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ARTICLE TYPE

Highly stable triple helix formation by homopyrimidine (L)-acyclic threoninol nucleic acids with single stranded DNA and RNA

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Acyclic (L)-threoninol nucleic acid (aTNA) containing thymine, cytosine and adenine nucleobases were synthesized and shown to form surprisingly stable triplexes with complementary single stranded homopurine DNA or RNA targets. The triplex structures consist of two (L)-aTNA strands and one DNA or RNA, and these triplexes are significantly stronger than the corresponding DNA or RNA duplexes as shown in competition experiments. As a unique property the (L)-aTNAs exclusively form triplex structures with DNA and RNA and no duplex structures are observed by gel electrophoresis. The results were compared to the known enantiomer (D)-aTNA, which forms much weaker triplexes depending upon temperature and time. It was demonstrated that (L)-aTNA triplexes are able to stop primer extension on a DNA template inferring the potential of (L)-aTNA for antisense applications.

Introduction

Fully modified acyclic nucleic acids are currently being intensely investigated owing to their potential for formation of highly stable duplex structures compared to DNA and RNA. Researchers have been synthesising various acyclic nucleic acids¹ and investigated their biophysical properties to form homoduplexes and heteroduplexes with DNA or RNA. These acyclic nucleic acids contain natural nucleobases and acyclic scaffold units, which are interconnected by phosphodiester linkages. The important goals for the construction of these acyclic oligonucleotides could be the development of nanostructures,^{2a} conformational switches^{2b} and bio-sensing technology. Amino acid nucleic acid (AANA) was first reported by Kanda *et al.* who showed that poly (T) of AANA (synthesized from L-serine) did not display hybridization with poly d(A).^{1a} Later, Asanuma *et al.* reported an acyclic threoninol nucleic acid (aTNA)^{1e} and acyclic serinol nucleic acid (SNA)^{1f} synthesized from D-threoninol and L-serinol respectively, which exhibited highly stable homoduplexes. SNA has been shown to make duplex with DNA and RNA while (D)-aTNA did not exhibit hybridization with DNA. Whereas based on the CD studies (D)-aTNA duplex was described as a right handed helical structure.^{1e}

However, to provide proof of the helical handedness of artificial oligonucleotides based on CD behaviour of the DNA or RNA is not straightforward.^{1c, 1h, 3} Therefore, we investigated the (L)-aTNA (Fig. 1a), because chirality plays a very important role in order to induce the handedness, that can facilitate the binding of acyclic nucleic acids towards DNA or RNA.

Homopyrimidine peptide nucleic acids (PNAs) have been intensively investigated as triplex forming oligonucleotides (TFOs) with DNA duplex or single stranded DNA.⁴ However, acyclic nucleic acids containing phosphodiester linkages have an advantage over PNA with regard to solubility and binding specificity based on the chiral monomers.⁵

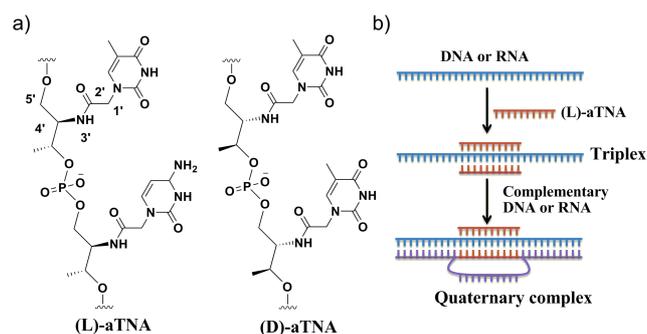


Fig.1 (a) Chemical structures of (L)-aTNA and (D)-aTNA. (b) Schematic model of binding of homopyrimidine (L)-aTNA to sequence complementary single stranded homopurine DNA or RNA target.

Until now there has been no report on acyclic nucleic acids which make a highly stable triple helix with DNA or RNA where two acyclic nucleic acid strands are hybridizing with single stranded DNA or RNA. Here we present the synthesis and biophysical properties that how homopyrimidine (L)-aTNA forms a highly stable triple helix with single stranded DNA or RNA in

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a (L)-aTNA-DNA-(L)-aTNA and (L)-aTNA-RNA-(L)-aTNA fashion (Fig. 1b).

Results and discussion

Synthesis of aTNA oligonucleotides

5 The (L)-aTNA phosphoramidites were synthesized starting from commercially available (L)-threonine amino acid. By using this synthetic methodology we obtained good yields compared to the reported synthetic procedure^{1e} (Supplementary Information S1, S2). The synthesis of (L)-aTNA oligonucleotides followed standard protocol of automatized oligonucleotide synthesis (Supplementary Information S3, S4). In order to investigate the binding properties of (L)-aTNA with DNA or RNA various oligonucleotide strands were synthesized (Table 1).

Table 1 Oligonucleotides used in the study

Entry	Oligonucleotide sequences ^a	Type
ON-1	5'-aaaaaaaaaaaaa-4'-dA-3'	(L)-aTNA
ON-2	5'-ttttttttttttt-4'-dT-3'	(L)-aTNA
ON-3	5'-aaaaaaaaaaaaa-4'-dA-3'	(D)-aTNA
ON-4	5'-ttttttttttttt-4'-dT-3'	(D)-aTNA
ON-5	5'-GATCCTTTTTTTTTTTTTTTG-3'	DNA
ON-6	5'-GATCCAAAAAAAAAAAAAAAAAG-3'	DNA
ON-7	5'-ttttctctctctc-4'-dT-3'	(L)-aTNA
ON-8	5'-tctctctctctttt-4'-dT-3'	(L)-aTNA
ON-9	5'-AGAGAGAGAGAAAAA-3'	DNA
ON-10	5'-ttttttt-4'	(L)-aTNA
ON-11	5'-ccccccc-4'	(L)-aTNA
ON-12	5'-aaaatttatattt-4'-dA-3'	(L)-aTNA
ON-13	5'-taataataaatt-4'-dT-3'	(L)-aTNA
ON-14	5'-AAAAAAAAAAAAAAAA-3	RNA
ON-15	5'-AGAGAGAGAGAAAAA-3	RNA

¹⁵ ^a Small letters dictate acyclic nucleotides. The dA and dT indicate for standard DNA nucleotides. The aTNA oligonucleotides are shown from 5' to 4' direction.

Biophysical properties of (L)-aTNA

20 First, we investigated the pairing properties of ON-1 and ON-2 with temperature-dependent UV spectroscopy at 260 nm in 10 mM phosphate buffer at pH 7 containing 100 mM sodium chloride. The spectrum showed a highly stable duplex ($T_m = 72$ °C) between ON-1 and ON-2 when comparing to the analogues DNA duplex ($T_m = 46$ °C, data not shown) (Fig. 2a). The CD profile of the ON-1/ON-2 duplex showed positive Cotton effects at 257 and 218 nm and negative Cotton effect at 241 nm (Fig. 2b). Furthermore single stranded ON-2 was observed as a random coil while ON-1 displayed a preorganized structure which showed CD signals at 267 nm and 218 nm as positive bands and negative bands at 247 nm and 203 nm (Figure. 2b). In addition UV melting of ON-1 showed very stable preorganized structure (Supplementary Information S5, Fig S1). Moreover, poly (A) of acyclic butyl nucleic acid (BuNA) has been shown preorganized structure at neutral pH.^{2b} It is important to note that both stereo isomers of aTNA containing a natural DNA nucleotide at 4' end. However, the presence of a natural nucleotide at the terminal position does not significantly affect the biophysical properties of acyclic oligonucleotides.^{1h} Next, we investigated the duplex formation of the (D)-aTNA, ON-3 and ON-4, which revealed similar duplex stability ($T_m = 71$ °C) with the inverse CD pattern

(Supplementary Information S5, Fig S2). These studies confirm that the inversion of the stereogenic center does not affect the duplex stability; rather the opposite helical structure (right handed or left handed) was obtained based on the stereochemistry.

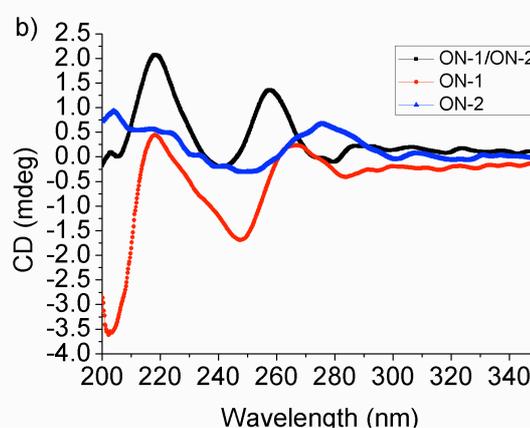
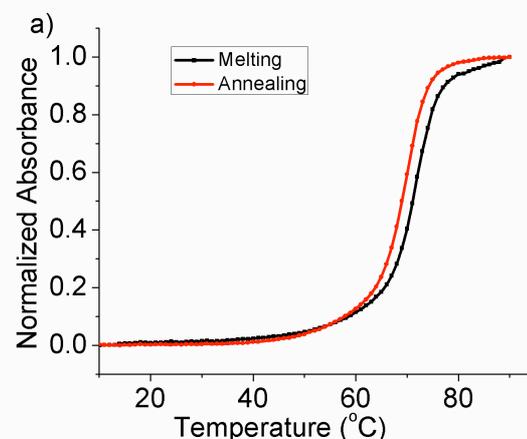


Fig. 2 (a) Melting profile of (L)-aTNA duplex (ON-1/ON-2) measured by UV at 260 nm. (b) CD profile of (L)-aTNA duplex (ON-1/ON-2), ON-1 and ON-2 at 20 °C. Experimental conditions: 10 mM phosphate buffer containing 100 mM sodium chloride at pH 7 with each oligo concentration of 2 μM for UV-melting and 0.5 μM for CD studies.

Triple helix properties of (L)-aTNA oligonucleotides with DNA

55 Our aim of the construction of the artificial nucleic acid was to target natural nucleic acids; therefore we investigated the hybridisation properties of aTNA with DNA and RNA. Interestingly, we observed very strong interactions when (L)-aTNA (ON-2) was mixed with DNA (ON-6), however in 2:1 stoichiometric ratio, where melting temperature was determined to be 82 °C (Fig. 3a). The CD analysis revealed positive bands at 255 nm with two shoulder peaks at 214 nm and 222 nm and negative bands at 272 nm and 240 nm with shoulder peak at 283 nm indicating the formation of triplex structure (Fig. 3b).

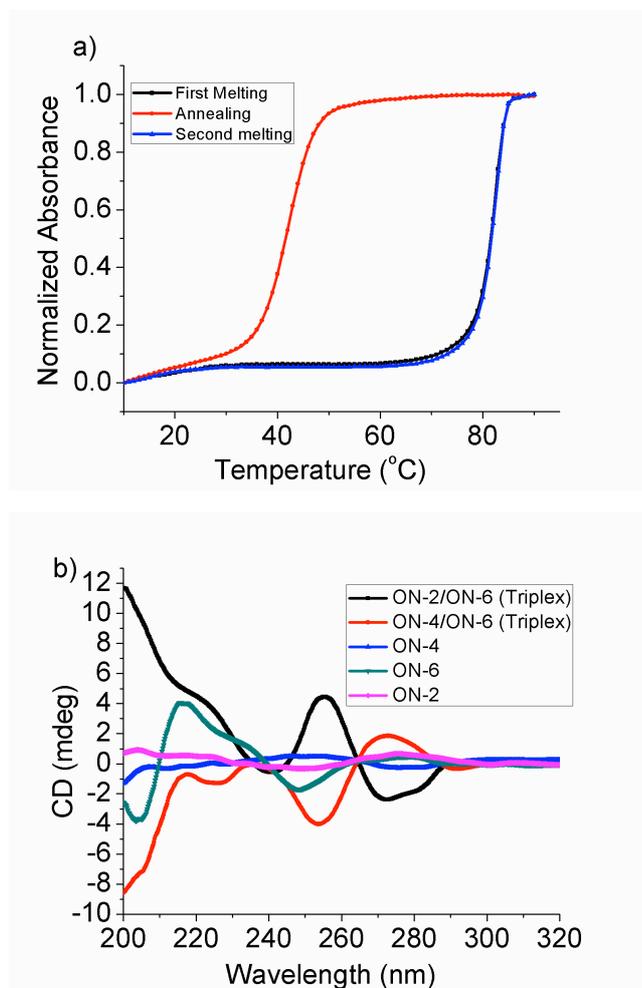


Fig. 3 (a) Melting-profile of triplex (ON-2/ON-6/ON-2) measured by UV at 260 nm. (b) CD profile of triplex formation of ON-2/ON-6/ON-2 and ON-4/ON-6/ON-4 (sample was incubated for 48 hr at 3 °C) at 20 °C.

Experimental conditions: 10 mM phosphate buffer containing 100 mM sodium chloride at pH 7, oligo concentration for (L)-aTNA (10 μ M for CD studies and 2 μ M for UV-melting) for DNA (5 μ M for CD studies and 1 μ M for melting experiment).

Table 2 Thermal stabilities of duplex and triplexes measured by UV at 260 nm^a

Oligonucleotides	T_m (°C) ^a
ON-5/ON-6 (DNA duplex)	46
ON-1/ON-2 (L-aTNA duplex)	72
ON-2/ON-6/ON-2 (L-aTNA-DNA triplex)	82
ON-4/ON-6/ON-4 (D-aTNA-DNA triplex)	47

^a Experimental condition: Each oligo with 1 μ M for triplexes and 2 μ M for duplexes in 10 mM phosphate buffer containing 100 mM sodium chloride at pH 7.

The CD obtained for (D)-aTNA (ON-4) with ON-6 demonstrated a similar triple helical structure with opposite handedness (Fig. 3b). However in this case it was required that the mixture was incubated at 3 °C for 48 hours prior to the CD experiment in order to obtain a strong CD signal. Moreover, the T_m profile showed weak interaction of ON-4 with the DNA strand (ON-6) with a melting temperature of 47 °C, where interactions were time dependent (Fig. 4). This study shows that the hybridization

behaviour is dependent on time and temperature. It was not possible to assign the handedness to the resulting (L)-aTNA-DNA-(L)-aTNA complex based on the CD studies. However, based on the stability of (L)-aTNA/DNA triplex as compared to the (D)-aTNA/DNA triplex it can be speculated that the highly stable triplex formation by (L)-aTNA with DNA could be due to the right handedness of the (L)-aTNA scaffold. These data indicate that triple helix structure made by (L)-aTNA is stronger than aTNA-aTNA or DNA-DNA homoduplexes (table 2).

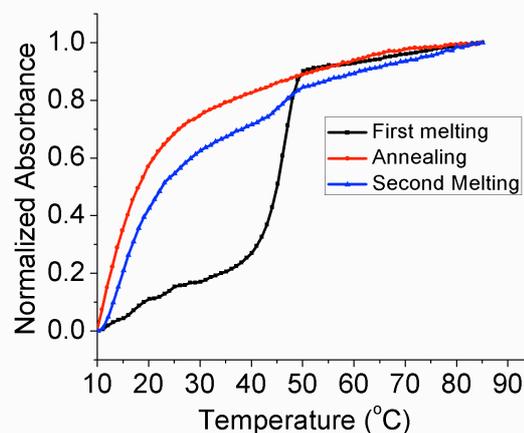


Fig. 4 Melting-profile of (D)-aTNA triplex (ON-4/ON-6/ON-4) at 260 nm in 10 mM phosphate buffer with 100 mM sodium chloride at pH 7 with each oligo concentration of 2 μ M for (D)-aTNA and 1 μ M for DNA. Sample was incubated for overnight at 3 °C before experiment.

Since, the melting transition of the (L)-aTNA/DNA complex measured by UV showed major hysteresis, it was important to investigate the nature of the complex formation. In the literature, similar results have been reported for the PNA-DNA-PNA triplex structure.^{4b} Therefore, we investigated the stoichiometric ratio between (L)-aTNA and DNA by CD spectroscopy and native gel electrophoresis. Moreover, we did not consider (D)-aTNA for further studies due the weak interaction with DNA. Fig. 5a shows the CD spectrum of DNA (ON-6) at fixed concentration, in the presence of different concentrations of ON-2 in 10 mM phosphate buffer with 100 mM sodium chloride at pH 7. CD titration clearly demonstrate that 5 μ M of DNA is saturated by 10 μ M of ON-2 (Fig. 5a). Further addition of ON-2 decreases the CD signal at 272 nm which is the characteristic negative band for this triplex structure. This study indicates that ON-2 hybridized with ON-6 in (L)-aTNA-DNA-(L)-aTNA fashion. Moreover the potential formation of triple helix structure like DNA-DNA-(L)-aTNA was analysed by gel mobility shift assay (Fig. 5b). ON-2 and ON-5 homopyrimidine strands were added together to the ON-6 and incubated for 12 hours at room temperature (25 °C). Surprisingly, we did not observe any band that corresponds to a DNA-DNA-(L)-aTNA triplex. Moreover, only two bands were observed which indicated only DNA duplex and (L)-aTNA-DNA-(L)-aTNA triplex (Fig. 5b, Lanes 2 and 3). These results clearly suggest that (L)-aTNA was unable to hybridize with DNA duplex in order to make conventional triple helix formation as observed with natural oligonucleotides.⁶ In addition when (ON-2) was added to the DNA duplex (ON-5/ON-6) then ON-2 was unable to displace the homopyrimidine DNA strand (data not

shown). Similarly, homopyrimidine DNA strand (ON-5) was unable to displace the ON-2 from triple helix (Fig. 5b, Lanes 4 and 5). These data revealed that homopyridine DNA strand cannot break the triple helix structure on the other hand homopyrimidine aTNA strand is unable to invade in the DNA duplex.

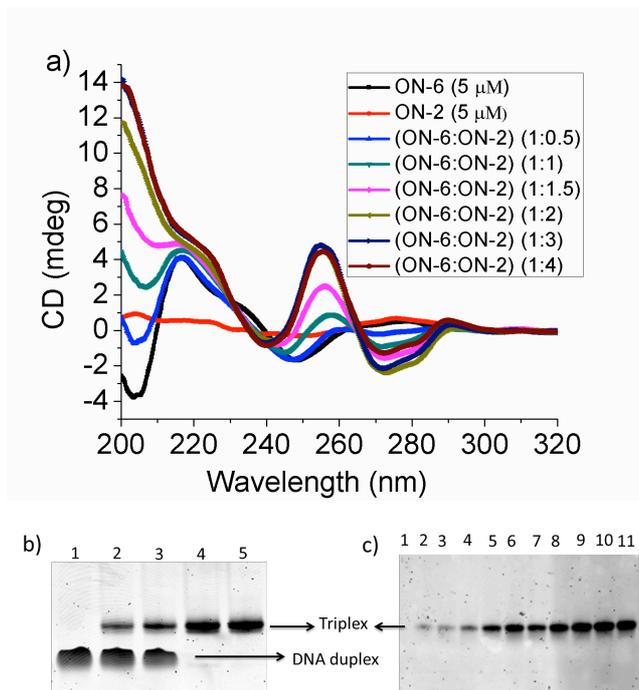


Fig. 5 Titration of DNA by (L)-aTNA. (a) CD titration for fixed concentration of ON-6 (5 μM) with increasing concentration of ON-2 at 20 $^{\circ}\text{C}$. (b) Non-denatured gel electrophoresis (each DNA strand with 10 pmol) Lane 1 = DNA duplex (ON-5/ON-6), Lane 2 = ON-5/ON-6/ON-2 (50 pmol), Lane 3 = ON-5/ON-6/ON-2 (100 pmol), Lane 4 = ON-6/ON-2 (50 pmol) + ON-5, Lane 5 = ON-6/ON-2 (100 pmol) + ON-5. (c) Titration of ON-6 (7 pmol) with increasing amount of ON-2 (0, 1.4, 2.8, 4.2, 5.6, 7, 8.4, 9.8, 11.2, 12.6, 14 pmol). "+" sign in figure caption indicate that (L)-aTNA or DNA was added after 1 hour incubation (10 mM phosphate buffer with 100 mM sodium chloride at pH 7) of first two strands at room temperature. Conditions: TBM running buffer, 12% non-denatured PAGE at constant power 3 watt.

Furthermore, the stoichiometry was confirmed by native gel electrophoresis. A fixed amount (7 pmol) of ON-6 was titrated with increasing amounts of ON-2. Figure 5c (Lanes 2 to 11) demonstrated that all the stoichiometric ratios of ON-2/ON-6 displayed only one band, which corresponds to the triple helix structure. Addition of more than two fold of ON-2 exhibited exclusively triple helix structure (data not shown). Collectively, all these studies clearly indicate that homopyrimidine (L)-aTNA does not make duplex with DNA while it exists only in the triple helix structure with single stranded DNA target in (L)-aTNA-DNA-(L)-aTNA fashion. This triple helix structure consists of Watson-Crick (2 hydrogen bonds) and Hoogsteen (2 hydrogen bonds) nucleobase pairing and therefore it was expected to show a single very sharp melting transition where three strands disrupt simultaneously (Fig. 3a).

Because two (L)-aTNA strands are involved in the triple helix structure, it is important to investigate the polarity⁷ of the (L)-aTNA strands in the duplex structure. Moreover, the presence of

cytosines is also important in order to target the homopurine rich tracts of DNA or RNA strands. For the formation of Hoogsteen bonds, homopyrimidine (L)-aTNA containing cytosines, require protonation. Therefore, pH of the buffer plays a very important role for the stability of the triple helix. To test this hypothesis we constructed two asymmetric (L)-aTNA strands (ON-7 and ON-8) containing thymine and cytosine nucleobases. Melting temperatures were measured in 10 mM phosphate buffer with 100 mM sodium chloride at pH 5.8 and 7. It was evident from the figure 6a that at pH 5.8, ON-7, which is oriented in an antiparallel direction relative to the complementary DNA strand (ON-9), resulted in a similar melting temperature as when ON-8, which is oriented in a parallel direction, was used. These results clearly indicate that the stability of the triple helix is independent of the polarity of the (L)-aTNA strands where two strands of (L)-aTNA are hybridizing with one DNA strand by Watson-Crick and Hoogsteen hydrogen bonds either in parallel or anti parallel direction.^{7a}

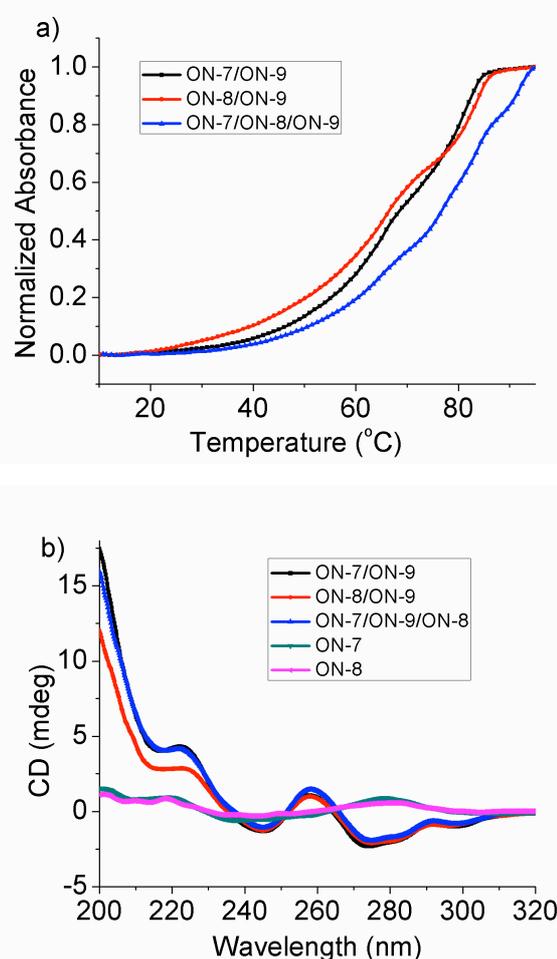


Fig. 6 (a) Melting profiles of triple helix at 260 nm. (b) CD spectra at 20 $^{\circ}\text{C}$. Experimental conditions: 10 mM phosphate buffer containing 100 mM sodium chloride at pH 5.8 for oligo concentration (L)-aTNA (10 μM for CD studies and 2 μM or 1 μM for UV-melting) for DNA (5 μM for CD studies and 1 μM for UV studies).

Next, we investigated the triple helix stability obtained by mixing both (L)-aTNA strands (ON-7, ON-8) with ON-9 at pH 5.8. This resulted in improved stability compared to the triplexes

formed with two of the same (L)-aTNA strand (Fig. 6a) (Table 3). This further demonstrates that a highly stable triplex can be obtained when ON-7 is in the antiparallel direction and ON-8 strand is in the parallel direction. Furthermore, we also investigated the triplex structure stability under neutral conditions (Supplementary Information S5, Fig. S3). These investigations revealed that low pH stabilizes the (L)-aTNA-DNA-(L)-aTNA structure to a higher extent than neutral conditions. Next, we investigated the conformational properties by CD spectroscopy and it showed positive peaks at 258 nm and 221 nm and negative peaks at 275 nm and 245 with shoulder peak at 283 (Fig. 6b).

Table 3 Thermal stabilities of (L)-aTNA/DNA triplexes measured by UV at 260 nm^a

Triplexes	T _m (°C) ^b
ON-7/ON-9/ON-7	65, 81
ON-8/ON-9/ON-8	66, 84
ON-7/ON-8/ON-9	76, >83

^a Experimental condition: Each oligo with 1 μM concentration in 10 mM phosphate buffer with 100 mM sodium chloride at pH 5.8. ^b Two melting transitions were observed because Watson-Crick interactions are stronger than Hoogsteen bonding in the case of G-C pairing.

We also investigated the short sequences (8 nucleotides) of (L)-aTNA for triplex formation with DNA. The melting temperature of (L)-aTNA (t)₈ with poly d(A)₈ was determined to be 44 °C (Supplementary Information S5, Fig. S4) (Table 4). In addition, (L)-aTNA (c)₈ showed the two melting transitions at 22 °C and 46 °C (Supplementary Information S5, Fig. S4).

Table 4 Thermal stabilities of triplexes measured by UV at 260 nm

Triplexes	T _m (°C) ^a
ON-10/d(A) ₈ /ON-10	44
ON-11/d(G) ₈ /ON-11	22, 46

^a Experimental condition: Each oligo with 1 μM concentration in 10 mM phosphate buffer with 100 mM sodium chloride at pH 7 for ON-10 and at pH 5.8 for ON-11.

Taken all together, these results suggest that for the design of homopyrimidine (L)-aTNA in order to capture single stranded homopurine DNA or RNA, a range of 8 to 15 nucleotides would be the optimum length which can be further anchor with DNA or RNA containing guanine and adenine nucleotides for excellent specificity.

In order to investigate any possibility to form duplex between (L)-aTNA and DNA a study of mixed sequences of (L)-aTNA nucleotides was carried out. We therefore synthesised ON-12 and ON-13 which are complementary to each other. Duplex stability (ON-12/ON-13) by UV was determined to be 70 °C (Supplementary Information S5, Fig. S5). The CD profile showed positive Cotton effects at 253 nm, 236 nm and 217 nm with negative Cotton effect at 271 nm (Supplementary Information S5, Fig. S5) which is inverse compared to the reported (D)-aTNA.^{1d} This data suggests that the CD profile obtained by duplex of poly (L)-aTNA (A) and poly (L)-aTNA (T) is completely different than mixed sequence of (L)-aTNA, which reveals the different

arrangement of nucleobases in their respective duplexes. On the other hand when ON-12 and ON-13 were mixed with their complementary DNA strands in 10 mM phosphate buffer containing 100 mM sodium chloride at pH 7 no hybridization was observed (data not shown). The data strongly support the interpretation that (L)-aTNA can interact with DNA or RNA only in the form of triplex structure.

Triple helix properties of (L)-aTNA oligonucleotides with RNA

Controlling and detecting the expression of single stranded RNA have wide applications in therapeutics and diagnostics.^{8a,b}

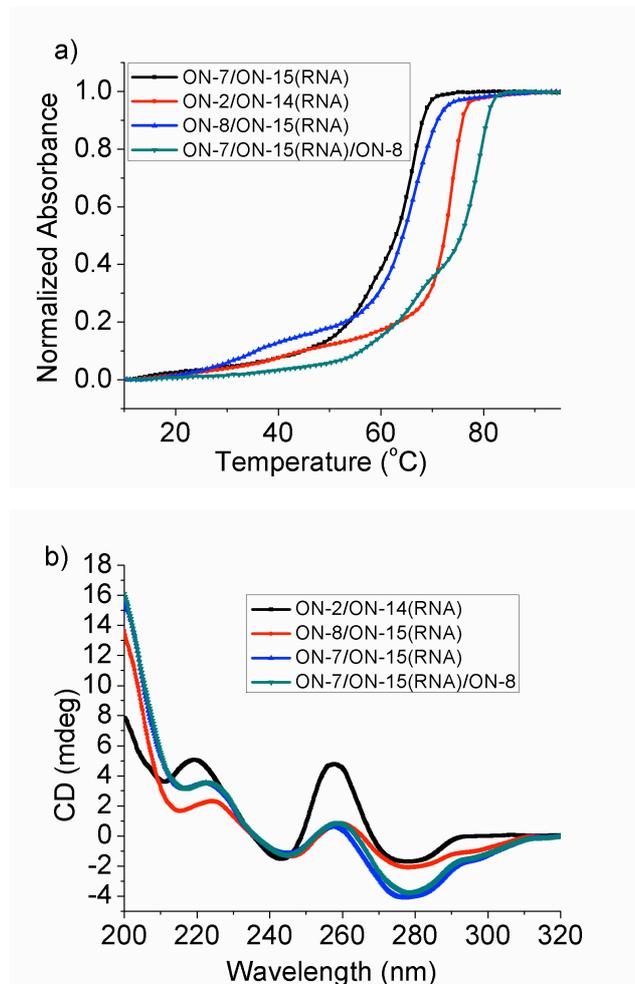


Fig. 7 (a) Melting profiles at 260 nm. (b) CD spectra at 20 °C.

Experimental conditions: 10 mM phosphate buffer containing 100 mM sodium chloride at pH 7 (ON-2) and pH 5.8 (ON-7 and ON-8), oligo concentration (L)-aTNA (10 μM or 5 μM for CD studies and 2 μM or 1 μM for UV-melting), RNA (5 μM for CD studies and 1 μM for UV studies).

Locked nucleic acid (LNA) has been utilized for the development of antisense against apolipoprotein-B mRNA.^{8c} Thus RNAs are important target molecules and it is important to investigate the binding properties of (L)-aTNA with RNA targets. Interestingly, when ON-2 was mixed with poly r(A)₁₅ (ON-14) a stable triple helix was observed by temperature dependent UV spectroscopy at 260 nm (T_m = 74 °C) in 10 mM phosphate buffer with 100 mM sodium chloride at pH 7 (Fig. 7a). The CD spectrum displayed

positive bands at 258 nm, 219 nm and 211 nm with negative bands at 277 nm and 243 nm (Fig. 7b). In addition; we investigated the triplex formation by ON-7 and ON-8 to its complementary strand of RNA target (ON-15). Temperature dependent UV spectroscopy at 260 nm of the triplexes ON-7/ON-15/ON-7 exhibited two melting transitions at 58 °C and 66 °C in 10 mM phosphate buffer containing 100 mM sodium chloride at pH 5.8 (Fig. 7a) (Table 5).

Table 5 Thermal stabilities of (L)-aTNA/RNA triplexes measured by UV at 260 nm^a

Triplexes	T _m (°C)
ON-2/ON-14/ON-2	74
ON-7/ON-15/ON-7	58, 66
ON-8/ON-15/ON-8	66
ON-7/ON-15/ON-8	66, 79

^a Experimental condition: Each oligo with 1 μM concentration in 10 mM phosphate buffer with 100 mM sodium chloride at pH 7 or pH 5.8.

In addition the ON-8/ON-15/ON-8 triplex stability was determined to be 66 °C indicating that either parallel or antiparallel orientations of both homopyrimidine (L)-aTNA strands towards homopurine RNA target does not affect the triplex stability as we observed for the (L)-aTNA/DNA triplex. Moreover, an increase in melting temperature (T_m = 66 °C and 79 °C) was observed when mixing ON-7, ON-8 and ON-15 in equal ratio at pH 5.8. This further proves that in order to obtain highly stable triplex structures one (L)-aTNA strand hybridizes in an antiparallel direction (Watson-Crick base pairing) and another (L)-aTNA strand binds in the parallel direction (Hoogsteen base pairing) with the RNA target. The CD profile at pH 5.8 shows positive Cotton effects at 258 nm and 224 nm and 215 nm with 277 nm and 244 nm (Fig. 7b). These results indicate that homopyrimidine (L)-aTNA also makes a stable triplex with a complementary homopurine RNA strand.

Mismatch study

We have illustrated that homopurine (L)-aTNA was capable of making a stable triple helix with complementary single stranded DNA or RNA. Thus, it was important to investigate the binding properties of mismatched DNA strands with (L)-aTNA. Therefore, we carried out temperature dependent UV spectroscopy studies of ON-2 with various DNA strands (Table 6) (Supplementary Information S5, Fig. S6). Incorporation of one thymine mismatch (T:T:T) in DNA strand resulted in a strong decrease in triplex stability ($\Delta T_m = -12$ °C), while two mismatches destabilized the triplex stability to 60 °C ($\Delta T_m = -22$ °C) as compared to fully complementary DNA strand (ON-6). Further, three mismatches displayed the triplex stability with 49 °C ($\Delta T_m = -33$ °C). Similar results were obtained when cytosines mismatch (T:C:T) were introduced in the DNA strands (Table 6, ON-6d to ON-6f). Incorporation of three cytosine mismatches exhibited less cooperative melting transition as compared to three thymine nucleotide mismatches (Supplementary Information S5, Fig. S6). Furthermore, one and two guanine nucleotide mismatches (T:G:T) also decreased the melting temperature with $\Delta T_m = -12$ °C and $\Delta T_m = -23$ °C respectively (Table 6, ON-6g and ON-6h). These data indicate that incorporation of one pyrimidine

or purine mismatch destabilizes the triple helix stability with similar fashion, which is independent of incorporation site in DNA strand. These results showed the sequence specificity of complementary homopyrimidine (L)-aTNA towards homopurine DNA strands.

Table 6 Thermal melting of triple helices of ON-2 with mismatched DNA strands measured by UV at 260 nm^a

Entry	DNA strands	T _m (°C)	ΔT_m (°C)
ON-6	5'-GATCCAAAAAAAAAAAAAAAAAG-3'	82	0
ON-6a	5'-GATCCAAAAAAAA T AAAAAAAAAG-3'	70	-12
ON-6b	5'-GATCCAA A TAAAAAAAA T AAAG-3'	60	-22
ON-6c	5'-GATCCAA A TAA A TAA A TAAAG-3'	49	-33
ON-6d	5'-GATCCAAAAAAAA C AAAAAAAAAG-3'	70	-12
ON-6e	5'-GATCCAA A CAAAAAAAA C AAAG-3'	60	-22
ON-6f	5'-GATCCAA A C A A A C A A A C A AG-3'	49	-33
ON-6g	5'-GATCCAAAAAAAA G AAAAAAAAAG-3'	70	-12
ON-6h	5'-GATCCAA A GAAAAAAAA G AAAG-3'	59	-33

^a Experimental condition: ON-2 with 4 μM and DNA strands with 2 μM concentration in 10 mM phosphate buffer containing 100 mM sodium chloride at pH 7. Bold and italic letters indicate mismatch nucleotides.

Quaternary complex formation of (L)-aTNA with DNA

Homopyrimidine PNA binds to double-stranded DNA containing a sequence complementary purine target which results in 'P-loop' triplex invasion structure.⁹ In order to investigate the strand invasion properties of (L)-aTNA, DNA strands of 55 nucleotides (ON-16 and ON-17) were synthesized containing d(A)₁₅ nucleotides in the middle. ON-2 was added to the DNA duplex in 10 mM tris buffer containing 100 mM sodium chloride and 10 mM MgCl₂ at pH 8.2 and incubated overnight at 25 °C. However, no invasion product was observed which was indicated by the non-denatured gel electrophoresis (Fig. 8a Lanes 5 and 6). On the other hand, when ON-2 was mixed with the DNA strand containing d(A)₁₅ and incubated for 2 hours followed by the addition of complementary DNA strand then a quaternary product was observed (Fig. 8a Lanes 7 and 8). Moreover, when all three strands were mixed at the same time only a small band of quaternary product was observed (Fig. 8a, Lane 9). For further confirmation we carried out titration of the triplex product with increasing amounts of ON-16.

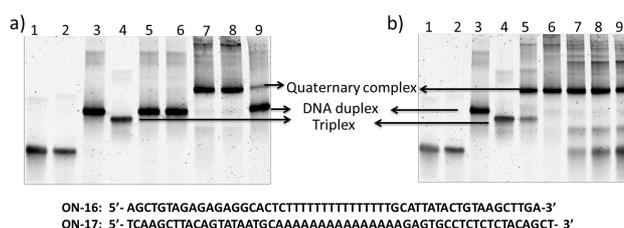


Fig. 8 Quaternary complex of (L)-aTNA with DNA duplex. (a)

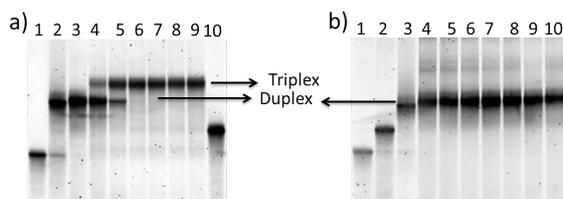
Experiment was carried out by 10 pmol of each DNA strand, Lane 1 = ON-16, Lane 2 = ON-17, Lane 3 = ON-16/ON-17, Lane 4 = ON-17/ON-2 (50 pmol), Lane 5 = ON-16/ON-17 + ON-2 (50 pmol), Lane 6 = ON-16/ON-17 + ON-2 (100 pmol), Lane 7 = ON-17/ON-2 (50 pmol) + ON-16, Lane 8 = ON-17/ON-2 (100 pmol) + ON-16, Lane 9 = ON-16/ON-17/ON-2 (100 pmol). (b) Each lane containing 10 pmol of ON-17 and 50 pmol of ON-2, Lane 1 = ON-16, Lane 2 = ON-17, Lane 3 = ON-16/ON-17, Lane 4 = ON-17/ON-2, Lane 5 = ON-17/ON-2 + ON-16 (5 pmol), Lane 6 = ON-17/ON-2 + ON-16 (10 pmol), Lane 7 = ON-17/ON-2 + ON-

16 (15 pmol), Lane 8 = ON-17/ON-2 + ON-16 (20 pmol), Lane 9 = ON-17/ON-2 + ON-16 (30 pmol). “+” sign in figure caption indicates that (L)-aTNA or DNA was added after 1 hour incubation (10 mM tris buffer with 100 mM NaCl, 10 mM MgCl₂ at pH 8.2) of duplex or triplex.
 5 Conditions: TBM running buffer, 10% non-denatured PAGE at constant power 3 watt.

Fig. 8b (Lanes 5 to 9) suggests the formation of the quaternary product preferably, which rules out the formation of a more complex product, where two strands of ON-16 can bind with triplex (ON-2/ON-17/ON-2). These results indicate that (L)-aTNA is unable to invade the DNA duplex, which might be due to the repulsion of negatively charged phosphate backbone. On the other hand due to the high stability of the triplex (L)-aTNA-DNA-(L)-aTNA, once formed, the complementary strand of the DNA was unable to displace the (L)-aTNA strands. This data indicate very strong interaction of (L)-aTNA towards DNA.

DNA polymerase stop assay

Acyclic oligonucleotides are very stable against nucleases.¹⁰ Construction of such type of fully modified nucleic acids could provide a tool for the development of antisense molecules. Recently, SNA nucleotides have been incorporated in siRNA which showed improved activity and stability. However fully modified SNA did not show any activity towards its target.¹⁰ Since, (L)-aTNA forms highly stable triple helix with DNA or RNA targets, we set out to investigate the DNA polymerase stop assay on a DNA template in the presence of (L)-aTNA. We chose a DNA strand of 55 nucleotides (ON-17) which is containing d(A)₁₅ in the middle of the strand. Interestingly, ON-2 was capable of stopping the primer (ON-18) extension by Bsu DNA polymerase (Fig. 9a Lanes 4 to 10). In order to investigate the specificity of the (L)-aTNA oligomers towards its DNA target we analysed the primer extension stop assay in the presence of ON-7 which was not fully complementary to its DNA target and as a result was unable to stop primer extension (Fig. 9b Lanes 4 to 10). This clearly shows the specificity of (L)-aTNA towards its target. In future (L)-aTNA could provide a platform in order to stop the translation of unwanted protein biosynthesis.



ON-16: 5'-AGCTGTAGAGAGAGGCACTCTTTTTTTTTTTTTTTTTCATTATACGTGAAGCTTGA-3'
 ON-17: 5'-TCAAGCTTACAGTATAATGCAAAAAAAAAAAAAAAAAAGAGTGCCTCTCTACAGCT-3' Template
 ON-18: 5'-AGCTGTAGAGAGAGGCACTC-3' Primer

Fig. 9 DNA polymerase stop assay. Primer extension studies were carried out by 5 pmol of primer (ON-18) and 5 pmol of DNA template (ON-17). (a) Lane 1 = ON-17, Lane 2 = ON-16/ON-17, Lane 3 = ON-17/ON-18 + Bsu Polymerase + dNTPs, Lane 4 = Lane 3 + ON-2 (5 pmol), Lane 5 = Lane 3 + ON-2 (10 pmol), Lane 6 = Lane 3 + ON-2 (20 pmol), Lane 7 = Lane 3 + ON-2 (40 pmol), Lane 8 = Lane 3 + ON-2 (60 pmol), Lane 9 = Lane 3 + ON-2 (80 pmol), Lane 10 = ON-17/ON-18. (b) Lane 1 = ON-17, Lane 2 = ON-17/ON-18, Lane 3 = ON-16/ON-17, Lane 4 = ON-17/ON-18 + Bsu Polymerase + dNTPs, Lane 5 = Lane 3 + ON-7 (5 pmol), Lane 6 = Lane 3 + ON-7 (10 pmol), Lane 7 = Lane 3 + ON-7 (20 pmol), Lane 8 = Lane 3 + ON-7 (40 pmol), Lane 9 = Lane 3 + ON-7 (60 pmol), Lane 10 = Lane 3 + ON-7 (80 pmol). “+” sign in figure caption indicate that (L)-aTNA added after 1 hour of incubation of primer and DNA template.

Conditions: TBE running buffer, 10% non-denatured PAGE at constant power 3 watt.

Conclusions

We have synthesised (L)-aTNA from (L)-threonine amino acid. Temperature dependent UV studies, CD spectroscopy and gel electrophoresis demonstrated that homopurine (L)-aTNA preferably bind with homopurine DNA or RNA targets in the form of triple helical structure. The most stable (L)-aTNA-DNA-(L)-aTNA or (L)-aTNA-RNA-(L)-aTNA triplexes were obtained when one (L)-aTNA strand is associated in anti-parallel orientation and the other (L)-aTNA strand is in the parallel orientation relative to the DNA or RNA strand. Furthermore, we demonstrated that (L)-aTNA was capable of blocking the DNA polymerase by making a triplex with the DNA template. These novel findings reveal the homopyrimidine (L)-aTNA as an efficient artificial nucleic acid for targeting single stranded homopurine DNA or RNA. In turn these properties may have potential for the development of biosensors, therapeutic molecules and it may also find application in DNA nanotechnology. The incorporation of pseudoisocytosine¹¹ in (L)-aTNA scaffold and construction of bis-(L)-aTNA is in progress to abolish the pH-sensitivity and improving the kinetics respectively.

Experimental Section

Circular dichroism (CD): CD experiments were performed in Milli-Q water using a Jasco model J-810 instrument. Quartz cuvettes (Hellma) were used for scanning the samples at path length of 1 mm or 10 mm at 20 °C. The concentration of each oligonucleotide was 5 μM (for 1 mm path length cuvette) or 0.5 μM (for 10 mm path length cuvette) in 10 mM phosphate buffer containing 100 mM sodium chloride at pH 7 or pH 5.8. Samples were scanned at 100 nm min⁻¹ with data pitch 0.2 nm and each CD spectrum was obtained by average of five scans. CD-titration (Fig. 5a) was carried out by making all the samples separately with different concentration ratios and incubated for overnight at 25 °C. CD spectra were baseline subtracted and smoothed. RNAs were purchased from Sigma-Aldrich.

Ultraviolet-melting: Temperature dependent UV spectroscopy experiments were carried out on Varian Cary 300 BIO instrument. Quartz cuvettes (Hellma) were used for scanning the samples at path length of 10 mm in 10 mM phosphate buffer with 100 mM sodium chloride at pH 7 or pH 5.8. Samples were scanned (heating and cooling ramp) at a rate of 1 °C/min at 260 nm. Melting temperature (*T_m*) was determined by first derivative of melting curve.

Electro mobility shift assay (EMSA): A known amount of (L)-aTNA was mixed with complementary single stranded DNA in 10 mM phosphate buffer containing 100 mM sodium chloride at pH 7 or 10 mM tris buffer containing 100 mM sodium chloride and 10 mM magnesium chloride at pH 8.2. Samples were incubated at room temperature (~25 °C). Loading buffer was added with final 1x concentration containing bromophenol blue

as tracking dye. The complexes were separated on 10 or 12% non-denaturing gel (polyacrylamide) at low temperature (3 °C) with 1x TBE (89 mM Tris-borate and 2 mM EDTA, pH 8.3) or 1 x TBM (100 mM Tris-borate and 5 mM MgCl₂, pH 8.2) running buffer at constant power 3 watt and visualized by fluorescent intercalating dye.

Primer extension studies: The inhibition of primer (5 pmol) extension on DNA template (5 pmol) by Bsu DNA polymerase (2 units) was carried out with dNTPs (200 μM) in 10 μl buffer. Increasing amount of (L)-aTNA was added in order to stop the DNA polymerase. The reaction mixtures were incubated overnight at 25 °C. Reaction mixture was analyzed by non-denature gel electrophoresis in TBE buffer. DNA polymerase and dNTPs were purchased from New England Biolabs.

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