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Modulation of the stability of i-motif structures using an acyclic threoninol cytidine derivative†

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The effect of aTNA (acyclic threoninol nucleic acids) units on the stability of intramolecular i-motifs was investigated by spectroscopic techniques. The replacement of selected positions of the C-core can modulate the stability at different pH ranges.

The i-motif is a secondary structure found in C-rich DNA sequences, which is stabilised under acidic conditions. The structure comprises two strands arranged in a parallel-type duplex with hemiprotonated $C \cdot CH^+$ base pairs, and two of these duplexes are held together in an antiparallel fashion by intercalation of each $C \cdot CH^+$ base pair (Fig. 1a).[1]

From the biological point of view, the presence of i-motif-forming sequences in telomeric and promoter regions supports the hypothesis that they may be functionally relevant.[2] Recently, the role of these structures as modulators of gene expression has been assessed.[3] Additionally, these structures are interesting in the field of nanotechnology, since for instance they have been used to control the assembly of supramolecular structures[4] or in the design of molecular motors[5] and sensing systems such as nanoswitches driven by pH changes to monitor biological processes.[6] Desirable properties regarding this last application points towards the necessity of nuclease resistant i-motifs and the availability of different pH-sensors suited to different pH regimes.[6a] The stability of i-motif structures is highly dependent of the base sequence.[7] It has been reported that simple manipulations of the C-core[8] or the loops[9] can modulate the pH sensitivity and also the introduction of chemical modifications.[2] In particular, sugar modifications of

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the cytidine core[4] have pointed the relevance of the sugar–sugar contacts in the stability of i-motifs.[10]

Threoninol is an acyclic scaffold that has been used to design new artificial nucleic acids (aTNA), capable of forming exceptionally stable homo-duplexes.[11]

Moreover, aTNA's can form stable hetero-duplexes with DNA and RNA in a parallel manner,[12] and also they are compatible with parallel G-quadruplex structures.[13] Recently, this acyclic scaffold has been used to design functional RNA/aTNA chimeras with improved nuclease resistance.[14] To date the effect of this modification in the formation of i-motifs has not been tested. In sight of these results, we are interested in study the effect of this sugar replacement on the thermal and pH stability.

The present work is based on a well described C-rich fragment of the vertebrate telomere $(C_3TA_2)_3C_3T$, which folds intramolecularly, where four cytidine stretches connected by three TAA loops form six $C \cdot CH^+$ base pairs.[15] In this work the loop bases were substituted by thymines and then, different positions of the C-core stretches of the 24 nt fragment $(C_3T_3)_4(Lt\_0)$ were systematically replaced by C_{LT} units (Fig. 1b), (Table 1). For detailed information see ESI† (Sections 6–8). Lt_0 was modified by a single C_{LT} substitution at various positions of the 5'-terminal stretch (Lt_1, Lt_2 and Lt_3) and more internal
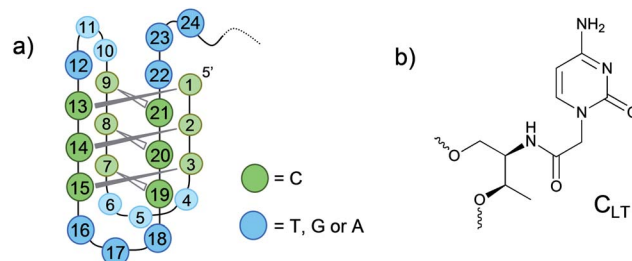

Fig. 1 (a) Schematic representation of a potential folding topology of the intermolecular i-motif studied in this work. (b) Chemical structure of C_{LT}.

positions (Lt_13, Lt_14 and Lt_15). Additionally, oligonucleotides containing two C_{LT} modifications involved in the same $C·CH^+$ base pair (Lt_1/13, Lt_2/14 and Lt_3/15) were studied. Finally, Lt_1/2/3 with three consecutive C modifications in the 5′-terminal stretch was included in the analysis. We assessed the thermodynamic stability of the modified 24 nt fragments by monitoring the absorbance at 295 nm as a function of temperature (Fig. S1, ESI†) and compared with the non-modified version (Table 1). For additional information see ESI† (Section 2).

At pH 5.3 the UV melting analyses reveal that all the C_{LT} substitutions reduce significantly the T_m, and the effect is highly dependent of the position. Single C_{LT} substitutions near the loops are less destabilizing than those in the middle of C-tracts. Similar effect has been observed with other acyclic backbone modifications.[10d] The effect of C_{LT} substitutions is not additive. When the two cytidines involved in a $C·CH^+$ base pair were replaced, the T_m is slightly lower than in single substitutions. The effect is opposite when the $C·CH^+$ is located in the center of the C-tracts (Lt_2/14). Finally, Lt_1/2/3 shows a higher thermal stability than some of the oligonucleotides containing one or two modifications. In all cases the values of Gibb's free energies follow the same pattern observed with the T_m values as expected because depend on the equilibrium constant. Analysing the enthalpic term for oligonucleotides containing one or two C_{LT} substitutions near the loops, it can be observed than the ΔH^0 exhibit a similar decrease indicating than the destabilization is mostly of enthalpic origin. The influence of the enthalpic change is compensated by opposite change in the entropy term leaving the influence on Gibbs's energy rather modest. A single replacement in central positions of a cytidine core causes a higher drop of ΔH^0, especially when is implicated the 5′-terminal stretch, suggesting that the $C·CH^+$ base pair is affected and additionally the distortion may be affecting the neighbouring base pairing. Surprisingly, when both modifications occupy the central part of the core, it leads to a more stable structure than the ones obtained with Lt_2 and Lt_14. Taking into account that in this case the enthalpic term is more

favourable, it can be reasoned that the pairing $dC–C_{LT}$ is less tolerated than the $C_{LT}–C_{LT}$ pair. Finally, Lt_1/2/3 has the highest enthalpic term, but at the same time the most unfavourable entropic term.

Also we investigated the i-motif formation by CD spectroscopy. In Fig. 2 there are depicted a selection of some CD spectra. For detailed information see ESI (Fig. S2 and Table S2†). Lt_0 has the characteristic i-motif signature,[16] with positive and negative peaks around 287 and 262 nm respectively and as well all modified sequences show this specific profile. Comparing with Lt_0, in general the modifications produce blue shifts of the positive and/or negative bands and a decrease in the molar ellipticity to a greater or lesser extent. The most affected is the version containing three C_{LT} residues, followed by the oligonucleotides containing a substitution in an internal position of the 5′-terminal stretch. As CD spectroscopy is sensitive to structural changes,[17] it can be reasoned that the deviations in the CD spectra from the natural sequence would be more pronounced as higher is the degree of distortion introduced in the i-motif structure.

Since structural changes may affect the pH stability, we decided to compare the pH dependence of two modified oligonucleotides with the unmodified version. We selected the one showing the most altered CD spectrum (Lt_1/2/3) and one showing moderate changes (Lt_3). The unfolding of the i-motif with pH at 25 °C was monitored at 295 nm. By plotting the unfolded fraction against pH and adjusting to a sigmoidal (see Section 4 ESI†) we obtained the titration curves showed in Fig. 3. The pH transition midpoint (pH_T) and the transitional range (R_T, range between 10 and 90% of unfolded structure) were calculated from the adjusted curves (Table 2). Comparing Lt_3 with Lt_0, the R_T has been slightly widened and also shifted toward acidic pH values, showing a pH_T around pH 6.00. Lt_1/2/3 as well shows a decrease in the pH_T, but the R_T is almost 3-fold wider. The presence of three consecutive C_{LT} in the 5′-external stretch introduces higher flexibility.

These effects may lead to the formation of several misfolded conformations provoking a decreased cooperativity.

Next, we performed 1D ^1H NMR experiments with Lt_0, Lt_3 and Lt_1/2/3 at different temperatures to obtain more detailed structural information (see Fig. S4, ESI†). Fig. 4 shows for each oligonucleotide the experiments recorded at 5 °C. In the three

Table 1 Thermal stabilities and thermodynamic parameters of Lt_0 and modified oligonucleotides at pH 5.3[a]

Name[b]	T_m	ΔT_m	ΔG^0_{298}	$\Delta\Delta G^0_{298}$	ΔH^0	$T\Delta S^0_{298}$
Lt_0	47.8	—	−5.7	—	−79.3	−73.7
Lt_1	39.5	−8.3	−2.9	2.8	−61.7	−58.8
Lt_2	29.1	−18.7	−0.6	5.1	−45.6	−45.0
Lt_3	38.4	−9.4	−2.6	3.1	−60.9	−58.4
Lt_13	37.4	−10.4	−2.7	3.0	−67.4	−64.8
Lt_14	29.3	−18.5	−0.8	4.9	−56.3	−55.5
Lt_15	37.9	−9.9	−2.7	3.0	−65.1	−62.4
Lt_1/13	35.4	−12.4	−2.3	3.4	−67.5	−65.2
Lt_2/14	32.4	−15.4	−1.6	4.1	−66.4	−64.8
Lt_3/15	34.7	−13.1	−2.1	3.6	−65.8	−63.7
Lt_1/2/3	35.6	−12.2	−2.4	3.3	−69.3	−67.0

[a] T_m is expressed in °C and ΔG^0_{298}, ΔH^0 and $T\Delta S^0_{298}$ in kcal mol^{-1}. Experimental errors are shown in Table S1 (see ESI). [b] The numbers indicate the positions substituted. $C_{oligonucleotide} = 2.7$ μM.
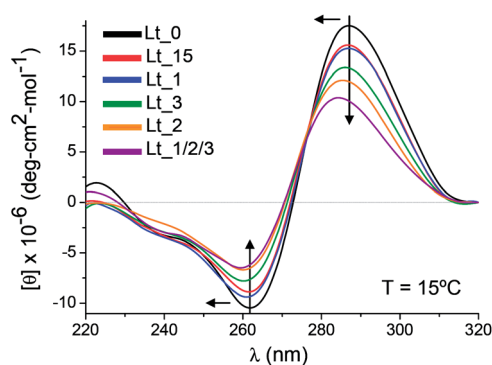

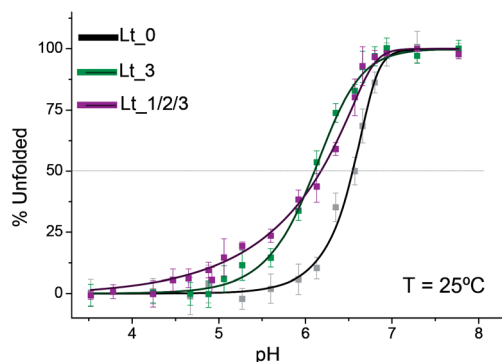**Fig. 2** CD spectra of Lt_0 and modified oligonucleotides at pH 5.3.

Fig. 3 pH titration of Lt_0, Lt_3 and Lt_1/2/3.

Table 2 pH transition midpoint and transitional range for Lt_0, Lt_3 and Lt_1/2/3

Name	pH_T	pH_{10}	pH_{90}	R_T
Lt_0	6.54 ± 0.02	6.05 ± 0.02	6.71 ± 0.02	0.66 ± 0.04
Lt_3	6.00 ± 0.08	5.42 ± 0.02	6.20 ± 0.07	0.78 ± 0.09
Lt_1/2/3	6.16 ± 0.02	4.80 ± 0.02	6.70 ± 0.02	1.90 ± 0.04

cases, the NMR spectra are consistent with i-motif formation. The characteristic imino signals of protonated cytosines are clearly observed at 15.0–16.0 ppm,[7,15] as well as the thymine imino protons in the loop regions (around 11.0 ppm), in agreement with previous studies reported in the literature.[15] The differences in the exchangeable protons spectra between the unmodified and Lt_3 and Lt1/2/3 are significant, indicating the effect of the acyclic threoninol backbone propagates up to the base pairs, affecting the core of the i-motif structure. Such effect is particularly pronounced in the case of Lt/1/2/3. The unfolding of the structure with increasing temperature can be followed in Fig. S4 in ESI.† In the unmodified oligonucleotide, the imino protons are observed up to 45 °C. In agreement with UV melting experiments, the exchangeable protons signals of Lt_3 and Lt_1/2/3 disappear at lower temperatures than the unmodified oligonucleotide.

In summary, we evaluated for the first time the effect of deoxy-sugar replacements by C_{LT} substitutions in the ability to form intramolecular i-motifs. Different positions of the C-core were replaced and the thermal and thermodynamic stability have been studied. In general the C_{LT} modifications reduced significantly the stability of the resulting structures and the effect is dependent of the position and the type of pair implicated, being the pairing dC–C_{LT} less favourable than the C_{LT}–C_{LT} pair. When a single substitution was introduced, the positions with a lesser decrease in stability are the positions near the loops.

In addition the pH stability of Lt_3 and Lt_1/2/3 was studied. In comparison with Lt_0, a single C_{LT} substitution produced a decrease of pH_T of about 0.54 units and maintained a comparable range of response. The substitution of three consecutive cytidines led to a less cooperative transition. Recently it has
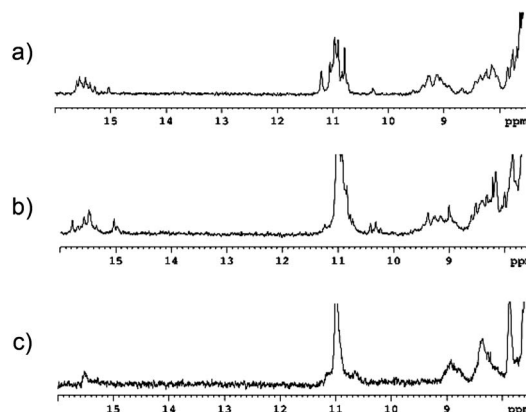

Fig. 4 NMR spectra at 5 °C and pH = 4.2 of (a) Lt_0, (b) Lt_3 and (c) Lt_1/2/3.

been described than the L-threoninol nucleic acids are more stable to nuclease degradation than unmodified oligonucleotides.[14] For this reason the possibility of modulating the pH stability of i-motifs by selected replacements of cytidines by C_{LT} modifications may allow the design of nuclease resistant i-motif structures suited for different pH ranges.

Acknowledgements

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