fluorescence intensity changes (135-138).

Chapter 2. Probing the effects of non-base-triple-forming bases on triplex formation

2.1 INTRODUCTION

The natural RNA-RNA₂ triplex structures with three consecutive U·A·U base triples were first discovered in the human telomerase RNA pseudoknot . The triplex structures with C⁺·G·C, U·A·U and U·G·C major-groove base triples (Figure 2.1) formed in telomerase RNA pseudoknot are highly conserved between human beings and other vertebrates, and also in ciliates and yeasts (64,139). RNA-RNA₂ triplex formation is also involved in many other functions (65,72,99,100). RNA-DNA₂ triplex has been found to be formed between microRNAs and DNA duplex for regulating gene expression (72,99,140). DNA-DNA₂ triplexes have been found to be present and involved in various biological processes (72,99,141).

Triplex-forming oligonucleotides (TFOs) are major groove ligands that can form Hoogsteen hydrogen bonds with the nucleic acid duplex regions. TFOs are very useful for the regulation of many biological processes such as controlling gene expression, catalyzing biological reactions, and sensing and communicating responses to cell signals (62-65,142-150). Many DNA and RNA duplex regions are important sites for triplex formation and protein binding (66-72). Therefore, it is important to have a better understanding of how the sequences near the triplexes (Figure 2.1 and 2.2) and other

conditions such as pH and salt concentration and composition affect the stability of nucleic acid triplexes.

Figure 2.1. (A) Chemical structures of nucleosides RNA U, DNA U, DNA T, DNA C, DNA pyrrolo C, RNA A, RNA 2-aminopurine and DNA 2-aminopurine. **(B)** Base triple structures of $C^+ \cdot G \cdot C$, $U \cdot A \cdot U$ and $U \cdot A \cdot T$.

2.2 MATERIALS AND METHODS

2.2.1 Oligonucleotides

DNA hairpins, RNA hairpins and Triplex-Forming Oligonucleotides (TFOs) with HPLC purification were purchased from Sigma-Aldrich in Singapore. All oligonucleotides were characterized by ESI-MS. The sequences of TFOs and hairpins are listed in Tables 2.1 and 2.2, respectively. A few examples of triplex structures are shown in Figure 2.2.

Table 2.1 TFO sequences studied in this thesis ^a

TFO	name	Sequences (5' - 3')													
1	rTFO-UC		U	C	U	C	U	C	U	C	U	U	U	C	
2	rTFO-5'A	A	U	C	U	C	U	C	U	C	U	U	U	C	
3	rTFO-5'G	G	U	C	U	C	U	C	U	C	U	U	U	C	
4	rTFO-5'U	U	U	C	U	C	U	C	U	C	U	U	U	C	
5	rTFO-5'2AP	P	U	C	U	C	U	C	U	C	U	U	U	C	
6	rTFO-3'A		U	C	U	C	U	C	U	C	U	U	U	C	A
7	rTFO-3'G		U	C	U	C	U	C	U	C	U	U	U	C	G
8	rTFO-5'ΔU			C	U	C	U	C	U	C	U	U	U	C	
9	rTFO-3'ΔC		U	C	U	C	U	C	U	C	U	U	U		
10	dTFO-tc		t	c	t	c	t	c	t	c	t	t	t	c	
11	dTFO-uc		u	c	u	c	u	c	u	c	u	u	u	c	
12	dTFO-5'2ap	p	u	c	u	c	u	c	u	c	u	u	u	c	
13	dTFO-5'ΔU		u	c	u	c	u	c	u	c	u	u	u		
14	rTFO-3'2AP		U	C	U	C	U	C	U	C	U	U	U	C	p
15	dTFO-3'2AP		u	c	u	c	u	c	u	c	u	u	u	c	p
16	rTFO-5'yC	y	U	C	U	C	U	C	U	C	U	U	U	C	

^a The RNA and DNA residues are shown in upper cases and lower cases, respectively.

The letters “P” and “p” represent RNA 2-aminopurine and DNA 2-aminopurine, respectively (Figure 2.1). “y” represents DNA pyrrolo C (Figure 2.1).

Table 2.2 DNA and RNA hairpin sequences studied in this thesis^a

HP	name	Sequences (5' - 3')
1	dHP	5' -tagagagagaaaagtttcgacttttctctctcta-3'
2	dHP-ΔtTA	5' - agagagagaaaagtttcgacttttctctctct -3'
3	dHP-tCG	5' -cagagagagaaaagtttcgacttttctctctctg-3'
4	dHP-ΔtTA-5'h	5' -hagagagagaaaagtttcgacttttctctctct -3'
5	rHP	5' -UAGAGAGAGAAAGUUUCGACUUUCUCUCUCUA-3'
6	rHP-ΔtUA	5' - AGAGAGAGAAAGTTTCGACTTTCTCTCTCT -3'
7	rHP-tCG	5' -CAGAGAGAGAAAGTTTCGACTTTCTCTCTCTG-3'
8	rHP-ΔtUA-5'h	5' -hAGAGAGAGAAAGTTTCGACTTTCTCTCTCT -3'

^a The RNA and DNA residues are shown in upper cases and lower cases, respectively.

The letter “h” represents 5' phosphate group (See Figure 1.4).

strand transition). Due to the hysteresis, we didn't get the equilibrium thermodynamic parameters for the triplex to hairpin transitions.

2.2.3 Dissociation constant measurement by steady-state fluorescence titration using 2-aminopurine-labeled TFOs

A Carry Eclipse fluorescence spectrophotometer was used for all 2-aminopurine steady-state fluorescence titration experiments. The buffer is 200 mM NaCl, 20 mM MES, 10 mM MgCl₂, 0.5 mM EDTA at pH 5.4, 5.6, or 6.4, or 200 mM NaCl, 20 mM MES, 10 mM MgCl₂, 0.1 mM spermine 0.5 mM EDTA at pH 6.4, or 200 mM NaCl, 20 mM MES, 0.5 mM EDTA at pH 6.4, or 200 mM NaCl, 20 mM MES, 0.1 mM spermine, 0.5 mM EDTA at pH 6.4. 80 μ L of the sample was used to take fluorescence measurements. The excitation wavelength was 303 nm. The emission spectra were taken from 330 nm to 420 nm. Each emission spectrum was scanned three times. The buffer was first measured to obtain background fluorescence. Then, 2AP labelled TFO alone sample (1 μ M) was put into fluorescence spectrometer to measure its fluorescence intensity. Finally, fluorescence emission spectra were taken for samples with different concentrations of hairpins mixed with 1 μ M 2AP labelled TFO. The fluorescence intensity of each sample was deducted by the fluorescence intensity of the buffer. The emission spectra were plotted by Origin8. The fluorescence intensity at 370 nm were plotted versus the concentration of hairpins.

The K_D values for the 2AP-labelled TFO binding to the hairpin were obtained using equation 1.

$$y = y_0 + \frac{B_{max}}{2R_0} [R_0 + x + K_D - \sqrt{(R_0 + x + K_D)^2 - 4R_0x}], \quad (1)$$

where y and y_0 refer to the fluorescence intensity with and without TFO, respectively. x refers to the concentrations of hairpin. B_{max} refers to the maximum change in fluorescence intensity upon the binding of DNA or RNA hairpins. R_0 refers to the concentration of 2AP labelled TFO (1 μ M). K_D refers to the equilibrium dissociation constant for 2AP labelled TFO and hairpins.

For the fluorescence measurement for the Pyrrolo-C labeled TFO, the excitation wavelength is 340 nm. The emission spectra were taken from 380 nm to 540 nm.

2.3 RESULTS AND DISCUSSION

2.3.1 Non-base-triple-forming nucleotides at the 3' end of an RNA TFO on the major groove can stabilize major-groove RNA-DNA₂ triplexes

We designed a series of TFOs and DNA/RNA hairpins to study how the nucleotides adjacent to a triplex affect the melting temperatures of a triplex (T_{ml}). The thermal melting data clearly suggest that removing a nucleotide on the 3' end of 12-nucleotide RNA TFO causes significant decrease of the T_{ml} of the triplexes (with the decrease of the T_{ml} values ranging from 4-16 °C, see Figure 2.3A-F). Thus, a base triple at the 3'

end of a TFO is critical in stabilizing RNA-DNA₂ and RNA-RNA₂ triplexes.

In the DNA/RNA hairpins studied here, a Watson-Crick T-A or U-A pair is located adjacent to the 3' end of a 12-nucleotide TFO. The effect of adding a nucleotide on the 3' end of the 12-nucleotide TFO (opposite to the T-A or U-A pair) is quite TFO sequence and hairpin dependent. The T_{ml} of dHP + rTFO-3'A (42.4 °C) is only slightly higher than the T_{ml} of dHP + rTFO-UC (41.5 °C). However, the T_{ml} of dHP + rTFO-3'G (45.0 °C) is much higher than the T_{ml} of dHP + rTFO-UC (41.5 °C) (Figure 2.3A). Similarly, rTFO-3'A (37.8 °C) and rTFO-3'G (39.3 °C) have a higher T_{ml} than the rTFO-UC (36.4 °C) with dHP-ΔtTA (Table 2.3). The results suggest that extending a TFO at the 3' end can generally stabilize RNA-DNA₂ triplexes.

For RNA-RNA₂ triplexes, the T_{ml} of rHP + rTFO-3'A (44.5 °C) is 4 °C lower than the T_{ml} of rHP + rTFO-UC (49.6 °C) (Figure S1). The T_{ml} of dHP + rTFO-3'G (51.1 °C), however, is slightly higher than the T_{ml} of rHP + rTFO-UC (Table 2.3). As expected, a similar trend was observed for the T_{ml} values of the three TFOs with rHP-ΔtUA (48.5 °C, 47.8 °C, and 50.5 °C for rTFO-UC, rTFO-3'A, and rTFO-3'G, respectively, see Table 2.3). It is possible that the added G residue at the 3' end can form a non-canonical G·U-A major-groove base triple and/or stacking interactions with the adjacent base in the TFO, and thus stabilize the RNA-RNA₂ triplexes. The added A residue at the 3' end, however, may not form favorable hydrogen bonding and/or stacking interactions in the RNA-RNA₂ triplexes.

2.3.2 Non-base-triple-forming nucleotides adjacent to the 5' end of a TFO can affect triplex formation

2.3.2.1 Adding a non-base-triple-forming base at the 5' end of a TFO can destabilize major-groove RNA-DNA₂ and DNA-DNA₂ triplexes

A series of TFOs (rTFO-UC, rTFO-5'A, rTFO-5'G, rTFO-5'U, rTFO-5'2AP, and rTFO-5'ΔU) and hairpins (dHP, dHP-ΔtTA, dHP-ΔtTA-5'h, dHP-tCG, rHP, rHP-ΔtUA, and rHP-ΔtUA-5'h) were designed to investigate the effects of the nucleotides adjacent to the 5' end of a TFO on the triplex formation (Figure 2.2). All the results are listed in Table 2.3. The T_{ml} of dHP + rTFO-5'A (39.7 °C) and dHP + rTFO-5'G (39.5 °C) and dHP + rTFO-5'U (40.0 °C) and dHP + rTFO-5'2AP (40.1 °C) are slightly lower than the T_{ml} of dHP + rTFO-UC (41.4 °C) (Figure 2.4) probably because the addition of the non-base-triple-forming nucleotides on the 5' end of the TFO does not give rise to favorable hydrogen bonding and stacking interactions for the RNA-DNA₂ triplex formation. The small destabilization effect is not due to the steric clash with the 5-methyl group of T base, because the similar effect is observed for DNA hairpin dHP-tCG in binding to the TFOs with variations at the 5' end (Table 2.3).

However, we didn't observe the same destabilizing effect in the rHP binding to rTFOs to form RNA-RNA₂ triplexes. The T_{ml} of rHP + rTFO-5'A (52.9 °C) is higher than the T_{ml} of rHP + rTFO-UC (49.5 °C), while the T_{ml} values of the rest TFOs are lower than rTFO-UC in binding to rHP (Figure 2.4C, Table 2.3). The results may suggest that a non-canonical A·U-A major-groove triple may form at the 5' end of the TFO, resulting in stabilization of the RNA-RNA₂ triplex. For the DNA-DNA₂ triplexes, the T_{ml} of dHP + dTFO-uc (31.4 °C) is similar to the T_{ml} of dHP + dTFO-5'2ap (30.5 °C) (Figure 2.4B).

As expected, the T_{ml} of dHP + rTFO-5'ΔU is only 35.6 °C (compared to a T_{ml} of 41.4 °C for dHP + rTFO-UC), presumably because there are 11 and 12 canonical base triples formed for rTFO-5'ΔU and rTFO-UC, respectively (Table 2.3). A similar destabilization effect is observed for DNA-DNA₂ triplexes. Surprisingly, only a small destabilization effect of removing a canonical U·A-U triple by removing the 5' terminal U residue in the TFO is observed for RNA-RNA₂ triplexes, rTFO-5'ΔU (48.8 °C) versus rTFO-UC (49.5 °C) in binding to rHP (Table 2.3).

2.3.2.2 A dangling DNA base pair adjacent to the 5' end of a TFO stabilizes major-groove RNA-DNA₂ and DNA-DNA₂ triplexes

The T_{ml} of dHP-ΔtTA + rTFO-UC (36.6 °C) is much lower than the T_{ml} of dHP + rTFO-UC (41.4 °C) (Table 2.3, figure 2.4A) even though the terminal T-A pair in the hairpin dHP is not involved in base-base hydrogen bonding interactions with the TFO. The

significant destabilization effect is probably due to the fact that a potential hydrogen bond formed between the 2'-OH group in 5' end of the TFO and a non-bridging oxygen in the hairpin (Figure 2.5) is disrupted upon removing the terminal T-A pair in the hairpin.

It is surprising that the T_{m1} of dHP- Δ tTA + rTFO-5' Δ U (36.7 °C) is the same as the T_{m1} of dHP- Δ tTA + rTFO-UC (36.6 °C), even though the former triplex has 11 base triples instead of 12 base triples in the latter triplex (Table 2.3, figure 2.4 A). The result indicates that a blunt-end terminal base triple at the 5' end of the TFO does not significantly stabilize a RNA-DNA₂ triplex probably due to the absence of the backbone-backbone hydrogen bond (formed between 2'-OH group in 5' end of the TFO and a non-bridging oxygen in the hairpin) as discussed above (Figure 2.5). Similar to RNA-DNA₂ triplexes, in the DNA-DNA₂ triplexes, the T_{m1} of dHP- Δ tTA + dTFO-uc (28.5 °C) is lower than the T_{m1} of dHP + dTFO-uc (31.4 °C) (Table 2.3, figure 2.4B), although the 2'-OH group is absent in the DNA TFO. It is important to note that one may stabilize a major-groove triplex with a DNA Watson-Crick duplex by simply extending the DNA Watson-Crick duplex adjacent to the 5' end of the TFO without even adding a base triple.

Surprisingly, we didn't observe in RNA-RNA₂ triplexes the stabilizing effect of a non-base-triple-forming Watson-Crick pair. The T_{m1} of rHP- Δ tUA + rTFO-UC (48.4 °C) is only slightly lower than the T_{m1} of rHP + rTFO-UC (49.5 °C) (Table 2.3, figure 2.4C)

suggesting that the backbone-backbone hydrogen bond (formed between 2'-OH group in 5' end of the TFO and a non-bridging oxygen in the hairpin) at the 5' end of the TFO is not as stabilizing in RNA-RNA₂ triplexes.

It was reported that adding a 5' phosphate in the purine strand in a DNA-DNA₂ triplex destabilizes the triplex (152). In this study, we found that a 5' phosphate can destabilize DNA-DNA₂, RNA-DNA₂, RNA-RNA₂ triplexes (Table 2.3). The most significant destabilizing effect is observed for RNA-RNA₂ triplexes, with the T_{m1} decreasing by about 10 °C upon the addition of a phosphate group (Figure 2.4).

2.3.3 Triplexes forming can enhance the fluorescence intensity of DNA pyrrolo C

Pyrrolo-C is a fluorescent C derivative and has been used to monitor RNA duplex and tertiary structure formation (153). We observe that the fluorescence intensity of DNA Pyrrolo C can be enhanced by dHP, dHP- Δ tTA, dHP-tCG, rHP, rHP- Δ tUA and rHP-tCG. While binding of dHP- Δ tTA-5'h does not affect the pyrrolo-C fluorescence. The fluorescence enhancement of pyrrolo-C upon binding to hairpins indicates that pyrrolo-C base is less stacked in triplexes than in single-stranded form. Due to the fact that the hairpins have intrinsic fluorescence when excited at 340 nm, we didn't obtain K_D values by titrating hairpins into pyrrolo-C labelled TFO.

Figure 2.3 Thermal melting curves of triplexes. The buffer for the triplexes containing dHPs is 200 mM NaCl, 20 mM MES, 10 mM MgCl₂, 0.5 mM EDTA, pH 6.4. The

buffer for triplexes containing rHPs is 200 mM NaCl, 20 mM MES, 10 mM MgCl₂, 0.5 mM EDTA, pH 5.4.

Figure 2.4. Effects of non-base-triple-forming nucleotides adjacent to the 5' end of a TFO on triplex formation. The values on the top and bottom are mean and standard

deviation values, respectively. **(A)** RNA-DNA₂ triplexes. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 6.4. **(B)** DNA-DNA₂ triplexes. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 6.4. **(C)** RNA-RNA₂ triplexes. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 5.4.

Figure 2.5 **(A)** Two U·A·U RNA-RNA₂ base-triples. There is a backbone-backbone hydrogen bond formed between 2'-OH in TFO and phosphate oxygen in purine strand. **(B)** Two U·A·T RNA-DNA₂ base-triples. There is a backbone-backbone hydrogen bond formed between 2'-OH in TFO and phosphate oxygen in purine strand. **(C)** Two T·A·T DNA-DNA₂ base-triples.

	rTFO- UC	rTFO- -5'A	rTFO- -5'G	rTFO- -5'U	rTFO- 5'2AP	rTFO- 5'ΔU	rTFO- 3'A	rTFO- - 3'G	rTFO- 3'2ap	rTFO- 3'ΔC	dTFO- tc	dTFO- uc	dTFO- 5'2ap	dTFO- 3'2ap	dTFO- 5'ΔU
dHP	41.4	39.7	39.5	40.0	40.1	35.6	42.3	44.7	42.0	32.5	33.0	31.4	30.5	31.0	25.5
	0.3	0.5	1.0	0.5	1.0	0.7	0.5	0.8	0.6	1.1	0.1	0.3	0.5	0.1	0.3
dHP- ΔtTA	36.6	38.2	36.1	36.3	38.4	36.7	37.5	39.4	39.0	29.1	31.0	28.5	32.7	28.7	27.6
	0.5	0.5	0.9	0.2	0.7	0.5	0.5	0.5	0.2	0.2	0.1	0.5	0.5	0.1	0.1
dHP- tCG	43.0	41.4	40.7	41.9	41.3	36.4	43.0	46.0	44.0	33.2	33.7	32.3	32.4	32.1	25.5
	0.4	0.3	0.7	0.5	0.8	1.3	0.6	0.5	0.4	0.2	0.1	0.4	0.2	0.1	0.3
dHP- ΔtTA -5'h	36.0	37.1	35.3	35.4	36.7	34.7	36.8	38.8	35.8	29.7	29.7	27.6	29.4	26.4	26.8
	0.2	0.1	0.2	0.3	0.1	0.2	0.2	0.4	0.3	0.5	0.3	0.1	0.2	0.3	0.1
rHP	49.5	52.9	48.3	47.8	47.3	48.8	45.2	51.1	42.6	35.0	<20	<20	<20	<20	<20
	0.1	1.3	0.3	1.1	0.6	0.7	0.6	0.5	0.1	0.6					
rHP- ΔtUA	48.4	52.7	50.0	50.2	50.7	50.2	48.1	51.0	40.8	35.4	<20	<20	<20	<20	<20
	0.2	0.7	0.2	1.4	0.9	0.9	0.6	0.9	0.5	0.7					
rHP- ΔtUA -5'h	38.8	42.5	40.6	38.1	42.0	40.0	38.0	41.1	37.2	32.1	-	-	-	-	-
	0.6	0.3	0.3	0.7	0.3	0.5	0.4	0.2	0.3	0.5					

Table 2.3 T_{ml} (°C) of the triplexes studied^a

The buffer for dHPs is 200 mM NaCl, 20 mM MES, 10 mM MgCl₂, 0.5 mM EDTA, pH 6.4. The buffer for rHPs is 200 mM NaCl, 20 mM MES, 10 mM MgCl₂, 0.5 mM EDTA, pH 5.4. The values on the top and bottom are mean and standard deviation values, respectively. “-” means the data were not measured.

Figure 2.6 Fluorescence of DNA Pyrrolo C labelled TFO (2 μ M) and the effect of DNA/RNA hairpins (2 μ M) binding. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 5.6.

2.3.4 Dissociation constant measurement by using TFOs with 5' 2-aminopurine steady-state fluorescence titration

We designed dTFO-uc-5'2ap and rTFO-5'2AP, with the DNA 2-aminopurine and RNA 2-aminopurine located at the 5' ends of the TFOs, respectively, to investigate the formation of DNA-DNA₂, RNA-DNA₂ and RNA-RNA₂ triplexes by steady-state fluorescence titration and to correlate our thermal melting data. The thermal melting data suggest that adding a 2-aminopurine residue at the 5' end of a TFO on the major groove destabilizes RNA-DNA₂ and DNA-DNA₂ triplexes. Our fluorescence titration data suggest that dHP binding to dTFO-5'2ap enhances the 2-aminopurine fluorescence intensity (Figure 2.7A). The fact that the fluorescence intensity of the 2-aminopurine is enhanced compared to single-stranded TFO suggests that the 2-aminopurine base

becomes less stacked upon the formation of both triplexes. The intrinsic fluorescence of hairpins is negligible (Figure S3). We can obtain a two-state titration curve (Figure 2.7E) with a K_D of $1.1 \pm 0.1 \mu\text{M}$ in the buffer of 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl_2 , pH 6.4. The titration of dHP-tCG with dTFO-5'2ap in the same buffer shows the similar fluorescence enhancement with a K_D of $1.2 \pm 0.2 \mu\text{M}$. Consistently, the T_{m1} values are similar for the two triplexes (30.5 °C for dHP and 32.4 °C for dHP-tCG), which are also similar to the T_{m1} values for dTFO-uc binding to dHP (31.4 °C) and dHP-tCG (32.3 °C). Thus, both non-base-triple-forming T-A and C-G base pairs show no significant interaction with the 2-aminopurine residue in the DNA TFO.

Our thermal melting data suggest that dTFO-5'2ap can bind to dHP- ΔtTA and dHP- ΔtTA -5'h to form triplexes ($T_{\text{m1}} = 32.7 \text{ °C}$ and 29.4 °C , respectively). However, no significant fluorescence intensity change was observed when we titrate dHP- ΔtTA and dHP- ΔtTA -5'h into dTFO-5'2ap (Figure 2.7B,F). It is probable that, in the absence of a terminal T-A or C-G base pair, the 2-aminopurine residue at the 5' end of the DNA TFO in the DNA-DNA₂ triplex experiences a local environment similar to that of a single-stranded TFO. The fact that, compared to dTFO-uc ($T_{\text{m1}} = 28.5 \text{ °C}$), dTFO-5'2ap ($T_{\text{m1}} = 32.7 \text{ °C}$) can form a relatively more stable triplex with dHP- ΔtTA suggests that the 5' terminal 2-aminopurine residue in the TFO may be pre-organized in binding to dHP- ΔtTA .

We observed the similar fluorescence enhancement for the RNA-DNA₂ and RNA-RNA₂ triplexes (Figures 2.8E,G and 2.9E,G). No significant fluorescence intensity change was observed upon the titration of dHP- Δ tTA, dHP- Δ tTA-5'h, rHP- Δ tUA and rHP- Δ tUA-5'h into rTFO-5'2AP (Figures 2.8B,F and 2.9B,F), although the thermal melting data suggest that the corresponding triplexes can form at the condition tested.

2.3.5 Dissociation constant measurement by using TFOs with 3' 2-aminopurine steady-state fluorescence titration

We also attached a DNA 2-aminopurine residue at the 3' ends of rTFO-UC and dTFO-uc, respectively, for the monitoring of triplex formation, and the effects of dangling Watson-Crick pairs and 5'phosphate on the purine strand.

Figure 2.7 Binding of dTFO-uc-5'2ap to DNA hairpins to form DNA-DNA₂ triplexes at pH 6.4 revealed by fluorescence enhancement. The buffer is 200 mM NaCl, 20 mM

MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 6.4. **(A,E)** Fluorescence titration of dHP with dTFO-5'2ap. The K_D is 1.1 μ M. **(B,F)** Fluorescence titration of dHP- Δ tTA with dTFO-5'2ap. There is no significant fluorescence change upon the titration of dHP- Δ tTA. **(C,G)** Fluorescence titration of dHP-tCG with dTFO-5'2ap. The K_D is 1.2 μ M, which is very close to the K_D for dTFO-5'2ap binding to dHP (1.1 μ M). **(D,H)** Fluorescence titration of dHP- Δ tTA-5'h with dTFO-5'2ap. There is no significant fluorescence change upon the titration of dHP- Δ tTA-5'h.

Figure 2.8 Binding of rTFO-5'2AP to DNA hairpins to form RNA-DNA₂ triplexes at pH 6.8 revealed by fluorescence enhancement. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 6.8. **(A,E)** Steady-state fluorescence titration of dHP with rTFO-5'2AP. The K_D is 5.0 μ M. **(B,F)** Steady-state fluorescence titration of dHP- Δ tTA with rTFO-5'2AP, there is no significant fluorescence change. **(C,G)** Steady-state fluorescence titration of dHP-tCG with rTFO-5'2AP. The K_D is 3.4 μ M, which is similar to dHP (5.0 μ M), probably because both dHP-tCG and dHP have a

dangling Watson-Crick pair for the triplex formation. **(D,H)** Steady-state fluorescence titration of dHP- Δ tTA-5'h with rTFO-5'2AP, there is no significant fluorescence change. The melting data is done in buffer: 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 6.4.

Figure 2.9 Binding of rTFO-5'2AP to RNA hairpins to form RNA-RNA₂ triplexes at pH 5.8 revealed by fluorescence enhancement. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 5.8. **(A,E)** Steady-state fluorescence titration of rHP with rTFO-5'2AP. The K_D is 1.2 μ M. **(B,F)** Steady-state fluorescence titration of rHP- Δ tUA with rTFO-5'2AP. There is no significant fluorescence change upon the titration of rHP- Δ tUA. **(C,G)** Steady-state fluorescence titration of rHP-tCG with rTFO-5'2AP. The K_D is 1.3 μ M, which is similar to rHP binding to rTFO-5'2AP (1.2 μ M). **(D,H)** Steady-state fluorescence titration of rHP- Δ tUA-5'h with rTFO-5'2AP. There is no significant fluorescence change upon the titration of rHP- Δ tUA-5'h. The

melting data is done in buffer: 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 5.4.

Figure 2.10 Binding of dTFO-3'2ap to DNA hairpins to form DNA-DNA₂ triplexes at pH 6.4 revealed by fluorescence enhancement. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 6.4. **(A,E)** Steady-state fluorescence titration of dHP with dTFO-3'2ap. The K_D is 1.1 μM. **(B,F)** Steady-state fluorescence titration of dHP-ΔtTA with dTFO-3'2ap. The K_D is 2.5 μM. **(C,G)** Steady-state fluorescence titration of dHP-tCG with dTFO-3'2ap. The K_D is 1.0 μM, which is similar to dHP binding to dTFO-3'2ap (1.1 μM). **(D,H)** Steady-state fluorescence titration of dHP-ΔtTA-5'ph with dTFO-3'2ap. The K_D is 4.1 μM.

Figure 2.11 Binding of rTFO-3'2ap to DNA hairpins to form RNA-DNA₂ triplexes at pH 6.8 revealed by fluorescence enhancement. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 6.8. **(A,E)** Steady-state fluorescence titration of dHP with rTFO-3'2ap. The K_D is 3.1 μM . **(B,F)** Steady-state fluorescence titration of dHP- ΔtTA with rTFO-3'2ap. The K_D is $> 5 \mu\text{M}$. **(C,G)** Steady-state fluorescence titration of dHP-tCG with rTFO-3'2ap. The K_D is 3.9 μM , which is similar to dHP binding to rTFO-3'2ap (3.1 μM). **(D,H)** Steady-state fluorescence titration of dHP- ΔtTA -5'ph with rTFO-3'2ap. The K_D is $> 5 \mu\text{M}$. The melting data is done in buffer: 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 6.4.

Figure 2.12 Binding of rTFO-3'2ap to RNA hairpins to form RNA-RNA₂ triplexes at pH 5.8 revealed by fluorescence enhancement. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 5.8. **(A,E)** Steady-state fluorescence titration of rHP with rTFO-3'2ap. The K_D is 1.1 μM. **(B,F)** Steady-state fluorescence titration of rHP-ΔtUA with rTFO-3'2ap. The K_D is 0.4 μM. **(C,G)** Steady-state fluorescence titration of rHP-tCG with rTFO-3'2ap. The K_D is 1.3 μM, which is similar to rHP binding to rTFO-3'2ap (1.1 μM). **(D,H)** Steady-state fluorescence titration of rHP-ΔtUA-5'ph with rTFO-3'2ap. The K_D is 6.2 μM. The melting data is done in buffer: 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 5.4.

2.3.6 Effects of pH, Mg^{2+} and spermine on triplex formation

Cytosine N3 atom needs to be protonated (Figure 2.1B) to form two Hoogsteen hydrogen bonds with a Watson-Crick G-C base pair (Figure 2.1B), which means low pH favors the triplex formation (73-82,129,154). Thus, we used 2-aminopurine fluorescence titration to measure the stabilities of the triplexes at varied pH. Multivalent cations such as Mg^{2+} and spermine are known to strongly stabilize triplex structures mainly through non-specific electrostatic effect (80,83-88,129,155) (83,92-98,156-162). To have a better understanding of how the Mg^{2+} and spermine affect the stability of triplex, we have used 2-aminopurine fluorescence titration to measure the stabilities of the triplexes in the presence and absence of Mg^{2+} and spermine.

For the DNA-DNA₂ triplex formed between dTFO-5'2ap and dHP, we obtained the K_D values of 1.1 and 4.7 μ M, respectively, at pH 6.4 and 6.6 (Figure 2.13D,E). Consistently, the T_{m1} values are 30.5 and <20 °C, respectively, at pH 6.4 and 6.6 (Figure 2.13D,E and Appendix Table S1). Thus, the fluorescence titration method is useful for studying the effect of pH on the triplex formation.

We used the 2-aminopurine fluorescence titration method to study the effect of pH on the triplex formation between rTFO-5'2AP and dHP and rHP to form RNA-DNA₂ and RNA-RNA₂ triplexes, respectively (Figures 2.14 and 2.15). As expected, both fluorescence titration and thermal melting data reveal the trend that increasing the pH

causes the destabilization of the RNA-DNA₂ and RNA-RNA₂ triplexes (Figures 2.14 and 2.15 and Appendix Table S1).

The fluorescence titration method was used for investigating the effects of Mg²⁺ and spermine on triplex formation. The K_D of dHP binding to dTFO-5'2ap in the NaCl only buffer (without MgCl₂ and spermine) is 5.0 μ M with a T_{ml} of 24.1 $^{\circ}$ C (Figure 2.16E). In the buffers with the addition of 0.1 mM spermine and 10 mM MgCl₂, we observed decreased K_D values of 2.2 μ M and 1.1 μ M, and increased T_{ml} values of 26.4 $^{\circ}$ C and 27.5 $^{\circ}$ C, respectively (Figure 2.16F,G). A correlation was observed between T_{ml} and K_D (Figure 2.17). Thus, the fluorescence titration experiments can be used to reveal the stabilization effect of MgCl₂ and spermine on the triplex formation (157,163-167).

Figure 2.13 Effect of pH on the binding of dTFO-5'2ap to dHP revealed by fluorescence titration. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂. (A,B,C) The emission spectra of steady-state fluorescence titration of dHP-1 with dTFO-5'2ap in different buffers (pH 6.4, 6.6, 6.8). (D,E,F) Fluorescence

intensity at 370 nm versus the concentrations of dHP (μM) in different buffers (pH 6.4, 6.6, 6.8). In panel F, the binding saturation is not reached. Thus, we only estimate the K_D to be $> 50 \mu\text{M}$.

Figure 2.14 Effect of pH on the binding of rTFO-5'2AP to dHP revealed by fluorescence titration. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl_2 . (A,B,C) Steady-state fluorescence titration of dHP with rTFO-5'2AP in different buffers (pH 6.4, 6.6, 6.8). (D) Fluorescence intensity at 370 nm versus the concentrations of dHP (μM) in pH 6.4. The binding dissociation constant is significantly lower than the concentration of rTFO-5'2AP. Thus, we only estimate the K_D to be $< 1 \mu\text{M}$. (E) Fluorescence intensity at 370 nm versus the concentrations of dHP (μM) in pH 6.6. The K_D is $0.8 \mu\text{M}$. (F) Fluorescence intensity at 370 nm versus the concentrations of dHP (μM) in pH 6.8. The K_D is $5.0 \mu\text{M}$.

Figure 2.15 Effect of pH on the binding of rTFO-5'2AP to rHP revealed by fluorescence titration. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl_2 . **(A-C)** The emission spectra of steady-state fluorescence titration of rHP with rTFO-5'2AP in different buffers (pH 5.8, 6.0 and 6.2). **(D)** Fluorescence intensity at 370 nm versus the concentrations of rHP (μM) in pH 5.8. The K_D is 1.2 μM . **(E)** Fluorescence intensity at 370 nm versus the concentrations of rHP (μM) in pH 6.0. The K_D is $> 5 \mu\text{M}$. **(F)** Fluorescence intensity at 370 nm versus the concentrations of rHP (μM) in pH 6.2. The K_D is $> 10 \mu\text{M}$.

Figure 2.16 Stabilization of triplexes by Mg^{2+} and spermine revealed by fluorescence titration. “NaCl only” buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, pH 6.4. “NaCl + Spermine” contains 0.1 mM spermine. “NaCl + MgCl_2 ” contains 10 mM MgCl_2 . “NaCl + MgCl_2 + spermine” contains both 0.1 mM spermine and 10 mM MgCl_2 . **(A,E)** The emission spectra and the fitting curves of steady-state fluorescence titration of dHP with dTFO-uc-5'2ap in the buffer without MgCl_2 or spermine. **(B,F)** The emission spectra and the fitting curves of steady-state fluorescence titration of dHP with dTFO-uc-5'2ap in the buffer with 0.1 mM spermine. **(C,G)** The emission spectra and the fitting curves of steady-state fluorescence titration of dHP with dTFO-uc-5'2ap in the buffer with 10 mM Mg^{2+} . **(D,H)** The emission spectra and the fitting curves of steady-state fluorescence titration of dHP with dTFO-uc-5'2ap in the buffer with 0.1 mM spermine and 10 mM MgCl_2 .

Figure 2.17 T_m versus K_D of different salt buffers. “NaCl only” buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, pH 6.4. “NaCl + Spermine” contains 0.1 mM spermine. “NaCl + MgCl₂” contains 10 mM MgCl₂. “NaCl + MgCl₂ + spermine” contains both 0.1 mM spermine and 10 mM MgCl₂.

2.4 CONCLUSIONS

In this study, we have found that non-base-triple-forming nucleotides can significantly enhance or weaken the stability of a triplex. For example, dHP with rTFO-UC is significantly more stable than the dHP-ΔtTA with rTFO-UC (T_{ml} difference of about 5 °C) even though 12 base triples are formed in these two triplexes. The energetic difference might be due to the fact that RNA-DNA₂ triplexes have backbone-backbone hydrogen bonds formed between 2'-OH in TFO and phosphate oxygen in purine strand

(168). Adding a 5' phosphate group adjacent to the 5' end of a TFO significantly destabilizes the triplex formation, suggesting that ^{32}P labeling may be used carefully in studying nucleic triplex formation (152). Furthermore, we have successfully developed a method of using the 2-aminopurine fluorescence titration to measure the K_D of the triplex forming reaction and study the effects of pH, MgCl_2 and spermine concentration on the triplex formation. Our results provide insights into how the triplex sequence and environmental factors may affect the stabilities of triplexes with varied compositions. Better understanding the factors affecting nucleic acid triplex structures may help develop an accurate method for the search for novel functional triplexes present in nature, and develop highly stable artificial triplex structures for biological applications (169).

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Supplementary Data

Probing the effects of non-base-triple-forming nucleotides on triplex formation

Triplexes samples of thermal melting were prepared as follows.

1. RNA/DNA hairpins dissolved in a buffer in 1.5 mL micro-tube, then heated by a heating block for 5 minutes at 95 °C.
2. The tube was then transferred into a 4 °C refrigerator for 10 minutes in order to cool down fast.
3. The dried triplex-forming oligonucleotide was dissolved in the same buffer and mixed with the RNA hairpin in the micro-tube, the final concentrations of the RNA hairpin and TFO were 2 µM.
4. The 1.5 mL micro-tube with 2 µM triplex was heated in the metal heating block for 5 minutes at 65 °C, then the heat block was powered off and the tube was left in the heat block until the temperature reaches room temperature (about 26 °C), and then the tube was kept in a 4 °C refrigerator overnight in order to form stable triplex structure.

Triplexes samples of fluorescence titration were prepared as follows.

- 1 The DNA and RNA hairpins were prepared at a total of 12 different concentrations, which are 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50 μM . All samples were put in an Eppendorf concentrator 5301 to remove the water in the stocks.
- 2 The DNA and RNA hairpins were dissolved in a buffer in 1.5 mL micro-tubes, then heated by a heating block for 5 minutes at 95 $^{\circ}\text{C}$.
- 3 The tubes were then transferred into a 4 $^{\circ}\text{C}$ refrigerator for 10 minutes in order to cool down fast.
- 4 The dried 2AP labelled TFO was dissolved in the same buffer and mixed with the DNA hairpins in the micro-tube, the final concentrations of the 2AP labelled TFO was 1 μM .
- 5 The 1.5 mL micro-tubes with triplex were heated in the metal heating block for 5 minutes at 65 $^{\circ}\text{C}$, then the heat block was powered off and the tube was left in the heat block until the temperature reach room temperature (about 26 $^{\circ}\text{C}$), and then the tube was kept in a 4 $^{\circ}\text{C}$ refrigerator overnight in order to form stable triplex structure.

Table S1. Melting temperatures of triplexes (T_{m1}) and hairpins (T_{m2}) and K_D obtained by fluorescence titration. All the buffers contain 200 mM NaCl, x mM MES and x.x mM EDTA. The melting transition with a melting temperature of $T_{m,3}$ may be due to the disruption of the top part (A-U pair rich region) of the RNA hairpin.

TFO	Hairpin	pH	[MgCl ₂] (mM)	[Spermin e] (mM)	Tm1(°C)	Tm2(°C)	KD (μM)
No TFO	dHP	7.0	10	0	NA	76.5	
No TFO	dHP	6.8	10	0	NA	75.8	
No TFO	dHP	6.6	10	0	NA	75.3	
No TFO	dHP	6.4	0	0	NA	74	
No TFO	dHP	6.4	10	0	NA	76.5	
No TFO	dHP	6.4	0	0.1	NA	74.5	
No TFO	dHP	6.4	10	0.1	NA	76.6	
No TFO	dHP	6.2	10	0	NA	75.6	
No TFO	dHP	6.0	10	0	NA	75.1	
No TFO	dHP	5.8	10	0	NA	76.1	
No TFO	dHP	5.6	10	0	NA	75	
No TFO	dHP	5.4	10	0	NA	73.5	
No TFO	dHP-ΔtTA	6.4	10	0	NA	75.2	
No TFO	dHP-tCG	6.4	10	0	NA	75.9	
No TFO	rHP	7.0	10	0	NA	88.4	
No TFO	rHP	6.8	10	0	NA	88	
No TFO	rHP	6.6	10	0	NA	87.9	
No TFO	rHP	6.4	0	0	NA	84.2	
No TFO	rHP	6.4	10	0	NA	88.5	
No TFO	rHP	6.4	0	0.1	NA	87.4	
No TFO	rHP	6.4	10	0.1	NA	88.9	
No TFO	rHP	6.2	10	0	NA	87.9	
No TFO	rHP	6.0	10	0	NA	87.5	
No TFO	rHP	5.8	10	0	NA	88.1	
No TFO	rHP	5.6	10	0	NA	87.2	
No TFO	rHP	5.4	0	0	NA	82.3	
No TFO	rHP	5.4	10	0	NA	86.2	
No TFO	rHP	5.4	0	0.1	NA	87.1	
No TFO	rHP	5.4	10	0.1	NA	86.8	
No TFO	rHP-ΔtTA	5.4	10	0	NA	86.3	
No TFO	rHP-tCG	5.4	10	0	NA		
rTFO-UC	dHP	7.0	10	0	20.9	77	
rTFO-UC	dHP	6.8	10	0	25.7	76.3	
rTFO-UC	dHP	6.6	10	0	30.3	75.5	
rTFO-UC	dHP	6.4	0	0	29.9	74.1	
rTFO-UC	dHP	6.4	10	0	41.4 (0.3)	76.7	
rTFO-UC	dHP	6.4	0	0.1	36.4	74.9	
rTFO-UC	dHP	6.4	10	0.1	39.7	76.8	
rTFO-UC	dHP	6.2	10	0	45.4	76.1	

rTFO-UC	dHP	6.0	10	0	49.6	75.2	
rTFO-UC	dHP	5.8	10	0	58	76.1	
rTFO-UC	dHP	5.6	10	0	About 70	74.6	
rTFO-UC	dHP	5.4	10	0	Overlap with hairpin	74.2	
rTFO-UC	dHP-ΔtTA	6.4	10	0	36.6 (0.5)	75.3	
rTFO-UC	dHP-tCG	6.4	10	0	43.0 (0.4)	76.4	
rTFO-UC	dHP-ΔtTA-5'h	6.4	10	0	36.0 (0.2)		
rTFO-UC	rHP	7.0	10	0	< 20	88.7	
rTFO-UC	rHP	6.8	10	0	< 20	88.4	
rTFO-UC	rHP	6.6	10	0	< 20	87.9	
rTFO-UC	rHP	6.4	0	0	< 20	84.2	
rTFO-UC	rHP	6.4	10	0	< 20	88.7	
rTFO-UC	rHP	6.4	0	0.1	< 20	87.7	
rTFO-UC	rHP	6.4	10	0.1	< 20	89	
rTFO-UC	rHP	6.2	10	0	< 20	88.2	
rTFO-UC	rHP	6.0	10	0	24.7	87.4	
rTFO-UC	rHP	5.8	10	0	27.9	88.3	
rTFO-UC	rHP	5.6	10	0	33.5	87.7	
rTFO-UC	rHP	5.4	0	0	42.2	82.6	
rTFO-UC	rHP	5.4	10	0	49.5 (0.1)	86.2	
rTFO-UC	rHP	5.4	0	0.1	49.5	87.3	
rTFO-UC	rHP	5.4	10	0.1	40.8	87.4	
rTFO-UC	rHP-ΔtUA	5.4	10	0	48.4 (0.2)	85.6	
rTFO-UC	rHP-ΔtUA-5'h	5.4	10	0	38.8 (0.6)		
dTFO-5'2ap	dHP	7.0	10	0	< 20	75.8	>50
dTFO-5'2ap	dHP	6.8	10	0	< 20	75.8	>50
dTFO-5'2ap	dHP	6.6	10	0	< 20	76.4	4.7 ± 0.4
dTFO-5'2ap	dHP	6.4	0	0	24.1	74.1	5.0 ± 0.3
dTFO-5'2ap	dHP	6.4	10	0	30.5 (0.5)	77	1.1 ± 0.1
dTFO-5'2ap	dHP	6.4	0	0.1	26.4	74.1	2.2 ± 0.1
dTFO-5'2ap	dHP	6.4	10	0.1	27.3	75.9	1.6 ± 0.2
dTFO-5'2ap	dHP	6.2	10	0	33.7	76.7	<1
dTFO-5'2ap	dHP	6.0	10	0	41.5	76.3	
dTFO-5'2ap	dHP	5.8	10	0	44.2	75.6	
dTFO-5'2ap	dHP	5.6	10	0	54	75	
dTFO-5'2ap	dHP	5.4	10	0	About 65	73.3	<1
dTFO-5'2ap	dHP-ΔtTA	6.4	10	0	32.7 (0.5)	75.3	No signal change
dTFO-5'2ap	dHP-tCG	6.4	10	0	32.4 (0.2)	76.8	1.2 ± 0.2
dTFO-5'2ap	dHP-tCG	6.2	10	0			<1
dTFO-5'2ap	dHP-ΔtTA-5'h	6.4	10	0	29.4 (0.2)		No signal

							change
dTFO-5'2ap	rHP	7.0	10	0	< 20	88	
dTFO-5'2ap	rHP	6.8	10	0	< 20	87.8	
dTFO-5'2ap	rHP	6.6	10	0	< 20	87.6	
dTFO-5'2ap	rHP	6.4	0	0	< 20	85	
dTFO-5'2ap	rHP	6.4	10	0	< 20	87.6	
dTFO-5'2ap	rHP	6.4	0	0.1	< 20	88.1	
dTFO-5'2ap	rHP	6.4	10	0.1	< 20	89	
dTFO-5'2ap	rHP	6.2	10	0	< 20	88.5	
dTFO-5'2ap	rHP	6.0	10	0	< 20	88.9	
dTFO-5'2ap	rHP	5.8	10	0	< 20	88.3	
dTFO-5'2ap	rHP	5.6	10	0	< 20	88	
dTFO-5'2ap	rHP	5.4	0	0	< 20	83.2	
dTFO-5'2ap	rHP	5.4	10	0	< 20	86.6	
dTFO-5'2ap	rHP	5.4	10	0	< 20	86.8	
dTFO-5'2ap	rHP	5.4	0	0.1	< 20	86	
dTFO-5'2ap	rHP	5.4	10	0.1	< 20	87.7	
dTFO-5'2ap	rHP	5.2	10	0			
dTFO-5'2ap	rHP	5.0	10	0			
dTFO-5'2ap	rHP-ΔtUA	5.4	10	0	< 20	86	
rTFO-5'A	dHP	6.4	10	0	39.7 (0.5)	75.7	
rTFO-5'A	dHP-ΔtTA	6.4	10	0	38.2 (0.5)	75.1	
rTFO-5'A	dHP-tCG	6.4	10	0	41.4 (0.3)	75.9	
rTFO-5'A	dHP-ΔtTA-5'h	6.4	10	0	37.1 (0.1)		
rTFO-5'A	rHP	6.4	10	0	< 20	87.6	
rTFO-5'A	rHP	5.4	10	0	52.9 (1.3)	86.1	
rTFO-5'A	rHP-ΔtUA	5.4	10	0	52.7 (0.7)	86.1	
rTFO-5'A	rHP-ΔtUA-5'h	5.4	10	0	42.5 (0.3)		
rTFO-5'U	dHP	6.4	10	0	40.0 (0.5)	76.1	
rTFO-5'U	dHP-ΔtTA	6.4	10	0	36.3 (0.2)	75.4	
rTFO-5'U	dHP-tCG	6.4	10	0	41.9 (0.5)	76.4	
rTFO-5'U	dHP-ΔtTA-5'h	6.4	10	0	35.4 (0.3)		
rTFO-5'U	rHP	6.4	10	0	< 20	87.9	
rTFO-5'U	rHP	5.4	10	0	47.8 (1.1)	86.5	
rTFO-5'U	rHP-ΔtUA	5.4	10	0	50.2 (1.4)	86.4	
rTFO-5'U	rHP-ΔtUA-5'h	5.4	10	0	38.1 (0.7)		
rTFO-3'A	dHP	6.4	10	0	42.3 (0.5)	76.5	
rTFO-3'A	dHP-ΔtTA	6.4	10	0	37.5 (0.5)	76	
rTFO-3'A	dHP-tCG	6.4	10	0	43.0 (0.6)	76.8	
rTFO-3'A	dHP-ΔtTA-5'h	6.4	10	0	36.8 (0.2)		

rTFO-3'A	rHP	6.4	10	0	< 20	88.5	
rTFO-3'A	rHP	5.4	10	0	45.2 (0.6)	87	
rTFO-3'A	rHP-ΔtUA	5.4	10	0	48.1 (0.6)	86.7	
rTFO-3'A	rHP-ΔtUA-5'h	5.4	10	0	38.0 (0.4)		
rTFO-5'ΔU	dHP	6.4	10	0	35.6 (0.7)	76.7	
rTFO-5'ΔU	dHP-ΔtTA	6.4	10	0	36.7 (0.5)	76	
rTFO-5'ΔU	dHP-tCG	6.4	10	0	36.4 (1.3)	77.1	
rTFO-5'ΔU	dHP-ΔtTA-5'h	6.4	10	0	34.7 (0.2)		
rTFO-5'ΔU	rHP	6.4	10	0	< 20	88.9	
rTFO-5'ΔU	rHP	5.4	10	0	48.8 (0.7)	87.2	
rTFO-5'ΔU	rHP-ΔtUA	5.4	10	0	50.2 (0.9)	85.4	
rTFO-5'ΔU	rHP-ΔtUA-5'h	5.4	10	0	40 (0.5)		
rTFO-3'ΔC	dHP	6.4	10	0	32.5 (1.1)	77	
rTFO-3'ΔC	dHP-ΔtTA	6.4	10	0	29.1 (0.2)	76.3	
rTFO-3'ΔC	dHP-tCG	6.4	10	0	33.2 (0.2)	77.2	
rTFO-3'ΔC	dHP-ΔtTA-5'h	6.4	10	0	29.7 (0.5)		
rTFO-3'ΔC	rHP	6.4	10	0	< 20	89	
rTFO-3'ΔC	rHP	5.4	10	0	35.0 (0.6)	87.5	
rTFO-3'ΔC	rHP-ΔtUA	5.4	10	0	35.4 (0.7)	85.1	
rTFO-3'ΔC	rHP-ΔtUA-5'h	5.4	10	0	32.1 (0.5)		
rTFO-5'G	dHP	6.4	10	0	39.5 (1.0)	76.6	
rTFO-5'G	dHP-ΔtTA	6.4	10	0	36.1 (0.9)	74.9	
rTFO-5'G	dHP-tCG	6.4	10	0	40.7 (0.7)	76.6	
rTFO-5'G	dHP-ΔtTA-5'h	6.4	10	0	35.3 (0.2)		
rTFO-5'G	rHP	5.4	10	0	48.3 (0.3)	87.4	
rTFO-5'G	rHP-ΔtUA	5.4	10	0	50.0 (0.2)	86.1	
rTFO-5'G	rHP-ΔtUA-5'h	5.4	10	0	40.6 (0.3)		
rTFO-3'G	dHP	6.4	10	0	44.7 (0.8)	76.1	
rTFO-3'G	dHP-ΔtTA	6.4	10	0	39.4 (0.5)	75.2	
rTFO-3'G	dHP-tCG	6.4	10	0	46.0 (0.5)	76.8	
rTFO-3'G	dHP-ΔtTA-5'h	6.4	10	0	38.8 (0.4)		
rTFO-3'G	rHP	5.4	10	0	51.1 (0.5)	87.6	
rTFO-3'G	rHP-ΔtUA	5.4	10	0	51.0 (0.9)	86.5	
rTFO-3'G	rHP-ΔtUA-5'h	5.4	10	0	41.1 (0.2)		
rTFO-5'2AP	dHP	6.4	10	0	40.1 (1.0)	75.1	<1
rTFO-5'2AP	dHP	6.6	10	0	29.3	75.3	<1
rTFO-5'2AP	dHP	6.8	10	0	27	75.7	5.0 ±0.5
rTFO-5'2AP	dHP	7	10	0	23.5	76.1	

rTFO-5'2AP	dHP-ΔtTA	6.4	10	0	38.4 (0.7)	75	
rTFO-5'2AP	dHP-ΔtTA	6.8	10	0			no signal change
rTFO-5'2AP	dHP-tCG	6.4	10	0	41.3 (0.8)	76.1	
rTFO-5'2AP	dHP-tCG	6.8	10	0			3.4 ±0.4
rTFO-5'2AP	dHP-ΔtTA-5'h	6.4	10	0	36.7 (0.1)		
rTFO-5'2AP	dHP-ΔtTA-5'h	6.8	10	0			no signal change
rTFO-5'2AP	rHP	5.4	10	0	47.3 (0.6)	86.7	
rTFO-5'2AP	rHP	5.8	10	0	46.9	86.3	1.2 ±0.4
rTFO-5'2AP	rHP-ΔtUA	5.4	10	0	50.7 (0.9)	85.7	
rTFO-5'2AP	rHP-ΔtUA	5.8	10	0	59.1	85.9	no signal change
rTFO-5'2AP	rHP-tCG	5.8	10	0	40.9	87.5	1.3 ±0.1
rTFO-5'2AP	rHP-ΔtUA-5'h	5.4	10	0	42 (0.3)		
rTFO-5'2AP	rHP-ΔtUA-5'h	5.8	10	0			no signal change
dTFO-uc	dHP	6.4	10	0	31.4 (0.3)	75.1	
dTFO-uc	dHP-ΔtTA	6.4	10	0	28.5 (0.5)	74.6	
dTFO-uc	dHP-tCG	6.4	10	0	32.3 (0.4)	75.8	
dTFO-uc	dHP-ΔtTA-5'h	6.4	10	0	27.6 (0.1)		
dTFO-uc	rHP	5.4	10	0	< 20	86	
dTFO-uc	rHP-ΔtUA	5.4	10	0	< 20	85.8	
dTFO-uc	rHP-tCG	5.4	10	0	< 20	87.4	
dTFO-tc	dHP	6.4	10	0	33.0 (0.1)	75.5	
dTFO-tc	dHP-ΔtTA	6.4	10	0	31.0 (0.1)	75.5	
dTFO-tc	dHP-tCG	6.4	10	0	33.7 (0.1)	76.6	
dTFO-tc	dHP-ΔtTA-5'h	6.4	10	0	29.7 (0.3)		
dTFO-tc	rHP	5.4	10	0	< 20	86	
dTFO-tc	rHP-ΔtUA	5.4	10	0	< 20	84.9	
dTFO-tc	rHP-tCG	5.4	10	0	< 20	87.1	
dTFO-5'Δu	dHP	6.4	10	0	25.5 (0.3)		
	dHP-ΔtTA	6.4	10	0	27.6 (0.1)		
	dHP-tCG	6.4	10	0	25.5 (0.3)		
	dHP-ΔtTA-5'h	6.4	10	0	26.8 (0.1)		
dTFO-3'2ap	dHP	6.4	10	0	31.0 (0.1)		1.1 ±0.2
	dHP-ΔtTA	6.4	10	0	28.7 (0.1)		2.5 ±0.2
	dHP-tCG	6.4	10	0	32.1 (0.1)		1.0 ±0.1
	dHP-ΔtTA-5'h	6.4	10	0	26.4 (0.3)		4.1 ±0.5

rTFO-3'2ap	dHP	6.4	10	0	42.0 (0.6)		
	dHP	6.8	10	0			3.1 ±0.8
	dHP-ΔtTA	6.4	10	0	39.0 (0.2)		
	dHP-ΔtTA	6.8	10	0			12.6 ± 4.2
	dHP-tCG	6.4	10	0	44.0 (0.4)		
	dHP-tCG	6.8	10	0			3.9 ±0.8
	dHP-ΔtTA-5'h	6.4	10	0	35.8 (0.3)		
	dHP-ΔtTA-5'h	6.8	10	0			10 ±4.6
	rHP	5.4	10	0	42.6 (0.1)		
	rHP	5.8	10	0			1.1 ±0.1
	rHP-ΔtUA	5.4	10	0	40.8 (0.5)		
	rHP-ΔtUA	5.8	10	0			<1
	rHP-tCG	5.8	10	0			1.3 ±0.2
	rHP-ΔtUA-5'h	5.4	10	0	37.2 (0.3)		
	rHP-ΔtUA-5'h	5.8	10	0			6.2 ±0.6

Figure S1 Thermal melting curves of triplexes. The buffer for the triplexes containing dHPs is 200 mM NaCl, 20 mM MES, 10 mM MgCl₂, 0.5 mM EDTA, pH 6.4. The buffer for triplexes containing rHPs is 200 mM NaCl, 20 mM MES, 10 mM MgCl₂, 0.5 mM EDTA, pH 5.4.

Figure S2 The intrinsic fluorescence of dHP when excited at 340 nm. The concentration of the dHP is 50 μ M. The buffer is 200 mM NaCl, 20 mM MES, 10 mM MgCl₂, 0.5 mM EDTA, pH 6.4.

Figure S3 The intrinsic fluorescence of dHP and rHP when excited at 303 nm. The buffer is 200 mM NaCl, 20 mM MES, 10 mM MgCl₂, 0.5 mM EDTA, pH 5.8. The values shown in the boxes are the concentrations (μ M) of the hairpins.

Figure S4 Binding of rTFO-3'2ap to RNA hairpins to form RNA-RNA₂ triplexes at pH 6.0 revealed by fluorescence enhancement. The buffer is 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 6.0. The melting data is done in buffer: 200 mM NaCl, 20 mM MES, 0.5 mM EDTA, 10 mM MgCl₂, pH 5.4.