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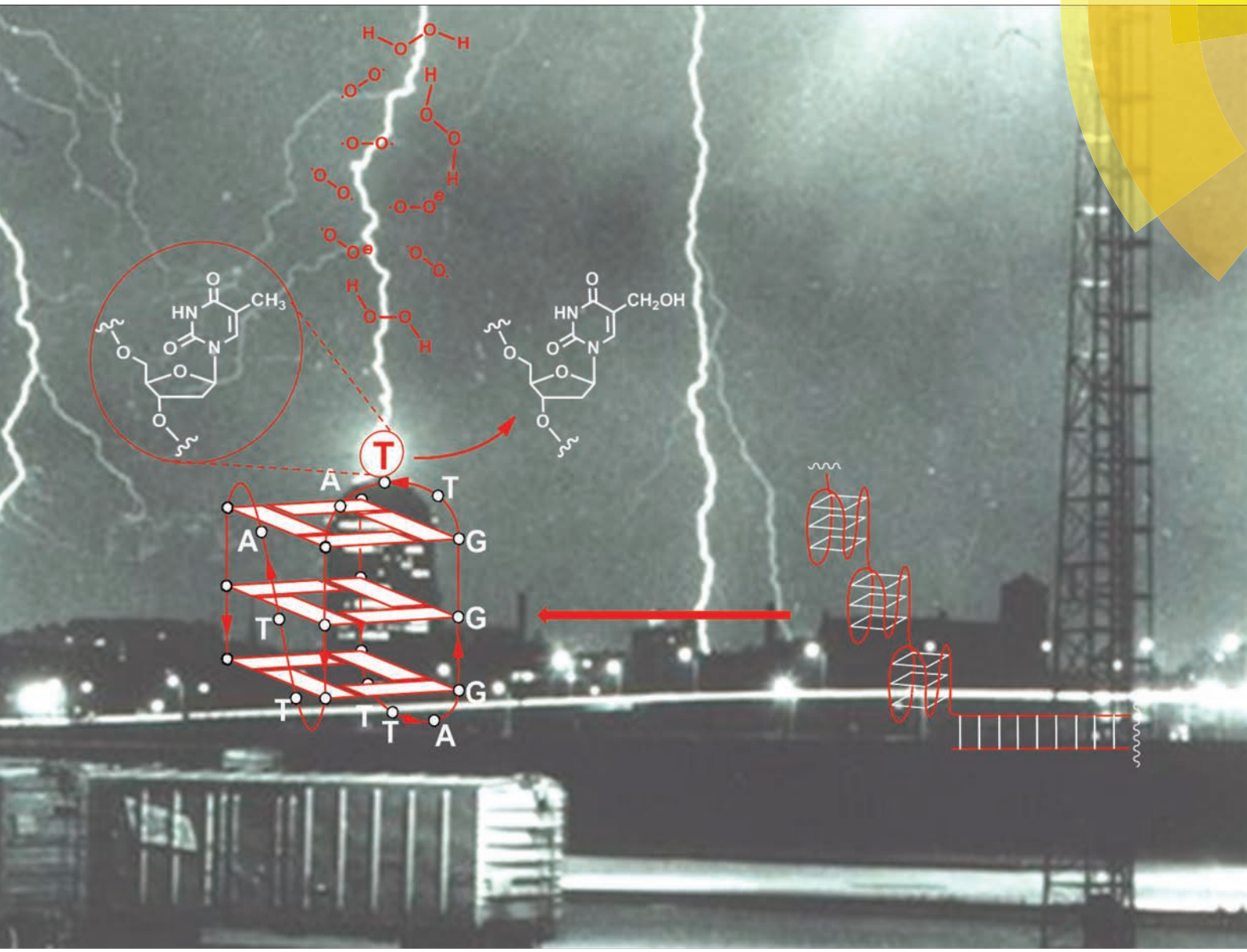

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The oxidative damage to the human telomere: effects of 5-hydroxymethyl-2′-deoxyuridine on telomeric G-quadruplex structures†

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As part of the genome, human telomeric regions can be damaged by the chemically reactive molecules responsible for oxidative DNA damage. Considering that G-quadruplex structures have been proven to occur in human telomere regions, several studies have been devoted to investigating the effect of oxidation products on the properties of these structures. However only investigations concerning the presence in G-quadruplexes of the main oxidation products of deoxyguanosine and deoxyadenosine have appeared in the literature. Here, we investigated the effects of 5-hydroxymethyl-2′-deoxyuridine (5-hmdU), one of the main oxidation products of T, on the physical–chemical properties of the G-quadruplex structures formed by two human telomeric sequences. Collected calorimetric, circular dichroism and electrophoretic data suggest that, in contrast to most of the results on other damage, the replacement of a T with a 5-hmdU results in only negligible effects on structural stability. Reported results and other data from literature suggest a possible protecting effect of the loop residues on the other parts of the G-quadruplexes.

Introduction

Nucleic acids are able to adopt several types of secondary non-B conformations. Among these, G-quadruplex structures, which can form in guanine-rich sequences, have received particular attention due to their dramatic stability and surprising polymorphism and because many sequences prone to form them have been identified in the human genome, particularly in telomeres,[1] gene promoters of oncogenes as *c*-kit, *c*-myc and KRAS[2] and untranslated regions.[3] As for the whole genome, these regions are under a constant barrage of chemically reactive molecules that can cause damage. Among these, the reactive oxygen species (ROS) are the most important contributors to harmful effects on DNA,[4] being continuously produced during normal metabolism and arising from environmental exposures to exogenous chemicals and ionizing radiation. Taking into account the increasing biological importance of G-quadruplex structures and, particularly, their relationship with cancer diseases, investigations on the effect of naturally occurring modified nucleotides originating from DNA lesions on their properties are considered an interesting research area. As a matter of fact, in G-quadruplex structures the presence of modified nucleotides or abasic sites,[5] often originating from them, could decrease their thermal stability, promote alternative conformations, shift the duplex/quadruplex equilibrium and negatively affect the interaction with the proteins involved in their biological functions. Due to their biological relevance, G-quadruplex structures formed by telomeric sequences containing modified bases originating from DNA damage have been first investigated, particularly taking into account that oxidative stress contributes to telomere shortening that, in turn, is directly associated with cell mortality and aging.[6–8]

Since G-quadruplexes are mostly composed of 2′-deoxyguanosines (dG), several investigations have been devoted to the effects of their oxidation products in G-quadruplex structures.[9–12] On the other hand, less attention has been paid to studying the effects of modified nucleotides originating from the oxidation of the loop residues, although, in principle, the loop bases could be more prone to damage because of their higher accessibility to exogenous harmful agents, alkylating ligands[13–16] and singlet oxygen sensitizers,[17,18] in comparison with the bases in the stem region involved in the G-tetrad formation. Interestingly, recent studies have clearly highlighted the importance of loop residues in the structural

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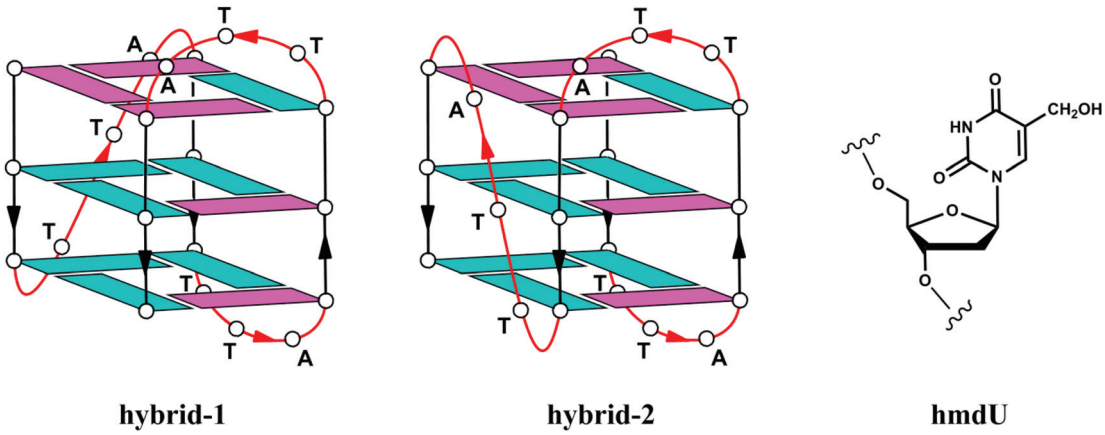

Fig. 1 Schematic representation of the G-quadruplex structures hybrid-1 and hybrid-2 occurring in human telomeric sequences (left and center). *Anti* and *syn* residues are in light blue and purple, respectively. Chemical structure of 5-hydroxymethyl-2′-deoxyuridine (5-hmdU) (right).

folding preference and stability of G-quadruplex structures.[19–22] The loop sequence of the telomeric G-quadruplex structures (TTA) is mostly composed by thymidines (T) but, curiously, only one study concerning the presence of the main oxidation product of the 2′-deoxyadenosine (dA), namely 8-oxo-7,8-dihydrodeoxyadenosine (8-oxo-dA), has appeared in the literature,[23] while investigations devoted to the presence in loops of T oxidation products are still lacking. The modified base 5-hydroxymethyluracil (5-hmUra, Fig. 1) has been identified as one of the major products of thymine oxidation,[24] however recent investigations suggest that this base could also originate from enzymatic oxidation and deamination of 5-methylcytosine.[25,26] 5-HmUra forms from hydroxyl radical attack on the 5-methyl position of thymine in DNA.[27] It has been estimated to form in humans approximately 620 times per cell per day, largely resulting from the reactivity of by-products from normal cellular metabolism.[28,29]

Increased 5-hydroxymethyl-2′-deoxyuridine (5-hmdU) formation in tissue DNA has been connected with human diseases, and 5-hmdU is considered a biomarker of oxidative DNA damage and of breast cancer.[30–32]

It is important to note that in a recent investigation concerning the G-quadruplex structures adopted by modified TBAs in which the loop thymidines were replaced, one at a time, by 5-hmdU residues, a position dependent effect on the thermal structural stability has been evidenced.[33]

For these reasons, in the present study we investigate the effects of 5-hmdU residues in the human telomeric sequence $(T_2AG_3)_4T_2$ (wtTel26) able to form a main monomolecular quadruplex in potassium ions solutions (hybrid-2, Fig. 1).[34] Furthermore, 5-hmdU containing analogues of the human telomeric sequence $AG_3(T_2AG_3)_3T_2$ (wtTel22)[35] have also been investigated in order to directly compare the effects of the presence of the T and dA oxidation products, considering that ODN wtTel22 has been already used in investigating 8-oxo-dA containing telomeric sequences.[23] Each of the sequences in which a thymidine has been replaced, one at a time, by a 5-hmdU residue (Table 1), has been studied by calorimetric (DSC: differential scanning calorimetry) circular dichroism (CD) and electrophoretic techniques.

Table 1 Sequences of the ODNs studied containing 5-hydroxymethyl-2′-deoxyuridine (H) and their natural counterparts

Name	Sequence
wtTel22	AGGGTTAGGGTTAGGGTTAGGG
Tel22-H5	AGGGHTAGGGTTAGGGTTAGGG
Tel22-H6	AGGGTHAGGGTTAGGGTTAGGG
Tel22-H11	AGGGTTAGGGHTAGGGTTAGGG
Tel22-H12	AGGGTTAGGGTHAGGGTTAGGG
Tel22-H17	AGGGTTAGGGTTAGGGHTAGGG
Tel22-H18	AGGGTTAGGGTTAGGGTHAGGG
wtTel26	TTAGGGTTAGGGTTAGGGTTAGGGTT
Tel26-H7	TTAGGGHTAGGGTTAGGGTTAGGGTT
Tel26-H8	TTAGGGTHAGGGTTAGGGTTAGGGTT
Tel26-H13	TTAGGGTTAGGGHTAGGGTTAGGGTT
Tel26-H14	TTAGGGTTAGGGTHAGGGTTAGGGTT
Tel26-H19	TTAGGGTTAGGGTTAGGGHTAGGGTT
Tel26-H20	TTAGGGTTAGGGTTAGGGTHAGGGTT

Results and discussion

Gel electrophoresis

The modified and unmodified telomeric sequences containing 5-hmdU were first analyzed by PAGE. Since the modified ODNs used in this study show the same length and have almost the same molecular weights as their natural counterparts wtTel22 and wtTel26, any difference in mobility should result from the conformational differences. However, the electrophoretic profile (Fig. 2) clearly shows that both wtTel22 and wtTel26 analogues are characterized by bands with electrophoretic motilities very similar to those of their natural telomeric sequences, thus strongly suggesting that the presence of a

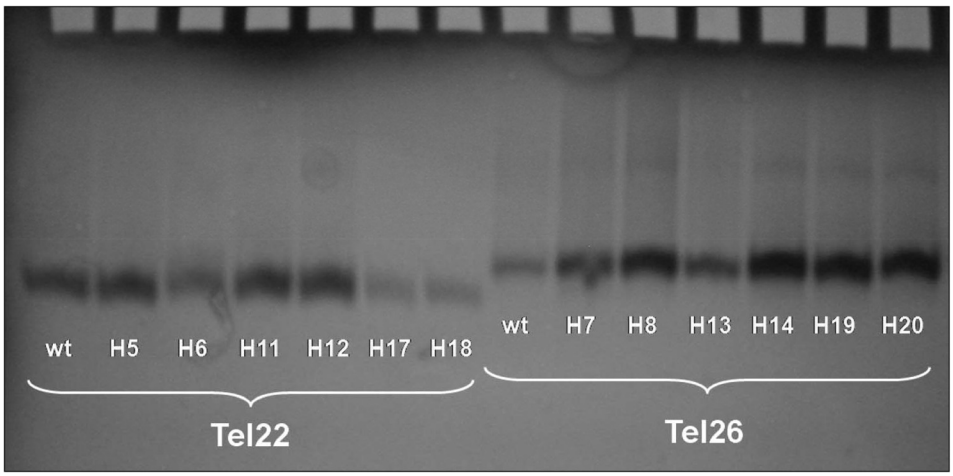

Fig. 2 Non-denaturing PAGE of the 5-hmdU containing ODNs investigated and their natural counterparts. Gel was performed at 8 V cm^{-1} and a temperature close to 5 °C. Bands were visualized by UV shadowing. See Experimental section for further details.

5-hmdU residue does not affect the monomolecular folding in the G-quadruplex structures distinctive of the natural sequences. As expected, wtTel26 and its analogues show slightly slower migrating bands than wtTel22 and its analogues, considering that they have two additional thymine residues at the 3′- and 5′-ends.

CD spectra and CD thermal analysis

Circular dichroism was used to explore the effect of the substitution of T with 5-hmdU residues on the telomeric G-quadruplex conformation. The CD spectra of wtTel22 and wtTel26 in potassium buffer solution are shown in Fig. 3 together with

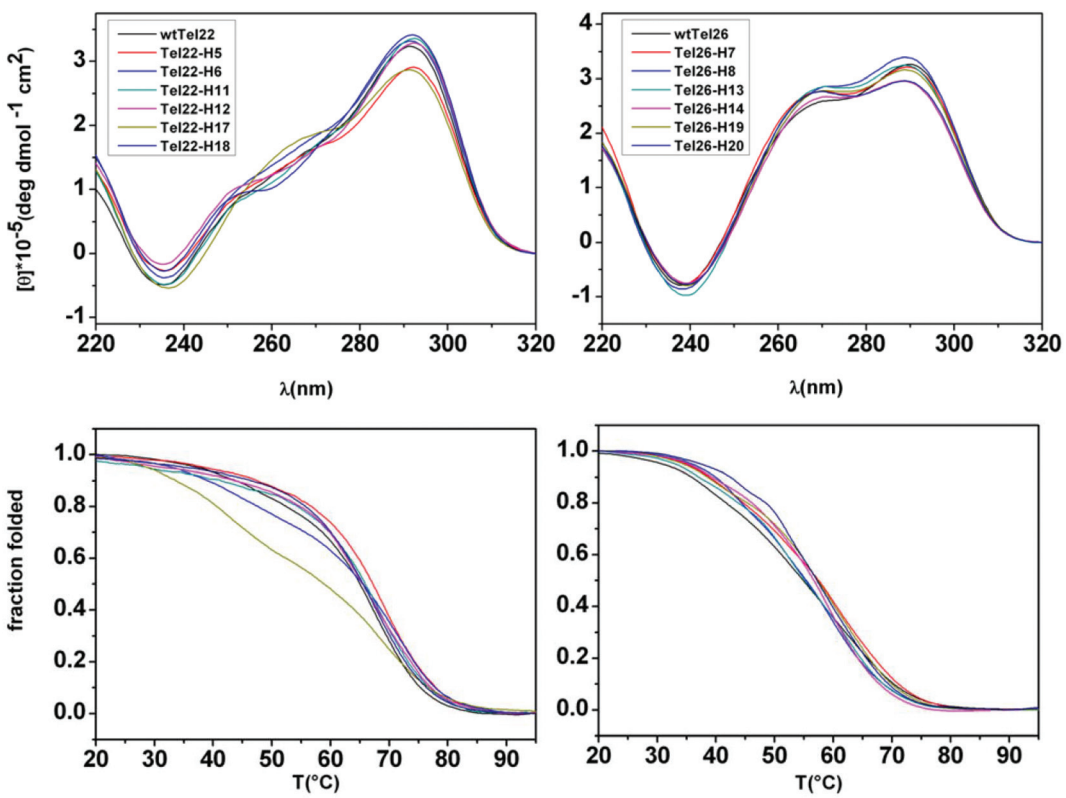

Fig. 3 CD spectra (top) and CD melting profiles (bottom) of the wtTel22 and its analogues (left) and wtTel26 and its analogues (right).

the CD spectra of the modified sequences. The CD spectra of both wtTel22 and wtTel26 show the characteristic profile of the (3 + 1) hybrid-type quadruplex conformation with a positive band around 290 nm, a shoulder around 265 nm and a negative band at 240 nm.[36] Most of the CD spectra for the sequences containing 5-hmdU show very similar profiles to the corresponding unmodified telomeric sequence, suggesting that the 5-hmdU introduction does not drastically change the quadruplex conformation. A noticeable change in the intensity of the 290 nm band was observed only for **Tel22-H5** and **Tel22-H17**, however the characteristic profiles of the hybrid-type quadruplex conformation were retained also for these two sequences.

To evaluate the effect of the introduction of 5-hmdU residues on the G-quadruplex thermal stability, the melting of the modified oligonucleotides was followed by CD experiments. The reversibility of the folding/unfolding process was verified by superimposition of the melting and annealing curves. The melting profiles of the modified sequences are quite similar to that of the relative natural sequence, revealing that the introduction of 5-hmdU has a slight impact on the G-quadruplex thermal stability (Fig. 3). However, CD melting profiles of **Tel22-H6** and **Tel22-H17** show two inflection points, thus suggesting the presence of an intermediate species in the unfolding process.

To obtain more insight into the temperature-induced denaturation process of the studied sequences, CD spectra were recorded on changing temperature in the range 20–95 °C (Fig. S1–S3 in the ESI†). The observed spectral evolution, for both sequences and their modified analogues, is consistent with the transition from the initial hybrid-type conformation, at low temperature, to the unfolded single strand at high temperature. However, no isoelliptic points were observed for all the studied sequences suggesting complex unfolding pathways.

To get more information on the number of species involved in the unfolding processes, the SVD analysis on CD data was performed (see Experimental section for details). Fig. 4 shows the difference between the primary CD melting data of **Tel22-H17** (Fig. S1 in the ESI†) and the reproduced CD spectra obtained by using the first two (Fig. 4, top) or three (Fig. 4, bottom) basis spectra obtained from SVD analysis. Inspection of Fig. 4 clearly shows that merely two spectral components are not enough to describe the whole spectral behavior (as the residual significantly deviates from the random noise) and at least a third spectral component must be added to have a residual near to the noise level. The first 6 singular values with the corresponding autocorrelation functions of the basis spectra (columns of *U*) and the coefficient vectors (columns of *V*) are reported in Tables S1 and S2 in the ESI† for all the studied sequences, together with the contribution of each singular value to the total variance. SVD results for the corresponding unmodified sequences wtTel22 and wtTel26 are reported in Table S3 in the ESI.† The magnitudes of the singular values provide the first indication of the number of significant spectral species. We found that, for the majority of the studied sequences, the first 3–4 singular values appear to

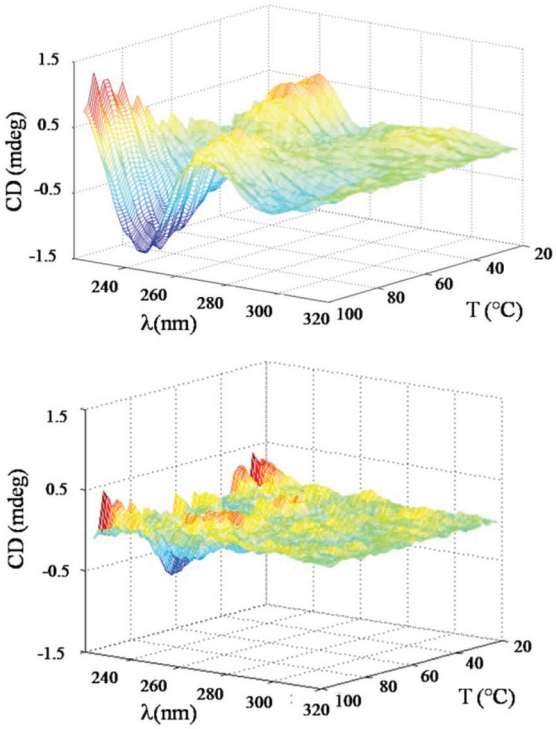

Fig. 4 3D residual plots obtained from the difference between the original experimental data matrix of the sequence **Tel22-H17** and data matrices computed by using the first two (top) and three (bottom) basic spectra obtained from SVD analysis of the CD spectra *vs.* temperature of **Tel22-H17** (reported in Fig. S1†). Red color represents regions with the larger positive deviation between experimental and computed data whereas blue color represents regions with the larger negative deviation.

significantly deviate from the remaining *S* values. This observation, together with the analysis of the magnitudes of the autocorrelation factors of the *V* and *U* matrices and of the relative variance (RV) associated with each *S* value (Tables S1–S3 in the ESI†) reveals that, in all cases, at least three main conformations are involved in the whole melting processes. These results are in agreement with previous studies reporting intermediate species formation in the folding/unfolding process of the unmodified human telomeric quadruplexes.[37–39]

DSC thermodynamic analysis

Differential scanning calorimetry was used to characterise the unfolding thermodynamics for all the studied sequences. Fig. 5 shows the calorimetric profiles for the wtTel22 and wtTel26 compared with DSC profiles of their modified analogues. All the sequences show wide DSC peaks spanning ~30–40 °C, thus suggesting the presence of multiple transitions. The DSC peaks of **Tel22-H5** and **Tel22-H6** clearly show a significant shoulder at lower temperatures, whereas two overlapping transitions are evident in the DSC profile of **Tel22-H17**. These observations are consistent with the SVD results showing the presence of an intermediate species in the unfolding pathway.

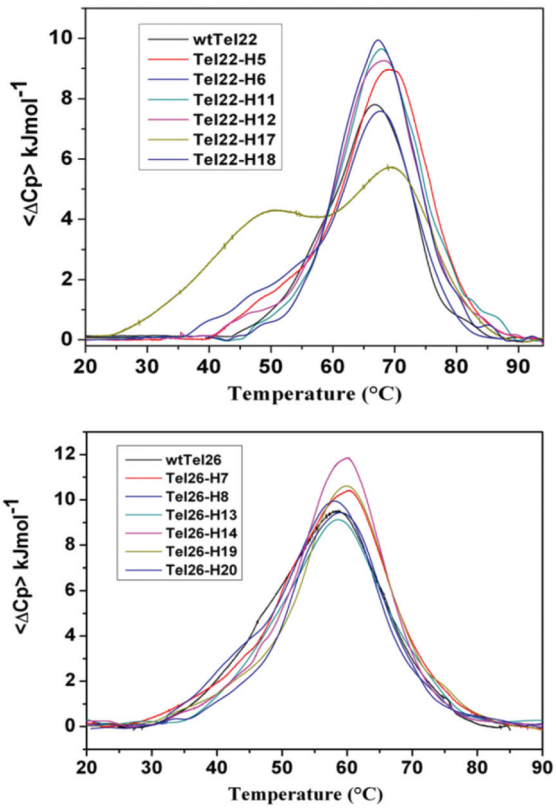

Fig. 5 Differential scanning calorimetry profiles for wtTel22 (top), wtTel26 (bottom) and their modified analogues. All the solutions contain 10 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0.

Table 2 Thermodynamic parameters for the unfolding processes of the studied G-quadruplexes measured by the differential scanning calorimetry

Sequences	ΔH_{cal} (KJ mol^{-1})	ΔS_{cal} (KJ K^{-1} mol^{-1})	T_m (°C)
wtTel22	150 ± 14	0.44 ± 0.04	67.0 ± 0.5
Tel22-H5	170 ± 16	0.49 ± 0.05	69.4 ± 0.5
Tel22-H6	141 ± 12	0.41 ± 0.04	68.3 ± 0.5
Tel22-H11	164 ± 15	0.47 ± 0.05	68.0 ± 0.5
Tel22-H12	162 ± 16	0.47 ± 0.06	67.5 ± 0.5
Tel22-H17	190 ± 19	0.55 ± 0.05	68.5 ± 0.5 48 ± 1
Tel22-H18	166 ± 15	0.47 ± 0.05	68.4 ± 0.5
wtTel26	213 ± 15	0.64 ± 0.06	58.0 ± 0.5
Tel26-H7	225 ± 18	0.67 ± 0.07	60.4 ± 0.5
Tel26-H8	208 ± 16	0.62 ± 0.06	58.2 ± 0.5
Tel26-H13	200 ± 16	0.60 ± 0.06	58.4 ± 0.5
Tel26-H14	230 ± 18	0.68 ± 0.07	60.3 ± 0.5
Tel26-H19	201 ± 16	0.60 ± 0.06	60.4 ± 0.5
Tel26-H20	223 ± 22	0.67 ± 0.07	59.0 ± 0.5

In Table 2 are collected the thermodynamic parameters for the whole unfolding process of the studied sequences. The melting temperatures are in good agreement with those obtained by CD measurements, considering that the DNA concentrations used in DSC measurements are approximately 4–5 times higher than those used in CD melting experiments; this observation is further evidence of the monomolecular nature of the observed transitions. The enthalpy values for wtTel22 and wtTel26 are in agreement with the values previously reported.[40–42] The enthalpy changes for the modified analogues are similar or slightly higher than the enthalpy change of the corresponding unmodified sequence. In particular, the sequences **Tel22-H5**, **Tel22-H17** and **Tel26-H14** show an enthalpy change 20–40 kJ mol^{-1} higher than the reference sequences. In addition, these sequences also show the highest values of the entropy change for the unfolding process, suggesting that their folded structures are more rigid and well-structured in comparison with the corresponding unmodified sequence and the other modified analogues. As noted before, **Tel22-H17** is the only sequence that shows two well resolved transitions in both CD and DSC profiles. It is noteworthy that **Tel22-H17** is also the only sequence that has the intermediate species thermodynamically relevant at physiological temperature. Beyond this exception, however, calorimetric and CD data taken together suggest that the presence of 5-hmdU does not perturb significantly the quadruplex structure.

Discussion

Several studies have been devoted to investigating the effects of oxidized bases in G-quadruplex structures whose presence has been ascertained in telomeres, particularly considering the sensitivity of these genome regions to oxidative stress.[43] Taking into account that dG is the most abundant nucleotide in the telomeric sequences, some studies have concerned the effect of both the main guanine oxidation product, namely 8-oxo-dG,[9,10] and abasic sites deriving from it,[44,45] on the physical–chemical properties of G-quadruplex structures adopted by these sequences. However, apart from guanosines, telomeric sequences are also composed of adenosines and thymidines forming the loop regions of the telomeric G-quadruplex structures. Two investigations have already been devoted to the effect on telomeric G-quadruplex structures of 8-oxo-dA residues,[23] on the one hand, and its corresponding abasic sites in the loop regions, on the other hand.[46] By investigating for the first time the effect of thymidine oxidation to 5-hmdU on the loop regions of telomeric G-quadruplexes, the present study completes the studies concerning the effects of oxidation products on the properties of these DNA secondary structures.

5-hmdU is one of the major products of thymidine oxidation and has been identified in DNA exposed to ionizing radiation[43] as well as to H_2O_2/Fe^{2+}/EDTA.[47,48]

The effect of this modified nucleoside on human telomeric regions has been approached by investigating two different sequences (Table 1) that have been suggested to form or a mixture of hybrid-1 and hybrid-2 G-quadruplexes (wtTel22) or hybrid-2 as the major structure (wtTel26) (Fig. 1). For both the human telomeric sequences, ODNs analogues each containing a 5-hmdU residue replacing a thymidine have been prepared (Table 1).

The electrophoretic analysis of the modified sequences in comparison with their natural counterparts has suggested that the presence of a 5-hmdU residue in a loop, regardless the position in the sequence, does not interfere with the monomolecular folding of the original sequence. Furthermore, CD investigations, besides confirming the monomolecular nature of the G-quadruplexes formed by the modified ODNs, clearly indicated that the replacement of a T with a 5-hmdU residue does not significantly alter the original G-quadruplex conformations adopted by the natural sequences and their thermal stabilities.

In view of the above described results, a comparison between data concerning the presence of 5-hmdU residues in human telomeric sequences with those obtained for other oxidative base lesions and abasic sites is of particular interest. In this frame, it can be useful to discuss separately and distinguish the effects of the oxidation on G-residues, thus forming the stacked G-tetrads in the G-quadruplex scaffold, from those on A- and T-residues in the loop regions of the structures.

According to investigations in K^+ solution concerning properties of the G-quadruplex structures adopted by modified human telomeric sequences 8-oxo-dG containing $T_2G_3(T_2AG_3)_3A$[9] and $G_3(T_2AG_3)_3$,[10] this type of oxidative modification is quite detrimental for the structural stability, particularly if the replacement concerns G-residues involved in the formation of the central G-tetrad. In a similar paper, the effect of abasic sites in the latter sequence has been also investigated, thus reaching analogous conclusions: the abasic sites are able to negatively affect structural stability, in particular if they occur in the central G-tetrad.[45] In a further investigation concerning the presence of abasic sites in a different human telomeric sequence, namely $TA(G_3T_2A)_3G_3$, similar data have been described.[46]

An interesting study relating to the site reactivity and reaction pathway of guanine oxidation carried out on the human telomeric sequence $(TAG_3T)_4T$ revealed that, on average, the G residues forming the external G-tetrads are more susceptible to oxidation, in comparison with those involved in the central G-tetrad,[49] thus shielding, in some way, that part of the G-quadruplex particularly critical for structural stability.

As far as the oxidative damage on the loop residues is concerned, an investigation regarding the presence of 8-oxo-dA residues has been reported.[23] In contrast to the destabilizing effects observed in the 8-oxo-dG containing G-quadruplex, the single replacement of the loop adenosines with 8-oxo-dA residues has proven to stabilize the G-quadruplex structures formed by the human telomeric sequence wtTel22 $AG_3(T_2AG_3)_3$ in K^+ solution, with slight differences among the positions in the sequences having 8-oxo-dA in the 2° loop, which show a stronger stabilizing effect compared to the other two loops. Surprisingly, an interesting investigation[46] concerning the same sequence containing abasic sites replacing adenosines has afforded partially contrasting results about thermal stability, the abasic site placed in the 2° loop being destabilizing and those in the 1° and 3° loop stabilizing. Furthermore, NMR results reported in the same study show that the sequences with the abasic site in the 1° and 3° loop adopt the hybrid-1 and hybrid-2 G-quadruplex topologies, respectively, although the biological consequences of this datum remain to be investigated.

Conclusion

In summary, the presence of oxidized guanosine products (8-oxo-dG residues or abasic sites deriving from them) in the stem moiety of a G-quadruplex structure significantly decreases the thermal stability, the guanosines involved in the formation of the central G-tetrad being more important for the structural stability than the others. In contrast, the presence of oxidation thymidine and adenosine products (5-hmdU and 8-oxo-dA, respectively, and the abasic sites deriving from the latter) is without particular effects on the G-quadruplex structure stability (5-hmdU) and in some cases even stabilizing (8-oxo-dA and abasic sites in two positions of three), while only the presence of an abasic site in the 3° loop has been shown to be destabilizing. Results described here and in the other papers allow us to hypothesize that thymidines and adenosines in the loops of human telomeric G-quadruplexes play a role in protecting those structural parts more important for their stability (the stacked G-tetrads) and then, their biological function. A further recent interesting investigation supporting this hypothesis concerns the effect of thymine dimers in the human telomeric sequence $T(AG_3T_2)_4$.[50] Although this UV-induced modification is rather more severe that those described before, the damage causes only slightly lower thermal stabilities, thus suggesting that the formation of thymine dimers is not detrimental to telomere structure and function. Fig. 6 shows a scheme resuming the main oxidative and photo-induced damage investigated in telomeric G-quadruplexes to date and its effects on structural stability.

Although 5-hmdU represents a type of DNA damage, it is an unusual nucleotide with several biological implications. Interestingly, it is not able to induce miscoding,[51] is not a polymerase blocking lesion[52] and does not perturb normal B-form DNA structure.[53,54] Furthermore, it completely replaces thymidine in the DNA of some bacteriophages,[55] as *e.g. B. subtilis* in which 5-hmUra:A base pair steps inherently increase DNA flexure, thus improving its interaction with type II DNA-binding protein TF1[56–58] and other similar proteins.[59,60] Furthermore, 5-hmUra:A base pair is a poor substrate for glycosylase activity[61] and recently it has proposed that 5-hmUra can be considered as an epigenetic mark.[62] These considerations strongly suggest non-detrimental and sometimes favourable effects of this modified nucleoside in several biological circumstances. Results concerning the effects of 5-hmdU in human telomeric sequences are in agreement with data collected from different biological systems.

Experimental section

Oligonucleotide synthesis and purification

The modified oligonucleotides reported in Table 1 were synthesized on a Millipore Cyclone Plus DNA synthesizer using

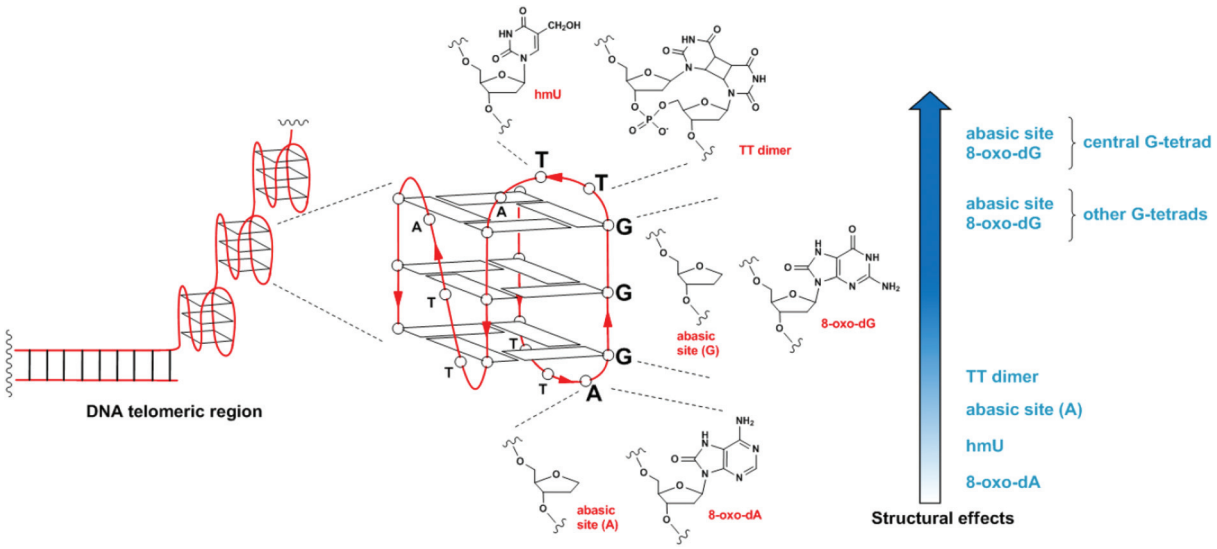

Fig. 6 Schematic illustration resuming the main oxidative and photo-induced damages investigated in telomeric G-quadruplexes to date. The arrow indicates roughly increasing destabilizing structural effects. To simplify, only the hybrid-2 G-quadruplex structure has been shown. See the Conclusion section for details.

solid phase β-cyanoethyl phosphoramidite chemistry on a 15 μmol scale. The synthesis of the suitably protected 5-hydroxymethyl-2′-deoxyuridine-3′-phosphoramidite was performed following the synthetic strategy proposed by Conte *et al.*[63] The oligomers were detached from the support and deprotected by treatment with concentrated aqueous ammonia at 55 °C overnight.

The combined filtrates and washings were concentrated under reduced pressure, redissolved in H_2O, analyzed and purified by high-performance liquid chromatography on a Nucleogel SAX column (Macherey-Nagel, 1000-8/46), using buffer A: 20 mM KH_2PO_4/K_2HPO_4 aqueous solution (pH 7.0) containing 20% (v/v) CH_3CN and buffer B: 1 M KCl, 20 mM KH_2PO_4/K_2HPO_4 aqueous solution (pH 7.0) containing 20% (v/v) CH_3CN; a linear gradient from 0 to 100% B for 45 min and flow rate 1 ml min^{-1} were used. The fractions of the oligomers were collected and successively desalted by Sep-Pak cartridges (C-18). The isolated oligomers proved to be >98% pure by NMR.

Gel electrophoresis

All oligonucleotides were analyzed by non-denaturing PAGE. Samples ([ODN] ≈ 1 mM) were annealed in the buffer 20 mM KH_2PO_4, 100 mM KCl, pH = 7 and loaded on a 20% polyacrylamide gel containing Tris-borate-EDTA (TBE) 2.5× and KCl 50 mM. The run buffer was TBE 1× containing 100 mM KCl. For all samples, a solution of glycerol/TBE 10× containing 100 mM KCl (2 : 1) was added just before loading. Electrophoresis was performed at 8 V cm^{-1} and a temperature close to 5 °C. Bands were visualized by UV shadowing.

Circular dichroism

CD (circular dichroism) spectra and CD melting curves were registered on a Jasco 715 circular dichroism spectrophotometer in a 0.1 cm pathlength cuvette and the wavelength varied from 220 to 320 nm. The spectra were recorded with a response of 16 s at 2.0 nm bandwidth and normalized by subtraction of the background scan with buffer. The temperature was kept constant at 20 °C with a thermoelectrically controlled cell holder (Jasco PTC-348). For all the sequences the strand concentration was 25 μM. CD melting curves were registered as a function of temperature from 20 to 95 °C at 290 nm at a scan rate of 1 °C min^{-1}. The melting temperature (T_m) was estimated from the inflection point of the melting profile. All the experiments were done in buffer solution containing 10 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0.

SVD analysis

Temperature-dependent multiple CD spectra were analyzed by singular value decomposition (SVD) to determine the number of significant spectral species involved in the observed CD variation.[64–66] SVD is a method from linear algebra for factoring a matrix.[67] Briefly, the matrix of the CD spectra A is decomposed by the SVD method into the product of three matrices:

$$A = USV^t \quad (1)$$

The matrix U contains the basis spectra which combine to form the experimentally observed CD spectra, S is a diagonal matrix that contains as many singular values as the number of basis spectra in the U matrix. V is a matrix containing the coefficient vectors as function of temperature. The magnitude of each singular value represents the contribution (weight) of the corresponding basis spectrum to the observed spectral behavior (contained in the data matrix). The singular values start relatively high and then decrease to a low noisy plateau which represents the noise level. Examination of the magni-

tudes of the singular values provided the first clue of the number of significant basis spectra. Another criterion used for determining the number of significant spectral components was the value of the first-order autocorrelation function for the columns of the U and V matrices. The shapes of both U and V columns should be nonrandom for a significant spectral component and the value of the autocorrelation function is a measure of the smoothness between adjacent row elements. Values near 1 indicate slow variation from row to row, or "signal". A value of the autocorrelation function higher than 0.7 for both the U and V matrices was selected as the cut off criterion for accepting a significant spectral species.[66,67] An additional indication of the number of significant spectral species was provided by the evaluation of the contribution of each singular value to the total variance in singular values. The relative variance (RV) of each singular value is given by:

$$RV = \frac{S_i^2}{\sum_i S_i^2} \quad (2)$$

where S_i^2 is the square of the singular value. Only the significant singular values will sum to contribute to >0.99 of the total variance.[64] Finally, to verify the chosen number of significant spectral species, the primary data set (D matrix) was reproduced by using only the selected basis spectra from the expression:

$$A' = US'V^t \quad (3)$$

where S' includes only significant (the ones judged above the noise) elements of S. Inspection of the residual ($A - A'$ matrix) allowed an evaluation of whether the chosen number of spectral components were sufficient to reproduce the observed spectral behavior.

Differential scanning calorimetry

Differential scanning measurements were performed on a last generation nano-DSC (TA Instruments). The excess molar heat capacity function ΔC_p was obtained after a baseline subtraction, assuming that the baseline is given by the linear temperature dependence of the native-state heat capacity. A buffer *versus* buffer scan was subtracted from the sample scan. All systems were tested for reversibility by running the heating and cooling curves at the same scan rate of 1° min^{-1}. The process enthalpies, $\Delta H°$, were obtained by integrating the area under the heat capacity *versus* the temperature curves. T_m is the temperature corresponding to the maximum of each DSC peak. Entropy values were obtained by integrating the curve $\Delta C_p/T$ *versus* T (where ΔC_p is the molar heat capacity and T is the temperature in Kelvin). The thermodynamic parameters in Table 2 represent averages of heating curves from three to five experiments. The reported errors for thermodynamic parameters were calculated on the average of 3–5 determinations. The measurements were performed with DNA strand concentrations in the range of 100–130 μM. All the experiments were done in buffer solution containing 10 mM phosphate with 100 mM KCl, 0.1 mM EDTA, at pH 7.0.

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