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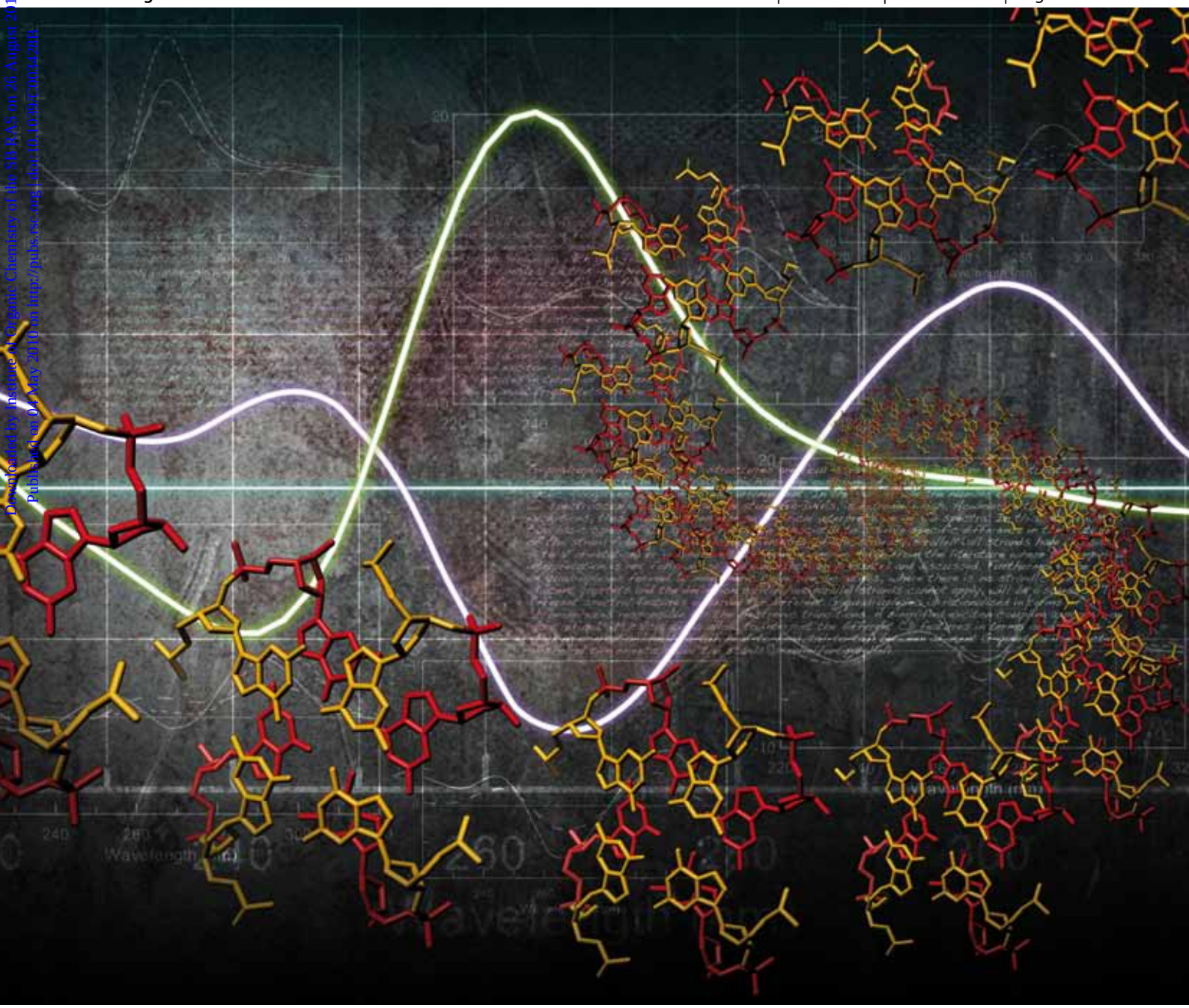

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A non-empirical chromophoric interpretation of CD spectra of DNA G-quadruplex structures†

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G-quadruplex DNA (G4-DNA) structures are four-stranded helical DNA (or RNA) structures, comprising stacks of G-tetrads, which are the outcome of planar association of four guanines in a cyclic Hoogsteen hydrogen-bonding arrangement. In the last decade the number of publications where CD spectroscopy has been used to study G4-DNAs, is extremely high. However, with very few exceptions, these investigations use an empirical interpretation of CD spectra. In this interpretation two basic types of CD spectra have been associated to a single specific difference in the features of the strand folding, *i.e.* the relative orientation of the strands, "parallel" (all strands have the same 5′ to 3′ orientation) or "antiparallel". Different examples taken from the literature where the empirical interpretation is not followed or is meaningless are presented and discussed. Furthermore, the case of quadruplexes formed by monomeric guanosine derivatives, where there is no strand connecting the adjacent quartets and the definition parallel/antiparallel strands cannot apply, will be discussed. The different spectral features observed for different G-quadruplexes is rationalised in terms of chromophores responsible for the electronic transitions. A simplified exciton coupling approach or more refined QM calculations allow to interpret the different CD features in terms of different stacking orientation (head-to-tail, head-to-head, tail-to-tail) between adjacent G-quartets irrespectively of the relative orientation of the stands (parallel/antiparallel).

1. Introduction

G-quadruplex DNA (G4-DNA) structures are four-stranded helical DNA (or RNA) structures, comprising stacks of G-tetrads (also known as G-quartets), which are the planar association of four guanines in a cyclic Hoogsteen hydrogen-bonding arrangement (Fig. 1).[1] G4-DNAs are formed by the folding of one DNA (or RNA) strand or by the association of two or more strands. This results in different combinations of relative strand orientations (see Fig. 2), with consequent formation of grooves of different widths, and different loops' arrangements.[2] From the biological point of view, G4-DNAs are widespread in the genome and it seems they play a role in a number of processes, such as replication, recombination transcription and translation[3] and are also found in telomeric regions.[4] Therefore, many efforts are devoted to investigate G4-DNAs along two strongly interconnected main lines: first, to understand their fine structures as a function of nucleotide sequence and external conditions (pH, temperature, solvent polarity, ionic strength, *etc.*) and, second, their identification as targets for chemotherapy.[5] As an example it should be mentioned that molecules that bind and stabilise G-quadruplexes have been shown to inhibit telomerase, an enzyme with high activity in human tumor cells, and are potential candidates for cancer therapy. In addition, several proteins have been found to have high affinity for G4-DNA in genomic regions and defects in these proteins can lead to errors in replication or transcription.

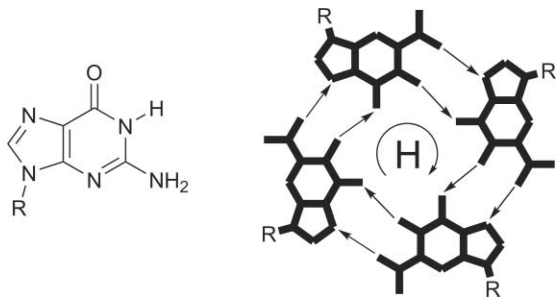

Fig. 1 The guanine moiety and the G-quartet. The G-quartet shows its "head" (H) side (from donor to acceptor H-bonding runs clockwise); the reverse side is referred as "tail" (T) (from donor to acceptor H-bonding runs counter-clockwise).

Chiroptical techniques, and in particular circular dichroism (CD),[6] were developed in the second half of the past century to study mainly steroidal ketones.[7] Subsequently they have been extended to other different chromophores with the main goal of assigning the absolute configuration of chiral molecules.[8] For its sensitivity to stereochemical variations, CD has emerged to be an important technique for studying subtle conformational changes and supramolecular interactions.[9] At the present time the

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† In memory of Professor Carlo Rosini (1948–2010), a pioneer of chiroptical techniques applied to organic stereochemistry. His human and scientific presence will always be with us.

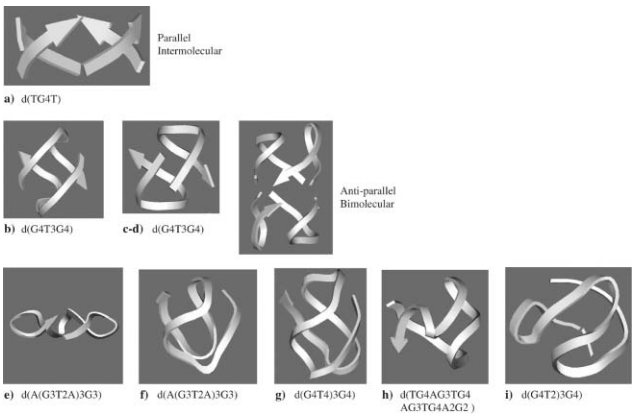

Fig. 2 Typical folding topologies of G4-DNA forming sequences: a parallel intermolecular structure (a), bimolecular antiparallel structural motifs (b–d), intramolecular parallel (e) and antiparallel (f–i) monomolecular structures. Arrows represent backbones running from 5′ to 3′ end. (Reproduced by permission of Royal Society of Chemistry from ref. 2).

outstanding sensitivity of CD is employed mainly to study biological macromolecules and their perturbation by external factors,[10] and among those G4-DNAs represent biological structures widely investigated. In the last decade the number of publications dealing with DNA G-quadruplex structures has increased exponentially, and the number of papers in which CD spectroscopy has been used to study quadruplex structures is extremely high.[11,12] CD has been used to study 3D-structures, ligand binding and effect of cations, to monitor the kinetics of quadruplex formation and the thermal melting, the effect of chemical modifications. However, with very few exceptions (see *infra*), these investigations use an empirical interpretation of CD spectra that relies on a relationship with spectra of known G-quadruplex structures.[11,13]

Although the topology of the folding of G4-DNA strands is very complex and many types of quadruplexes have been reported, there are two basic types of CD spectra, which have been associated to a single specific difference in the features of the strand folding, *i.e.* the relative orientation of the strands, parallel (all strands have the same 5′ to 3′ orientation) or antiparallel.

The spectra of "parallel" quadruplexes (a typical example is represented by the tetramolecular quadruplex formed by $d(G_4)$, see Fig. 3 (left side), in which four strands with all glycosyl bonds in *anti* conformation run parallel to each other) have a dominant positive band at *ca.* 260 nm, and a negative peak at *ca.* 240 nm.[14,15] On the contrary, the spectra of "antiparallel" quadruplexes (a typical example is represented by two folded-back strands of $d(G_4T_4G_4)$, see Fig. 3 (right side), where guanines alternate *syn* and *anti* glycosyl conformation along each strand) have a negative band at 260 nm and positive band at 290 nm.[14,16] Both quadruplex types display an additional characteristic positive peak at 210 nm.

Although this empirical simple relationship (positive CD at 260 nm and negative CD at 240 nm → "parallel strands"; positive CD at 290 nm and negative CD at 260 nm → "antiparallel strands") holds for many real cases and is currently the

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Antonio Randazzo graduated in Pharmaceutical Chemistry in 1995 at the University of Naples "Federico II" with a thesis on the synthesis of modified oligonucleotides. In 1998 he got his PhD in Bioactive Natural Compound. He joined the Faculty of Pharmacy of the University of Naples "Federico II" in 1999 as Assistant Professor of Organic Chemistry. He became Associate Professor in 2006 in Organic Chemistry at the same Faculty. His research activity is mostly in the fields of the synthesis and structural studies of modified oligonucleotides. Particularly, he focused his attention (i) on the studies of the three-dimensional structures of modified quadruplexes, and (ii) on the study of the interaction of small organic molecules with different quadruplex structures. He is the author of more than 60 papers in peer-reviewed international journals. Roberta Trotta and Stefano De Tito are PhD students at the Faculty of Pharmacy in Naples.

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Gian Piero Spada obtained his doctoral degree in Organic Chemistry in 1980 at the University of Bologna with a thesis on the chiroptical techniques. After a research fellowship period, he joined the Faculty of Pharmacy of the University of Bologna in 1983 as Assistant Professor of Organic Chemistry. He became Associate Professor in 1992 and since 2000 he has been full Professor of Organic Chemistry. His research activity is in the fields of stereochemistry and supramolecular chemistry; in particular, his current interest focuses on (i) the study of the supramolecular architectures obtained through self-recognition and self-assembly via *hierarchical organisation of non covalent interactions, (ii) the chiral transfer from molecular to supramolecular level and (iii) the study of photoresponsive molecules and the control of nanoarchitectures and partially ordered systems through chemical and physical stimuli. He is member of the Management Committee of the COST Action MP0802 (financed by FP7-RTD), the European Network on G-quartets and Quadruplex Nucleic Acids. He is author of more than 120 papers on peer-reviewed international journals. Stefano Masiero (Associate Professor) and Silvia Pieraccini (Assistant Professor) are senior members of his research group while Rosaria Perone joined the group as PhD student.*

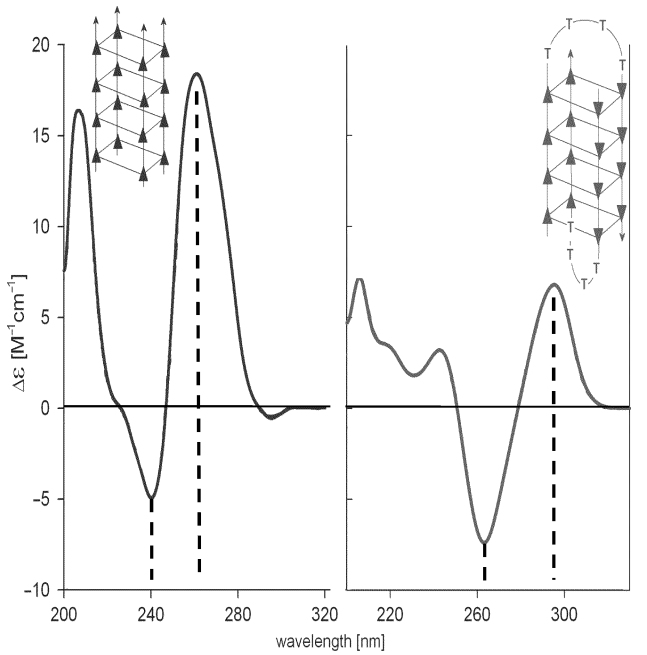

Fig. 3 CD spectra of guanine quadruplexes. Left side: the parallel-stranded quadruplex $[d(G_4)]_4$ stabilized by 16 mM K^+; right side: Na^+-induced antiparallel bimolecular quadruplex of $[d(G_4T_4G_4)]_2$. The triangles in the sketches indicate guanines and point in the 5′–3′ direction. $\Delta\varepsilon$ is expressed in terms of nucleotide molar concentration (adapted from ref. 13).

interpretation key for most papers dealing with CD of G4-DNAs,[12,14,16,17] it cannot be considered of general validity and it cannot be enforced for more complex quadruplexes forming systems and in several cases (see *infra*) where strand polarity is meaningless.

To have a better understanding of CD spectra of G4-DNAs it is desirable to rationalise CD signals in terms of chromophores responsible for electronic transitions in the UV-Vis range. Starting from the "chromophore" it would be then possible to justify how different folding patterns (characterised by, *inter alia*, different strand polarities) origin different CDs.

2. The origin of CD in G4-DNA

In general, for solutions, there is a direct correlation between the regions of absorption and CD. In the case of non-coupled chromophores, the shapes of CD and absorption spectra are similar, although the vibrational fine structure can be different. If two or more strongly absorbing chromophores are chirally oriented with respect to each other, one observes an exciton spectrum characterized by the presence of two bands with opposite signs, where λ_{max} in absorption corresponds, or nearly corresponds, to zero CD intensity.[18,19] In general, the two bands of an exciton spectrum do not necessarily have the same intensity: a conservative exciton CD spectrum (equal intensity of the two bands) occurs only if the coupled chromophores possess a well isolated allowed transition. Non-degenerate couplings with other transitions present in the same chromophore may alter the relative intensity of the two bands. An exciton CD spectrum is rather commonly observed during the formation of helical polymers or helical self-assembled aggregates. For example, CD spectra of α-helix of peptides in the absorption region of the amide chromophore have been interpreted as due to exciton coupling between π–π* transitions located in two adjacent amide groups. Similar explanations have been given for explaining the CD spectrum of B-DNA.

In the case of G4-DNA the chromophores absorbing in the UV-Vis region with $\lambda > 210$ nm are represented by guanines while contributions from the rest of the biopolymer are negligible. Guanine has in fact two well-isolated absorption bands in the 240–290 nm region which are connected to two well characterised π–π* transitions at *ca.* 279 nm and 248 nm;[20] the two transitions are roughly short and long axis polarised, respectively (Fig. 4).‡ In G4-DNAs, G-quartets are piled one on the top of the other and rotated one with respect to the adjacent one: this rotation causes chiral exciton coupling between transition dipole moments located in near-neighbour guanines. The coupling of these moments can be calculated for plausible models and compared with experimental data.

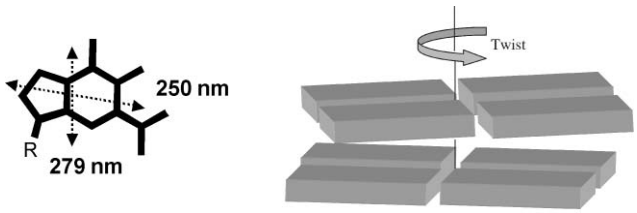

Fig. 4 Orientation of the two most relevant electric transition moments (dotted double-head arrows) of the guanine chromophore (left) and a sketch of the chiral arrangement of two adjacent G-quartets (each parallelepiped represents a guanine base).

The first non-empirical interpretation of the CD of a G4-DNA has been reported by Spada *et al.*[21] by modelling the spectrum of polyguanilic acid, poly(G) (Fig. 5). Poly(G) shows the typical CD spectrum of "parallel" G4-DNA dominated by a non-conservative positive exciton couplet characterised by a positive band at 260 nm and a negative one at 240 nm. This spectrum has been reproduced satisfactorily by an exciton calculation considering only near-neighbour interactions between the guanine transitions (completely neglecting the contribution from the sugar-phosphate backbone) of two stacked G-quartets.[21,22]

Poly(G), like $d(TG_4T)$ and other G_n strands, is known to form tetramolecular four-stranded helices based on G-quartets. The four strands run parallel in a right-handed quadruplex; the quartets are highly planar, perpendicular to the helix axis and connected to the phosphate-sugar backbone by glycosyl bonds that are exclusively *anti*. Due to the heterotopic nature of the two faces of the G-quartet (Fig. 1), when the G-tetrads are piled in a columnar structure, each quartet can stack onto the adjacent one through the same (head-to-head or tail-to-tail) or the opposite

‡ Also the higher energy absorption at *ca.* 200 nm is well characterised,[20] however, as often happens with higher-energy transitions, the CD connected to this transition cannot easily be interpreted due to the presence of several chromophores which absorb in the same spectral region. Nevertheless, this spectral region, so far not well studied, might give additional information complementary to that obtained from the low-energy region; in particular, it might be directly sensitive to the structure of the sugar-phosphate backbone.

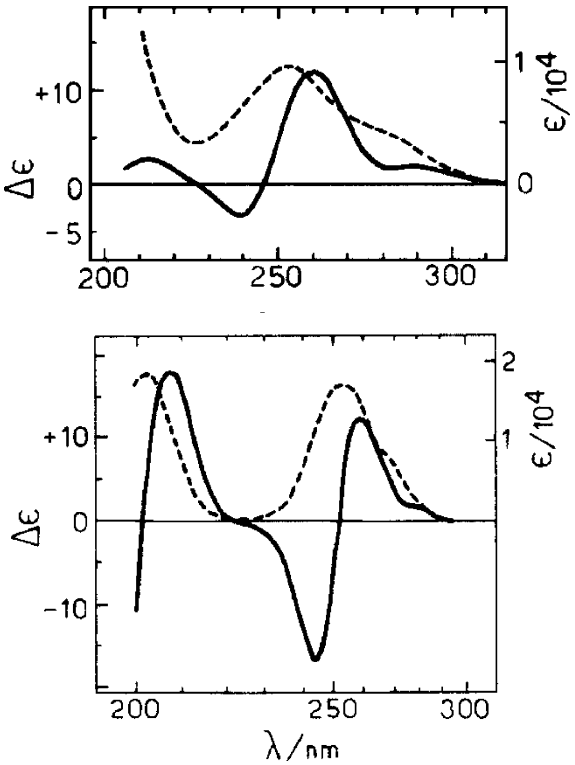

Fig. 5 Top: absorption (dashed) and CD (solid) solution spectra of poly(G) ($\Delta\varepsilon$ is expressed in terms of nucleotide molar concentration). Bottom: calculated absorption (dashed) and CD (solid) spectra for a system composed of two G-quartets oriented as in Fig. 6, left side (reproduced by permission of Società Chimica Italiana from ref. 21).

(head-to-tail) face leading to a heteropolar or homopolar stacking, respectively (Fig. 6). In poly(G) the quartets are stacked in a right-handed way and all tetrads have the same orientation, *i.e.* a head-to-tail (homopolar) stacking.

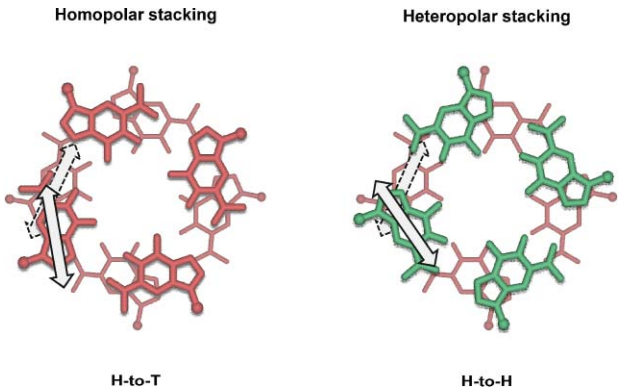

Fig. 6 Top view of the heteropolar and homopolar stacking of two G-quartets: the "head" and the "tail" sides of the G-quartets are represented in red and green, respectively (the double-head arrows represent the transition moments corresponding to the absorption band at *ca.* 250 nm).

From a qualitative point of view it is not necessary to perform a detailed QM calculation. One can simply explain the positive sign of the exciton coupling corresponding to the 250 nm (*i.e.* positive and negative branches at 260 and 240 nm, respectively) transition of the guanine looking at the relative disposition of the two transition moments located in the closest guanines (full and dotted arrows in Fig. 6). In fact, in the framework of the chiral exciton coupling, the sign of the couplet is determined by the sign of the product $[\mathbf{r}_{ij} \cdot (\boldsymbol{\mu}_i \times \boldsymbol{\mu}_j)\ V_{ij}]$ where $\mathbf{r}_{ij}$ is the interchromophoric distance vector from i to j, $\boldsymbol{\mu}_i$ and $\boldsymbol{\mu}_j$ are the transition dipole moments on groups i and j, respectively, and V_{ij} is the interaction energy between the two dipoles:[18] knowing the position and orientation of the transition dipoles the sign of the exciton couplet can be inferred. Generally speaking, the Rotational Strength R_{0a} (a quanto-mechanical quantity that is proportional to the band area of the CD spectrum) of a specific electronic transition from ground state *0* to excited state *a* can be expressed as the scalar product of the electric ($\boldsymbol{\mu}_{0a}$) and magnetic ($\mathbf{m}_{0a}$) transition dipoles of the transition:[8,23]

$$R_{0a} = \boldsymbol{\mu}_{0a} \cdot \mathbf{m}_{0a} \quad (1)$$

Therefore, according to eqn (1), an electronic transition must posses both electric and magnetic transition moments to be CD active.

Electric allowed transitions (as is the case for π–π^* transitions in locally achiral planar aromatic chromophores) are associated to high electric dipole transition moments but negligible magnetic transition moments: the magnetic moment necessary for observing intense CD arises from the exciton coupling of two non-coplanar electric moments. The two possible coupling modes for the two electric moments (see, for example, Fig. 7) on the two chromophores are non-degenerate and give rise to magnetic moments parallel or antiparallel with respect to the total electric moment: therefore the two combinations generate positive or

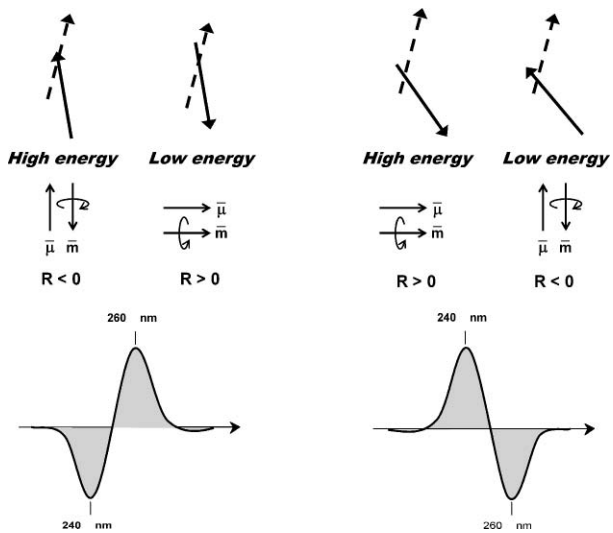

Fig. 7 A simplified model for the origin of the positive (left side) and negative (right side) exciton couplets for the head-to-tail (H-to-T) and head-to-head (H-to-H) G-quartet stacking, respectively. Top: the arrangement of two 250 nm electric transition moments (full line: front vector; dashed line: rear vector) located in two closest guanines. Middle: the magnetic (**m**) and electric (**μ**) moments generated by the coupling of the two guanine chromophore (more in details, in the high energy coupling of the left-side panel, the two electric transition moments–top–sum to a total electric vector pointing upward–middle–and generate a charge rotation with a resulting magnetic moments pointing downward, that is antiparallel). Bottom: the predicted CD spectra.

negative rotational strength, respectively, the component at higher wavelength being that due to the lower energy coupling mode.

Considering the case of "parallel" G4-DNAs, the disposition of two adjacent G-quartets in a H-to-T orientation is that reported in Fig. 6 where the electric moments (of the 250 nm transition) of a couple of near-neighbour guanines have been superposed. Applying the simplified model of the exciton coupling it emerges that this chiral arrangement (see Fig. 7, left side panel) is expected to exhibit a positive exciton couplet centred at around 250 nm. This is indeed in agreement with the experimental data and the result of the QM calculations.[21,22]

Going back to the CD of "antiparallel" G4-DNA (Fig. 3), one can notice immediately that, apart from the 290-300 nm positive band, the other parts of the spectrum show a nearly mirror-image relationship of the bands in respect of "parallel" G4-DNA (although the quadruplex is still right-handed). In 1998 Spada *et al.*,[24] comparing CD spectra of different G4-DNA forming strands, proposed for the first time that this "*is probably due to the inversion of polarity of the quartets in the hairpin structures*". Inversion of polarity (from head-to-tail to head-to-head or tail-to-tail) could cause a different arrangement of the near-neighbour transition dipole moments and consequently an oppositely signed exciton couplet corresponding to the 250 nm transition. A few years later Wen and Gray gave the same explanation.[25] They specifically proposed that a positive CD band near 260 nm arises from the stacking of quartets of the same polarity while a band at longer wavelength arises from the stacking of quartets with alternating polarities. When the glycosyl bonds of the guanines alternate in *syn* and *anti* conformations along each strand for quadruplexes having antiparallel strands, the G-quartet polarity also alternates, while quadruplexes with parallel strands and all *anti* glycosyl bonds have non-alternating G-quartet polarity. Since CD is very sensitive to base stacking geometry, quadruplexes with alternating *vs.* non-alternating G-quartet polarity are expected to show different CD spectra.

The polarity of stacked quartets, not the relative orientations of the backbone strands, is likely to be the determining factor for G-quadruplex CD spectra.

It should be noticed that in the heteropolar H-to-H stacking of two quartets, the relative orientation of the closest dipole moments is, in fact, quite different from the case above. In Fig. 6, right side, the quartets are represented in a geometry observed in many "antiparallel" G4-DNA. Using the qualitative approach to the exciton coupling, this chiral arrangement is expected to give a negative couplet centred at around 250 nm (Fig. 7, right side panel) as experimentally observed.

Only recently Gray *et al.* performed a CD spectral calculation with the dipole–dipole approximation of two G-quartets stacked with the same or opposite polarity (Fig. 8).[22] Their results confirm the late computations by Spada *et al.*[21] on the homopolar stacked system and show how heteropolar stacking explains the emergence of a positive CD signal at *ca.* 290 nm.

The very few theoretical computations so far published, although performed in the dipole–dipole approximation, clearly support the non-empirical explanation for the origin of the CD bands in G4-DNAs as due to the stacking interactions of neighboring G-quartets with the same or opposite polarity. Therefore, the kind of CD spectrum is actually not directly related to the relative strands orientation: the stacking orientation of the

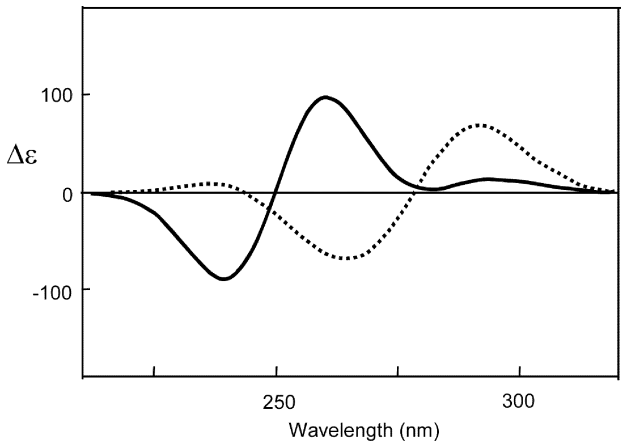

Fig. 8 Superimposed calculated CD spectra of two G-quartets stacked in the H-to-T (solid line) or H-to-H (dashed line) orientation as shown in Fig. 6. The relative orientation of the G-quartets for the calculation were extracted from the solution structure of $d(G_3T_4G_3)$ that present mixed polarities of stacked G-quartets.[22]

G-tetrads obviously depends on the folding of the strand (and hence on the parallel/antiparallel strand polarity); however, no direct relationship can be established between the two topological features.

3. A case study: $d(G_3T_4G_3)$

The oligomer $d(G_3T_4G_3)$ has been shown by NMR studies[26] to form a quadruplex, consisting of three stacked quartets, generated by two folded (antiparallel) oligomer strands. The arrangement of the stacked quartets is sketched in Fig. 9: the cartoon shows that the two lower adjacent G-quartets exhibit the homopolar H-to-T stacking, while the other two adjacent G-quartets (the upper two quartets) have the heteropolar H-to-H stacking. This mixed stacking arrangement is due to the fact that the conformations of glycosyl bonds along a given G_3 tract are not all the same, being either *syn-syn-anti* or *syn-anti-anti* (in the 5′ to 3′ direction). The experimental CD spectrum of $[d(G_3T_4G_3)]_2$, see Fig. 10, is characterised by two positive bands above 240 nm, the higher intensity one at *ca.* 290 nm and the weaker one at 250–260 nm. Gray *et al.*[22] calculated the CD spectrum for the three stacked tetrads of guanines of the $[d(G_3T_4G_3)]_2$ structure containing two adjacent G-quartets of the same polarity and two adjacent G-quartets of opposite polarities. The general features of the experimental CD spectrum are all predicted well, including the two positive bands. Interestingly, the average of the spectra calculated for a pair of homo- or heteropolar stacked quartets (calculated spectra in Fig. 8) has the same spectral shape of the more refined calculated spectrum for a stack of three quartets of alternating polarities, further supporting the hypothesis that CD spectra of G-quadruplexes have shapes dominated by the stacking polarities of nearest-neighboring G-quartets.

4. The CD of chemically modified G4-DNA revisited

In the following, we will give a critical appraisal of a number of non-commented CD spectra reported in the literature, which can be interpreted on the basis of the kind of stacking interactions

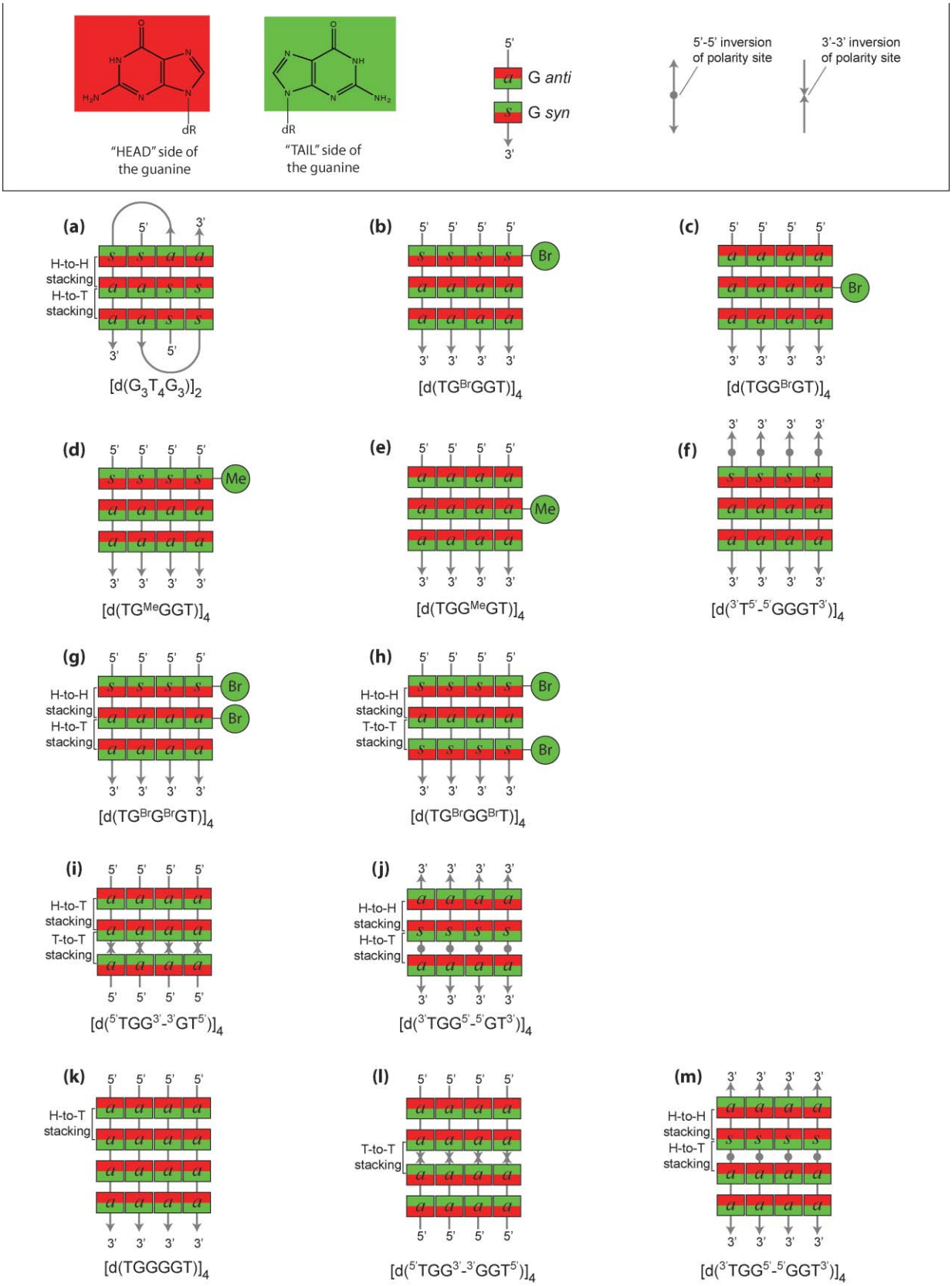

Fig. 9 A sketch of the stacking arrangement of the G-quartets observed in G-quadruplexes obtained from different oligonucleotides. Each G residue is represented by a bi-coloured rectangle and the head (H) and the tail (T) faces (as defined in Fig. 1) are in red and green, respectively; *s* and *a* refer to the *syn* and *anti* conformation around the glycosyl bond, respectively; the arrow represents the 5′-to-3′ direction of the strand. A graphic legend is reported in the box on the top.

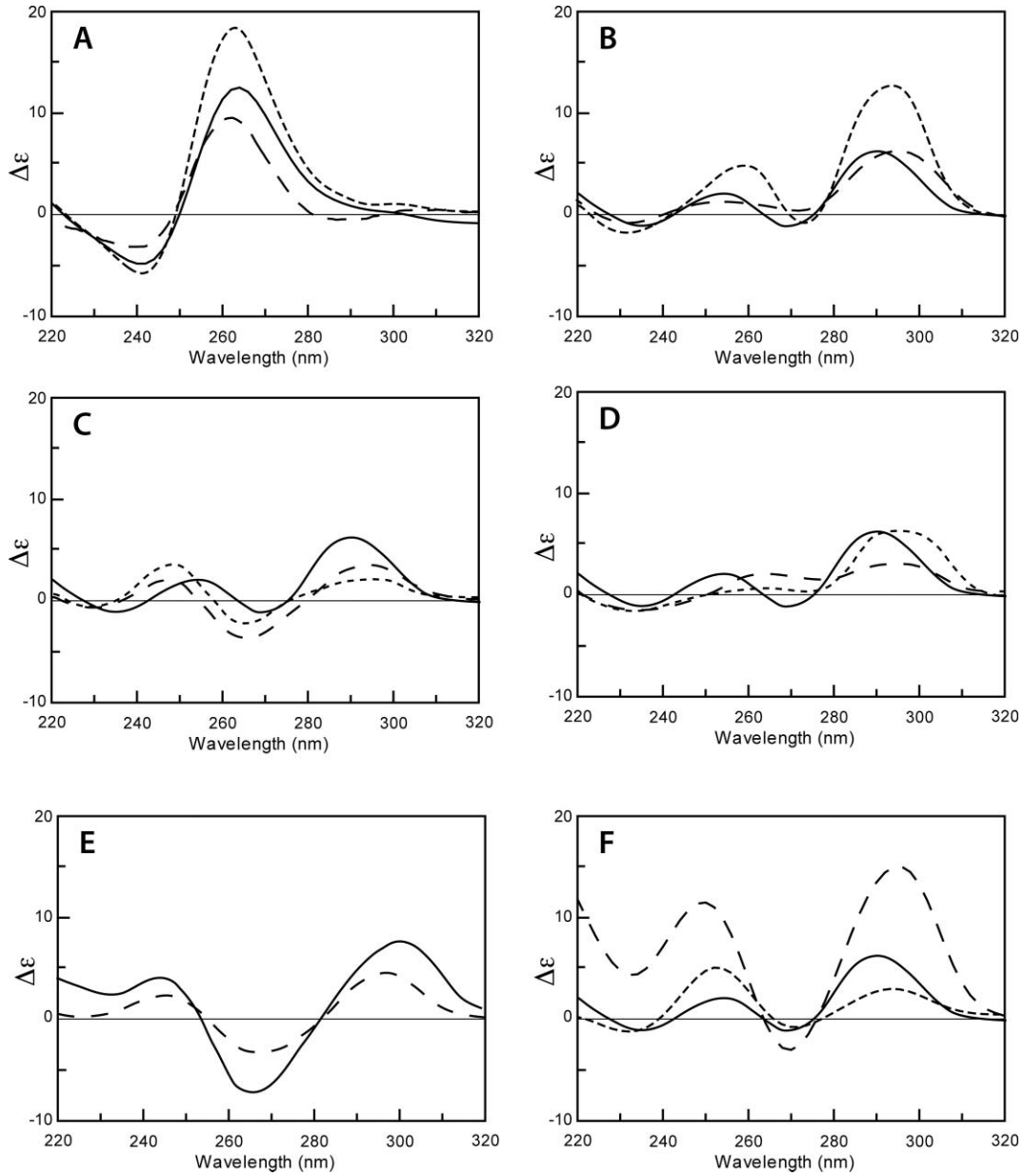

Fig. 10 CD spectra of selected G4-DNA mentioned in the text. **A**: $[d(TGGGT)]_4$ (solid), $[d(TGG^{Me}GT)]_4$ (long-dashed) and $[d(TGG^{Br}GT)]_4$ (short dashed); **B**: $[d(G_3T_4G_3)]_2$ (solid), $[d(TG^{Me}GGT)]_4$ (long-dashed) and $[d(TG^{Br}GGT)]_4$ (short dashed); **C**: $[d(G_3T_4G_3)]_2$ (solid), $[d(^{3'}TG^{5'}\text{-}^{5'}GGT^{3'})]_4$ (long-dashed) and $[d(^{5'}TG^{3'}\text{-}^{3'}GGT^{5'})]_4$ (short dashed); **D**: $[d(G_3T_4G_3)]_2$ (solid), $[d(^{3'}T^{5'}\text{-}^{5'}GGGT^{3'})]_4$ (long-dashed), $[d(TG^{Br}G^{Br}GT)]_4$ (short dashed); **E**: $[d(G_4T_4G_4)]_2$ (solid), $[d(TG^{Br}GG^{Br}T)]_4$ (short dashed); **F**: $[d(G_3T_4G_3)]_2$ (solid), $[d(^{3'}TGG^{5'}\text{-}^{5'}GGT^{3'})]_4$ (long-dashed) and $[d(^{5'}TGG^{3'}\text{-}^{3'}GGT^{5'})]_4$ (short dashed). CD spectra were drawn from data of ref. 27, 29–33 ($\Delta\varepsilon$ is expressed in terms of nucleotide molar concentration).

between adjacent G-tetrads constituting the G-quadruplexes, irrespectively of the relative strand orientation. In particular, we will focus our attention to the CD spectra of quadruplexes containing chemical modification, that lead to the formation of unusual folding and G-tetrad stacking arrangements, for which the definition of "parallel" or "antiparallel" is not completely appropriate.

We have found the observations described in the previous section particularly useful to interpret some CD spectra of modified quadruplexes reported in the literature. For example, three modified DNA quadruplexes have been recently studied, namely $[d(TG^{Br}GGT)]_4$, $[d(TGG^{Br}GT)]_4$ and $[d(TGGG^{Br}T)]_4$, where G^{Br} indicates an 8-bromoguanine residue.[27] All three quadruplexes are characterised by a 4-fold symmetry with all strands parallel to each other. The whole of the reported data demonstrates that the replacement in turn of different guanine residues with G^{Br} in the sequence 5′-TGGGT-3′ affects the resulting quadruplex structures, leading to dissimilar CD profiles. Differently from $[d(TG^{Br}GGT)]_4$ and $[d(TGG^{Br}GT)]_4$, $[d(TGGG^{Br}T)]_4$ turned out to be not perfectly structured. Interestingly, $[d(TG^{Br}GGT)]_4$ exhibited two positive CD bands at 296 and 263 nm, and one negative band at 233 nm (see Fig. 10B), whereas, the spectrum of $[d(TGG^{Br}GT)]_4$ was very similar to that of unmodified $[d(TGGGT)]_4$ and, therefore, characterised by maximum and minimum Cotton effects

at 263 and 243 nm, respectively (Fig. 10A). These data suggest that G^{Br} residues in $[d(TG^{Br}GGT)]_4$ do affect the structure of the quadruplex, most probably because, due to the presence of the bulky bromine substituent in the C-8 position, these nucleobases adopt *syn* glycosyl conformations.[28] On the other hand, the G^{Br} residue in $[d(TGG^{Br}GT)]_4$ seems to adopt an *anti* glycosidic conformation, since the CD spectrum is basically superimposable to that of the unmodified quadruplex $[d(TGGGT)]_4$ (Fig. 9, sketch *c*). Fascinatingly, the "parallel" quadruplex $[d(TG^{Br}GGT)]_4$ possesses the same CD spectrum of the "antiparallel" quadruplex $[d(G_3T_4G_3)]_2$. This apparent contradiction can be explained looking at the relative orientation of the three tetrads of the two molecules (Fig. 9, sketch *a* and *b*). Unfortunately, the NMR analysis has not been able to unambiguously determine the glycosyl bond angles of G^{Br}, due to the lack of the hydrogen in position 8 of the nucleobase. However, if we assume that the G^{Br} residues adopt a *syn* glycosidic conformation, $[d(TG^{Br}GGT)]_4$ is characterized by the two lower adjacent G-quartets exhibiting a homopolar H-to-T stacking, whereas the two upper quartets have the heteropolar H-to-H stacking (Fig. 9, sketch *b*), as exactly observed in $[d(G_3T_4G_3)]_2$ (Fig. 9, sketch *a*). Therefore, in this case, the analysis of CD data could be really useful to determine the right structure of the complex. Furthermore, in a following investigation, the same authors used 8-methyldeoxyguanosine residues (G^{Me}) in place of G^{Br}, taking into consideration that the methyl group has a steric size comparable to that of the bromine atom, and therefore is similarly inclined to promote the *syn* glycosyl conformation.[29] Moreover, the use of G^{Me} residues provides additional structural information based on NOE contacts involving the methyl protons in the C-8 position. As expected, the spectroscopic data of $[d(TG^{Me}GGT)]_4$ (Fig. 10B), $[d(TGG^{Me}GT)]_4$ (Fig. 10A) and $[d(TGGG^{Me}T)]_4$ turned out to be very similar to those of their analogues $[d(TG^{Br}GGT)]_4$, $[d(TGG^{Br}GT)]_4$ and $[d(TGGG^{Br}T)]_4$. However, in this case, NMR analysis was able to definitively prove that dG^{Me} adopts a *syn* glycosidic conformation in $[d(TG^{Me}GGT)]_4$ (Fig. 9, sketch *d*) and an *anti* glycosidic conformation in $[d(TGG^{Me}GT)]_4$ (Fig. 9, sketch *e*), thus inferring the interpretation of the CD spectra.

Interesting results can also be found in other investigations. Short oligonucleotides containing a site of inversion of polarity have been studied.[30] Among the six reported oligonucleotides, the sequence $^{3'}T^{5'}$-$^{5'}GGGT^{3'}$ turned out to fold into a tetramolecular quadruplex having two "parallel" subunits joined together through the inversion of polarity sites. However, one G-tetrad adjacent to the inversion of polarity site is characterized by having all G in *syn* glycosyl conformation. Consequently, the quadruplex $[d(^{3'}T^{5'}$-$^{5'}GGGT^{3'})]_4$ (Fig. 9, sketch *f*) is characterized by the same number of G-tetrads having the same relative orientation as observed in $[d(G_3T_4G_3)]_2$, $[d(TG^{Br}GGT)]_4$ and $[d(TG^{Me}GGT)]_4$ (Fig. 9, sketches *a*, *b* and *d*). Thus, although $[d(^{3'}T^{5'}$-$^{5'}GGGT^{3'})]_4$ can be considered to be a parallel quadruplex, its CD spectrum is very similar to those of the quadruplexes indicated above (some of which are antiparallel), showing a negative band at 233 nm and two positive bands at 265 and 295 nm (see Fig. 10D).

Another example reported in the literature deals once again with the study of modified d(TGGGT).[31] In this case, two or three Gs have been replaced by G^{Br} residues. In particular, four modified oligonucleotides have been studied, namely $d(TG^{Br}G^{Br}GT)$, $d(TG^{Br}GG^{Br}T)$, $d(TGG^{Br}G^{Br}T)$ and $d(TG^{Br}G^{Br}G^{Br}T)$. Only the pentamers $d(TG^{Br}G^{Br}GT)$ and $d(TG^{Br}GG^{Br}T)$ were able to fold into a well-defined quadruplex structure. The CD spectrum of parallel quadruplex $[d(TG^{Br}G^{Br}GT)]_4$ (Fig. 10D) closely resembles that of the antiparallel quadruplex $[d(G_3T_4G_3)]_2$, and therefore strongly suggests that the G^{Br}-tetrad at the 5′ edge is characterized by G^{Br} in a *syn* glycosyl conformation, whereas the G^{Br} residues of the central tetrad are supposed to adopt an *anti* glycosyl conformation (Fig. 9, sketch *g*). Instead, an unprecedented CD spectrum is reported for $[d(TG^{Br}GG^{Br}T)]_4$ (Fig. 10E). In particular, the presence of a positive band at 295 nm indicates that there is at least a heteropolar stacking, presumably (and realistically) due to the presence of at least one *syn* G^{Br}-tetrad. It is interesting to note that if we assume that the G^{Br}-tetrad at the 5′ edge is characterized by G^{Br} in *syn* glycosyl conformation and the one at the 3′ edge is in *anti* conformation, the overall arrangement of the tetrads should resemble that of $[d(G_3T_4G_3)]_2$ (Fig. 9, sketch *a*), and therefore it should give rise to very similar CD spectrum. However, the presence of a negative band at 263 nm clearly indicates that this is not the case. Another possible arrangement should be that with a *syn* G^{Br}-tetrad at 3′ edge and an *anti* G^{Br}-tetrad at the 5′ edge. However, this arrangement would lead to one homopolar H-to-T and one heteropolar T-to-T stacking. Unfortunately no calculations have been reported so far in the literature for a T-to-T stacking, therefore, for the time being, we cannot rule out this possible arrangement. Nevertheless, the hypothesis of having both *syn* G^{Br}-tetrads at 3′ and 5′ edges, creates the classical alternation of orientation of the tetrads (H-to-H and T-to-T) (Fig. 9, sketch *h*) found in antiparallel quadruplexes like $[d(G_4T_4G_4)]_2$ (Fig. 9, sketch *a*), whose CD spectrum closely resembles that of parallel quadruplex $[d(TG^{Br}GG^{Br}T)]_4$ (Fig. 10E).

Other examples reported in literature of parallel quadruplexes having heteropolar (H-to-H and/or T-to-T) stacking come from other oligonucleotides having inversion of polarity sites in the middle of the G tracts. In particular, among others, the sequences $^{5'}TG^{3'}$-$^{3'}GGT^{5'}$, $^{3'}TG^{5'}$-$^{5'}GGT^{3'}$, $^{5'}TGG^{3'}$-$^{3'}GGT^{5'}$ and $^{3'}TGG^{5'}$-$^{5'}GGT^{3'}$ have been analyzed by both CD and NMR spectroscopy.[30,32] $^{5'}TG^{3'}$-$^{3'}GGT^{5'}$ forms a quadruplex structure composed by two "parallel" subunits having all the residues in *anti* glycosyl conformation. This arrangement leads to a typical H-to-T stacking with a T-to-T stacking at the inversion of polarity site (Fig. 9, sketch *i*). On the other hand, $[d(^{3'}TG^{5'}$-$^{5'}GGT^{3'})]_4$ is characterized by two "parallel" subunits and by a *syn* G-tetrad that lead to a heteropolar H-to-H stacking (Fig. 9, sketch *j*) and to a H-to-T stacking at the inversion of polarity site. Interestingly, $[d(^{5'}TG^{3'}$-$^{3'}GGT^{5'})]_4$ and $[d(^{3'}TG^{5'}$-$^{5'}GGT^{3'})]_4$ are characterized by a very similar CD profile (Fig. 10C). This means that the contribution to the CD spectrum of the heteropolar stacking H-to-H is very similar to that of T-to-T stacking. The very little difference of the two spectra could be ascribed to the fact that the T-to-T stacking in $[d(^{5'}TG^{3'}$-$^{3'}GGT^{5'})]_4$ is right across the inversion of polarity site, where the twist angle is slightly different from the one of a regular quadruplex.[33] This latter observation could also explain why their CD spectra are similar but not identical to that of $[d(G_3T_4G_3)]_2$, which is composed of three G-tetrads having the same relative orientation.

The arrangements of $^{5'}TGG^{3'}$-$^{3'}GGT^{5'}$ and $^{3'}TGG^{5'}$-$^{5'}GGT^{3'}$ closely resemble those of $^{5'}TG^{3'}$-$^{3'}GGT^{5'}$ and $^{3'}TG^{5'}$-$^{5'}GGT^{3'}$, respectively. $^{5'}TGG^{3'}$-$^{3'}GGT^{5'}$ turned out to be characterized by two "parallel" subunits with all G-tetrads having G residues in *anti*

glycosyl conformation (Fig. 9, sketch *l*). On the other hand, $^{3'}$TGG$^{5'}$-$^{5'}$GGT$^{3'}$ is characterized by one G-tetrad containing all Gs in *syn* glycosyl conformation adjacent to the 5′–5′ inversion of polarity site, although it maintains a parallel organization (Fig. 9, sketch *m*). The interpretation of the CD spectra of [d($^{5'}$TGG$^{3'}$-$^{3'}$GGT$^{5'}$)]$_4$ and [d($^{3'}$TGG$^{5'}$-$^{5'}$GGT$^{3'}$)]$_4$ are more complicated, due to the presence of inversion of polarity sites and the lack of a reference CD spectrum for these particular distributions and orientations of G-tetrads. Nevertheless, it is noteworthy that although both quadruplexes can be considered parallel, both of them are characterized by CD spectra having a positive band at *ca.* 295 nm (Fig. 10F).

5. Non-covalent G-quadruplexes

G-quartet formation is neither limited to G-rich oligonucleotides nor to aqueous solutions. It is well known, in fact, that guanylic acids, despite their being monomeric derivatives, self-organise in water to give elongated objects formed by G-quartets stacked one on the top of the other: no covalent bond connects the adjacent quartets and the structure is stabilised by π–π interactions, dipole-ion interactions and H-bonding.[34] Analogously, monomeric guanosine compounds functionalised in order to allow them to dissolve in organic solvents (lipoGs) form a variety of stacked G-quartet based supramolecular species in the presence of metal ions.[35,36] We will limit here the discussion to the simplest supramolecular species obtained from lipoGs, the so called "octamers" formed by two stacked G-quartets. As commented before, the two quartets in the octamer can be arranged in three different orientations: H-to-T (C_4 symmetry), H-to-H and T-to-T (D_4 symmetry). While 3′,5′-*O*-didecanoyl-2′-deoxyguanosine forms in solution a K^+-templated C_4-symmetric octamer structure,[37,38] other lipophilic guanosine derivatives (*e.g.* 5′-*O*-adamantanoyl-2′,3′-di-*O*-isopropylidene guanosine) can give a different stereoregular octamer with a D_4-symmetry.[39,40] It is relatively easy to assign the symmetry of the octamers to the C_4 or D_4 point group: in fact, while in the former case two sets of signals are observed in the 1D ^{1}H NMR spectrum (the two G-quartets are diastereotopic), in the latter case only a single set of signals is observed for the two homotopic G-quartets. Furthermore, circular dichroism is diagnostic of the stacking polarity of two contiguous G-quartets.

The C_4-symmetric octamer (homopolar H-to-T stacking) exhibits opposite signed bands at *ca.* 260 and 240 nm while in D_4-symmetric octamer (heteropolar T-to-T stacking) both bands are blue-shifted by 20–30 nm (with the opposite signed bands at *ca.* 290 and 260 nm), see Fig. 11. The situation recalls the issue discussed above for homopolar/heteropolar stacking in G4-DNA despite the absence of any "strand" connecting the guanosine units.§

6. Conclusions

Although the empirical, simplistic, rule relating the "parallel strands" of G4-DNA to a positive CD at 260 nm and a negative one at 240 nm and "antiparallel strands" to positive CD at 290 nm and negative at 260 nm is very popular and generally adopted by research groups using chiroptical techniques for studying G-quadruplex formation, its rational had never been clarified. Furthermore, in different examples presented here and taken from the literature the aforementioned rule is not followed or is meaningless. In guanine modified oligonucleotides or in the presence of inversion sites in the strands a feature of "antiparallel strands" (positive CD at 290 nm) is commonly observed in spite of the fact that all the four strands run in a parallel fashion. Furthermore, for monomeric guanosine compounds (guanylic acid derivatives), there is no strand connecting adjacent quartets and the concept of parallel/antiparallel strands cannot apply.

The different spectral features observed for different G-quadruplexes can be rationalised in terms of chromophores responsible for electronic transitions. Starting from the "chromophore" it would then be possible to justify how different folding patterns (characterised by, *inter alia*, different strand polarities) origin different CDs. A simple exciton coupling approach or more refined QM calculations allow to interpret the different CD features in terms of different stacking orientation (head-to-tail, head-to-head, tail-to-tail) between adjacent G-quartets irrespectively of the relative orientation of the stands (parallel/antiparallel)

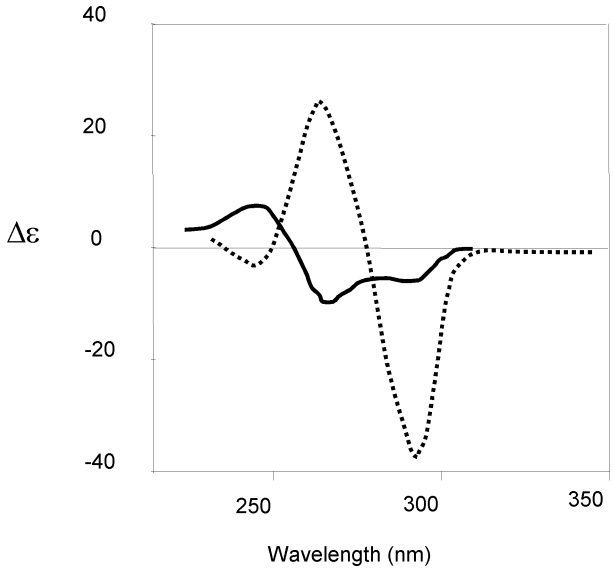

Fig. 11 Comparison between CD spectra of C_4- (solid line)[38] and D_4-symmetric (dotted line)[40] octamers lipoG$_8$·M$^+$.

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