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Higher-Order Quadruplex Structures

Luigi Petraccone

Abstract Structural studies have shown that four G-tracts along a DNA strand are the minimal requirement for intramolecular G-quadruplex formation. Longer DNA sequences containing multiples of four G-tracts could, in principle, form higher-order structures based on multiple G-quadruplex blocks. This latter condition is abundantly verified for the telomeric single-stranded overhang (~200 nt) consisting of tens of TTAGGG repeats, thus opening new interesting questions about the structure of the "real" telomeric DNA. How many quadruplex units form in the human telomeric overhang? Which type of quadruplex topologies? Do they interact or not? What about their binding properties? Although many of these questions are still unanswered, recent experimental and computational studies have begun to address them. The existence and relevance of these higher-order quadruplex structures in the human genome is now an interesting and stimulating research topic in the quadruplex field. The recent results, the unsolved problems, and the future prospects for understanding higher-order telomeric quadruplex structures are the main topics of this review. Other studies on long telomeric RNA sequences and on other intramolecular (non telomeric) DNA higher order quadruplex structures are also presented.

Keywords G-quadruplex · Higher order structure · Molecular modeling · Telomeric DNA · Telomeric RNA · Thermodynamic stability

Contents

L. Petraccone (✉)
Dipartimento di Scienze Chimiche, Università degli Studi di Napoli "Federico II", Via Cintia, 80126 Napoli, Italy
e-mail: luigi.petraccone@unina.it

1 Introduction

Single quadruplex units are building blocks for higher-order quadruplex structures both intramolecular and intermolecular, depending on whether or not the quadruplex blocks belong to the same DNA (or RNA) strand. In the last 5 years, intramolecular higher-order quadruplex structures have been the subject of increasing interest due to their relevance as a model for the full length telomeric DNA structure [1–9]. Indeed, structural studies have shown that four G-tracts along a telomeric DNA strand are the minimal requirement for intramolecular G-quadruplex formation but that the telomeric single-stranded overhang consists of tens of TTAGGG repeats (~200 nt) and may form higher order structures involving multiple quadruplex units. As telomeric DNA is a promising anti-cancer drugs target, detailed knowledge of its physiological relevant structure is of paramount importance for structural-based drug design.

Despite this, so far structural studies on human telomeric DNA have been limited to short DNA sequences (no more than four G-tracts) able to form individual quadruplexes (Fig. 1a). The first topology for a human telomeric quadruplex sequence to be reported in a K^+ environment was determined by crystallographic analysis [10]. This structure – called "parallel" or "propeller" – has all four G-tracts parallel and connected by three propeller loops. Later, two distinct (3+1) topologies, termed hybrid-1 and hybrid-2, have been reported using NMR methods in solution [11–13]. Both structures have three G-tracts oriented in one direction and the fourth in the opposite direction, but differ in the order of the loop arrangements. These two forms appear to coexist in K^+ solution and the equilibrium between them is governed by the presence of 3′-flanking bases [6]. Recently, an antiparallel basket type form in K^+ solution – called "Form-3" – have been reported [14]. This is distinct from the known antiparallel topology in Na^+ solution [15] and contains just two G-quartets. This structural polymorphism has stimulated wide debate: which of these topologies is the most biologically relevant? Which is the prevalent quadruplex structure in physiological conditions?

Although there is not yet a final answer, it is clear from these structural studies that the particular truncation of the full length telomeric DNA employed dictates the structure that is observed. Several four-repeat human telomeric sequences which start with a G (i.e., there are no flaking bases at the 5′-extremity) adopt

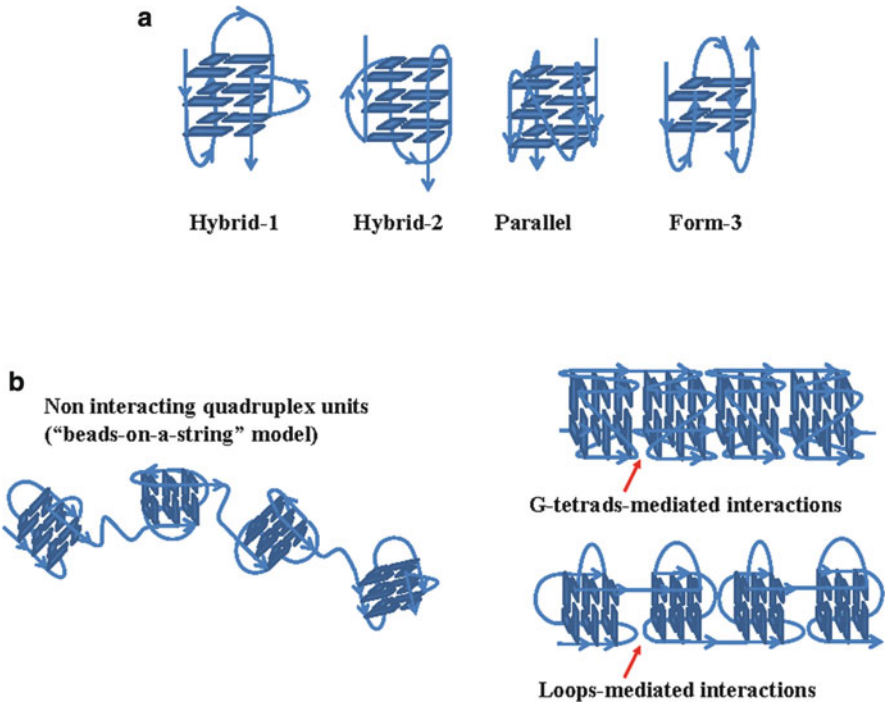

Fig. 1 (**a**) Schematic structures of the single quadruplex topologies (hybrid-1, hybrid-2, Form-3 and Parallel) determined for the four-repeat human telomeric sequences in K^+ solution (the *arrows* are in the 3′–5′ direction). (**b**) Possible structures for long telomeric sequences based on the single quadruplexes as building blocks. The quadruplex subunits could be not interacting (beads-on-a-string model) or could interact through staking interactions involving directly the G-tetrads cores or the loop bases

form-3 in K^+ solution [14]. Sequences with both 5′-flaking and 3′-flaking sequences adopt the hybrid conformation; hybrid-2 is favored by the presence of 3′-flaking bases [6]. What about the "real" telomeric overhang whereby each four-repeat unit has tens of TTAGGG repeats at both its extremities? When considering a quadruplex formed inside the full length telomeric DNA, the presence of flanking bases seems to be more realistic in mimicking the physiological condition. However, the possibility of forming consecutive quadruplex blocks adds another dimension to this already complex problem, while opening new interesting questions about the structure of the "real" telomeric DNA (Fig. 1b). How many quadruplex units form in the human telomeric overhang? What types of quadruplex topologies exist? Do the contiguous quadruplex units interact or not? What about their binding properties? Although many of these questions are still unanswered, recent experimental and computational studies have begun to address them. The question about the existence and relevance of these higher-order quadruplex structures in the human telomere is now an interesting and stimulating research topic in the quadruplex field. The recent results, the unsolved problems, and the future

prospects for understanding higher-order telomeric quadruplex structures are the main topics of this review. Other studies on long telomeric RNA sequences and on other intramolecular (non telomeric) DNA higher order quadruplex structures are also presented.

2 Experimental Evidence of Multi-quadruplex Structures in Human Telomeric DNA

In the last 5 years the properties of long telomeric sequences have been the subject of several experimental studies. Telomeric sequences with 8, 12, and 16 repeats, that potentially can form 2, 3, and 4 consecutive quadruplexes, have been employed (Table 1). What experimental evidence exists to show that these sequences really form intramolecular structures based on the single quadruplexes as building blocks? Data collected with different methodologies strongly point to this direction. Gel electrophoresis and analytical ultracentrifugation (AUC) data demonstrated that longer telomeric sequences form mainly compact intramolecular structures consistent with the formation of contiguous quadruplex units [1, 5, 8, 20, 21]. A further indication of the multi-quadruplex nature of these structures has been inferred from the comparison of their circular dichroism (CD) spectra with the CD spectrum of the standard single quadruplex forming sequences [such as $(TTAGGG)_4$ or slight variants thereof]. It has been observed that the CD spectrum of sequences formed by integral multiples of the four G-tracts [such as $(TTAGGG)_n$ with $n = 8, 12, 16$] has a similar shape to the CD spectrum of the single quadruplex but with an intensity that approximately corresponds to an integral multiple of the monomer intensity [1, 2, 5]. The simplest interpretation of this observation leads to the multi-quadruplex structure of the longer sequences: another quadruplex unit is added to the previous one when the DNA sequence is elongated.

Other indirect evidence of the existence of these multi-quadruplex structures comes from the analysis of their melting behavior. Van't Hoff analysis of the UV melting process for the $(TTAGGG)_n$ with $n = 8$ and 12 revealed a ΔH° values close to the value expected for a quadruplex melting (~200 kJ/mol). This observation suggests that the $(TTAGGG)_8$ and $(TTAGGG)_{12}$ structures are divided into more cooperative units which melt independently, each unit having (approximately) the "size" of a quadruplex [1, 22]. A similar result has been obtained for the eight-repeat sequence in the presence of 40% (w/v) PEG200 [23]. On the other hand, the total enthalpy values for the melting of 8-repeat and 12-repeat sequences has been measured directly by means of differential scanning calorimetry (DSC) yielding values more consistent with the presence of 2 and 3 quadruplex units, respectively [5]. The thermodynamics of these higher-order structures' melting will be discussed in more detail later in the chapter (see Sect. 5).

Several experimental studies were done on the eight-repeat sequences [such as $(TTAGGG)_8$ or slight variants thereof] [2, 9, 16]. In one of the early studies, the ability

Table 1 Studied DNA telomeric sequences with an integral multiple of the four G_3 blocks

		Solution conditions			
Telomeric sequences	N°[a]	Conc. (mM)	Salt	Subunit structure[b]	Reference
$G_3(T_2AG_3)_7$	n.d.[e]	100	KCl	Various[c]	[8]
$G_3(T_2AG_3)_7$	1–2	2.5	KCl	Hybrid	[9]
$G_3(T_2AG_3)_7$	2	100.0	NH_4OAc	Hybrid	[16]
$AG_3(T_2AG_3)_7$	2	100.0	KCl	Hybrid	[4]
$AG_3(T_2AG_3)_7$	2	10	KCl	Hybrid	[17]
		90.0	LiCl		
$(T_2AG_3)_8$	2	100.0	KCl	Hybrid[d]	[1]
$(T_2AG_3)_8$	2	100.0	KCl	Hybrid	[5]
$(T_2AG_3)_8$	1–2	50	KCl	n.d.	[18]
		10	$MgCl_2$		
$(T_2AG_3)_8\ T_2$	2	100	KCl	Hybrid	[2, 5, 19]
$G_3(T_2AG_3)_{11}$	1–3	2.5	KCl	Hybrid	[9]
$G_3(T_2AG_3)_{11}$	n.d.	100	KCl	Various[c]	[8]
$AG_3(T_2AG_3)_7$	2–3	10	KCl	Hybrid	[17]
		90	LiCl		
$(T_2AG_3)_{12}$	3	100	KCl	Hybrid[d]	[1]
$(T_2AG_3)_{12}$	3	100	KCl	Hybrid	[5]
$(T_2AG_3)_8\ T_2$	3	100	KCl	Hybrid	[5]
$G_3(T_2AG_3)_{15}$	n.d.	100	KCl	Various[c]	[8]
$(T_2AG_3)_{16}$	4	100	KCl	n.d.	[4]
		10	$MgCl_2$		
$(T_2AG_3)_{16}$	2	500	KCl	n.d.	[18]
		10	$MgCl_2$		

[a]Number of quadruplex subunits; more than one integral number indicates that a distribution has been observed (i.e., 2–3 means that DNA strands containing 2 or 3 quadruplex subunits were observed)
[b]Suggested quadruplex structure as inferred from the CD spectrum
[c]The quadruplex conformation was found to be dependent on DNA concentration (going in the direction antiparallel – hybrid – parallel on increasing DNA concentration)
[d]In the original paper, the authors suggested a mix of parallel and antiparallel as the hybrid structure and its CD spectrum was unknown
[e]n.d. stands for "not determined"

of the eight-repeat telomeric sequence to form a two-quadruplex structure has been demonstrated by means of CD, AUC, steady-state fluorescence measurements and molecular modeling [2]. Later, the formation of two contiguous quadruplexes in the $GGG(TTAGGG)_7$ DNA telomeric sequence has been confirmed on the basis of ESI–MS experiments by counting the number of ammonium ions selectively trapped in the structure [16]. Other authors demonstrated by using FRET that two contiguous quadruplexes are formed in the $AGGG(TTAGGG)_7$ sequence [4].

Another remarkable demonstration of a multi-quadruplex structure was obtained by means of atomic force microscopy (AFM) experiments on the $(TTAGGG)_{16}$ sequence [4]. AFM images showed that four contiguous quadruplexes, separated by only a short single-stranded TTA linker, readily formed in the 96 nt telomeric

repeat sequence, although the resolution of the method could not definitively assign the conformations of the individual quadruplex units.

In contrast to these studies, there are scattered reports on sequences with 8, 12, and 16 repeats that suggest that the number of quadruplex subunits formed in these sequences is lower than the value expected on the basis of their length [9, 17, 18]. For example, it has been reported that significant fractions of a 16-repeat and 12-repeat form only 2 quadruplex subunits (instead of 4 and 3, respectively) [17, 18]. However, the source of this discrepancy is not clear and should be further explored. The lower KCl concentrations employed in these studies could be a key element in explaining the observed results.

3 Toward a Molecular Model of Long Human Telomeric Sequences

Experimental evidence suggests that long human telomeric sequences form intramolecular structures formed by consecutive blocks of single quadruplex-like structures. However, the way from this "qualitative" information to a high-resolution structure of the full human telomeric overhang is long. What is the folding of the individual quadruplex units within the multi-quadruplex structure? How do they interact? Regarding this latter issue, there are two opposite possibilities: (1) the G-quadruplex blocks do not interact and can move relatively independently of each other, constrained only by the connecting TTA linkers (this model is called "beads-on-a-string") [1] or (2) G-quadruplex blocks strongly interact through stacking interactions involving directly the G-tetrads cores or the loop bases (Fig. 1b).

Recently, molecular modeling studies have provided some useful suggestions in addressing this and other questions [3, 5, 19]. The basic idea of these studies is that the most "natural" candidates for building blocks of a multi-quadruplex structures are the known individual quadruplex structures formed by the four-repeat human telomeric sequences. Among these, the hybrid and parallel quadruplexes provide efficient scaffolds for a compact-stacking multi-quadruplex structure of human telomeric DNA. Indeed, the 5′ end and the 3′ end of these quadruplex structures point in opposing directions and additional hybrid-type or propeller quadruplexes can be linked end-to-end to form linear multi-quadruplex structures [6, 24]. Following this point of view, several higher order quadruplex structures have been built by joining hybrid and parallel quadruplex as building blocks (Fig. 2). Molecular modeling techniques have been employed to evaluate which molecular models were stereochemically and energetically "plausible" higher-order quadruplex structures. Although experimental validation of the models is always needed, all-atom molecular dynamics (MD) simulations in explicit solvent have provided interesting information on the dynamics and stability of the quadruplex subunits, highlighting the specific implications of each structural model.

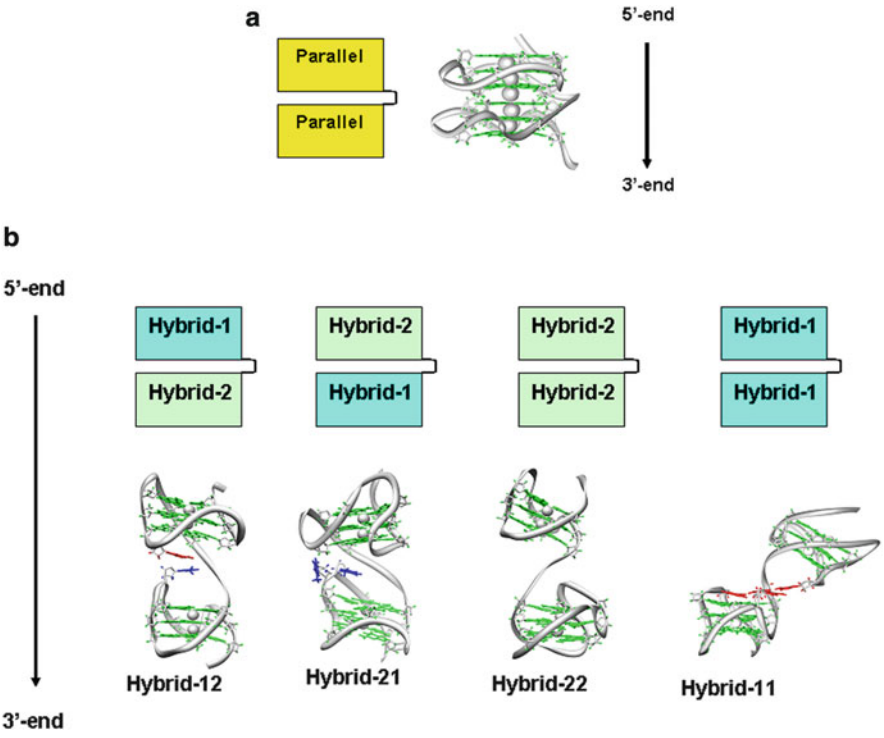

Fig. 2 Schematic representation and computed structures of the parallel (**a**) and hybrid-based (**b**) models of the eight-repeat human telomeric sequence containing two quadruplex subunits. For clarity, in the computed structures loop bases not involved in a quadruplex–quadruplex interaction are not shown. The 5′–3′ orientation is indicated by the *black arrows*. Adapted from [19]

3.1 Parallel-Based Higher Order Quadruplex Structures

A multi-quadruplex structure formed by consecutive parallel quadruplexes was first proposed by Neidle and coworkers [3]. In this model consecutive quadruplexes are connected by a TTA loop in a 5′–3′ orientation and the terminal G-tetrad of each quadruplex subunit stacks over the initial G-tetrad of the subsequent quadruplex subunits, forming extended stacks of such quadruplex structures (Fig. 2a). MD simulations of such molecular structures containing two, three, or four consecutive parallel quadruplexes have been carried out by two groups reaching similar results: the parallel-based higher order quadruplex structures are stable and particularly rigid as expected due to the large stacking interactions existing between each subunits [3, 5, 19]. Flexibility of the models decreases on increasing the number of G-quadruplex subunits (from two to four) due to the greater number of stacking interactions between the G-tetrads. The most flexible part of the model are the loops; however the conformational changes observed in the loops do not have any

impact on the structure of the central G-tetrads core. All the TTA loops in the structural models have similar conformation and are arranged diagonally, external to the guanine tetrads in a propeller-like manner characteristic of the parallel quadruplex topology. The dA nucleotide in each TTA sequence is swung back such that it intercalates between the two T bases and makes stacking interactions with one of the T bases. The contributions of the TTA loops to the total free energy, as estimated by molecular mechanics and Poisson–Boltzmann surface area calculations (MM–PBSA), are very similar [3].

There is no doubt that the parallel quadruplex with its external propeller loops and its exposed terminal G-tetrads seems an ideal topology with which to form extended stacked structures and these MD studies have shown that it can produce stereochemically and energetically plausible higher-order structures.

Nevertheless, experimental evidence of parallel-based higher order quadruplex structures formation is still absent and this model appears inconsistent with the CD spectra in solution reported for longer telomeric sequences (Table 1). Further, experimental AFM images of the $(TTAGGG)_{16}$ sequence clearly show peaks and valleys corresponding to the quadruplex subunits and the TTA linkers connecting them [4], whereas a compact cylindrical structure with contiguous quadruplexes stacked upon one another (without knots) should be expected on the basis of a parallel quadruplex higher-order model [3]. However, recent gel electrophoresis and CD data performed on the eight-repeat sequence in solution with a greatly diminished water activity [in buffers containing 40% (w/v) PEG200] suggest that, in this condition, the parallel-based structure could be slightly favored over other higher order structures (as was previously shown for the four-repeat sequence) [23, 25]. Whether or not, these experimental conditions (of low water activity) are representative of the cellular environment is still matter of debate [26–28].

3.2 Hybrid-Based Higher Order Quadruplex Structures

Various structural models have been built by combining in different ways the hybrid-1 and hybrid-2 conformations to form a two-quadruplex structure [19]. Four possibilities exist in combining the two hybrid-type quadruplex in a 5′–3′ orientation to form a two-quadruplex high-order structure: two consecutive hybrid-1 (Hybrid-11) or hybrid-2 (Hybrid-22) quadruplexes, one hybrid-1 followed by a hybrid-2 (Hybrid-12) or one hybrid-2 followed by a hybrid-1 quadruplex (Hybrid-21). Molecular modeling studies have shown that each of these possibilities results in a quadruplex–quadruplex junction with different structural and dynamic properties (Fig. 2b) [19]. For example, the Hybrid-12 and Hybrid-21 models are not equivalent. In the Hybrid-12 model an interaction between the second loop of the hybrid-1 quadruplex and the second loop of the hybrid-2 quadruplex has been observed, whereas in the Hybrid-21 model there is an interaction between the third loops of both quadruplex units. In the same way, the Hybrid-11 and Hybrid-22 structures are quite different from each other and from the mixed-type structures (Hybrid-12 and

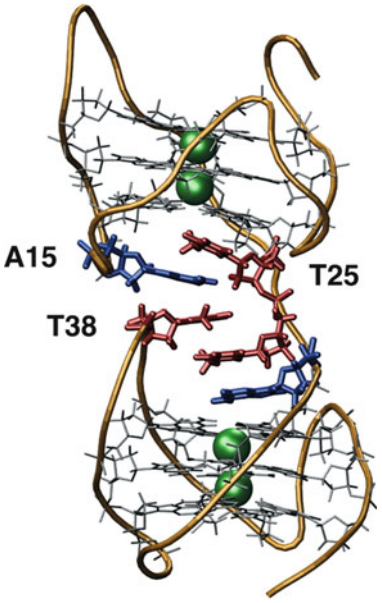

Fig. 3 Average structure from MD simulation of the Hybrid-12 model. The *spheres* are the potassium ions coordinated between the G-tetrads. The loops residues (A15, T25 and T38) involved in the quadruplex–quadruplex interaction are also shown (from [2])

Hybrid-21). Hybrid-11 shows a bending between the quadruplex units stabilized by a specific interaction between dA residues belonging to different subunits. In contrast, the Hybrid-22 model does not show any significant interaction between the quadruplex units. Further, all the hybrid-based structures are clearly different from the parallel model that has extensive quadruplex–quadruplex stacking interactions directly mediated by G-tetrads. The parallel model is the most compact one with an average 5′–3′ distance of 32 Å whereas for all the other models this distance is in the range 42–52 Å. Among all the hybrid-like models, the Hybrid-12 is the one with the most stable linkage between the two quadruplex units within the structure (Fig. 3). In particular, the adenine A15 (belonging to the first quadruplex) forms stable stacking interactions with the thymines T38 and T25 (belonging to the second quadruplex) for 95% of the MD trajectory. Further, it has been shown that, when the MD simulation starts from an initial conformation in which these bases do not interact, the A15-T38 is recovered over the course of the simulation, thus suggesting that this interaction is stable and characteristic of the Hybrid-12 model [2, 19].

As each quadruplex–quadruplex junction is a unique conformation, the molecular surfaces are considerably different, which could have significant implications for structure-based drug design. For instance, Hybrid-11, because of the bending of the two quadruplex units, presents an accessible planar surface corresponding to an A-triad structural motif that is not present in the other models. Despite these structural differences the free energies of these models, as evaluated by MM–PBSA calculations, are very similar, suggesting that the free energy barriers between the different hybrid-based two-quadruplex structures are very small, which is also

consistent with the low free energy barrier reported for the different conformations of the single quadruplex unit folding [29].

What these results bring to light is that, because of the asymmetry of the hybrid-types G-quadruplex structure, four different quadruplex–quadruplex junctions are possible, each characterized by specific shape, electrostatic, and dynamic properties [19]. In this regard it should be noted that a multi-quadruplex structure formed by only one type of hybrid should have only one type of junction (Hybrid-22 or Hybrid-11). The presence of the Hybrid-12 motif in telomeric sequences with more than two quadruplex units implies the presence of either both the Hybrid-12 and Hybrid-21 junctions. Thus, the existence of the mixed-hybrid higher-order quadruplex structure instead of one built from a single type of hybrid structure could provide a better means to modulate protein recognition and regulation of telomere biology. Further, the small free energy barriers between the different conformations (Hybrid-11, Hybrid-22, Hybrid-12, Hybrid-21, and all-parallel) suggests that an equilibrium between different forms could be very sensitive to the local microenvironment as well as to the binding of small molecules to a particular quadruplex–quadruplex junction.

A three-quadruplex structure formed by alternating hybrid-1 and hybrid-2 quadruplex subunits (corresponding to the model 5′-hybrid-1-hybrid-2-hybrid-1-3′, called Hybrid-121) has recently been simulated and proposed as representative of the 12 repeat $(TTAGGG)_{12}$ structure [5]. In this model, there are two different quadruplex junctions (following the DNA strand in the 5′–3′ direction): the hybrid-1-hybrid-2 and the hybrid-2-hybrid-1. The interactions between the two quadruplex units at the hybrid-1-hybrid-2 junction are retained during 10 ns of MD simulation of the Hybrid-121 model as it was observed for the two-quadruplex Hybrid-12 model confirming that this type of quadruplex–quadruplex interaction (QQI) is particularly stable. To date no other models combining hybrid with parallel or antiparallel or other quadruplex folding types have been subject of modeling studies and proposed as "plausible" alternative structures. As will be shown later, the hybrid-based models are, at present, the most consistent with the available data on longer telomeric sequences.

4 How to Discriminate Between Alternative "Plausible" Higher Order Quadruplex Models?

As described above, different higher order quadruplex structures have been proposed for longer DNA telomeric sequences depending on the type of quadruplexes involved and on the kind of interactions between the quadruplex units. All the proposed models are in principle potential candidates to represent the "real" DNA telomeric structures. How is it possible to distinguish these models experimentally? Which one might be most representative of a high-resolution structure of the telomeric DNA? Resolution of this problem is not an easy task for standard

structural techniques like X-ray crystallographic and NMR because of their inherent difficulty of coping with longer DNA sequences.

To address this issue, an innovative approach combining different experimental and computational approaches to discriminate among several plausible structures has been reported [19]. The basic idea is to validate the structural models by means of experimental measurements. To apply the method, one should look at some physico-chemical properties that can be readily computed from the structural models and, at the same time, can be measured experimentally with enough accuracy. The comparison of the predicted and the measured values of these properties can then be employed as test of validity of the structural models. This approach has been pioneered by the groups of Prof. J.B. Chaires and Prof. J.O. Trent (University of Louisville, KY, USA) to show that the parallel conformation was not the dominant structure adopted in solution by the four-repeat human telomeric sequence [30]. They employed the program HYDROPRO [31] to compute from the crystallographic atomic coordinates of the parallel quadruplex the expected value of the sedimentation coefficient and found it significantly different from the value experimentally measured in K^+ solution by means of AUC experiments. The same methodology was later employed to discriminate between two structural models for the three-quadruplex forming sequence $(TTAGGG)_{12}$, one involving all parallel quadruplex and the other containing both hybrid-1 and hybrid-2 quadruplexes (the Hybrid-121 model discussed in Sect. 3.2). The expected sedimentation coefficients distributions were computed from the MD trajectories of these two higher order quadruplex models and were compared with the experimental sedimentation coefficient distribution [5]. The measured sedimentation coefficient has been found to be consistent with the predicted value of the hybrid-based model whereas it has been found to be significantly different from the value predicted on the basis of the parallel-based model (Fig. 4a). On the other hand, the CD spectra of $(TTAGGG)_{12}$ were also characteristic of the hybrid-type structures (Table 1). Although these results do not allow unequivocal proof of the three-quadruplex structure, it clearly suggests that a major fraction of $(TTAGGG)_{12}$ can fold in three contiguous quadruplex units and that these units are most likely a mixture of hybrid conformations. The same results were obtained with the $(TTAGGG)_{12}TT$ sequence, suggesting that the presence of the 3′-flanking bases are less important in determining the quadruplex units folding in comparison with their drastic effect on the folding of the short telomeric sequences [5, 6].

Another useful experimental property able to discriminate between different higher order quadruplex models is the degree of solvent accessibility of the adenine residues involved in the quadruplex loops. Indeed, this property can be easily predicted from the atomic coordinates of each model by computing the solvent accessibility surface area (SASA) of each dA residues which can then be experimentally investigated by quantitative fluorescence studies using systematic single-substitutions to replace each of the adenine bases with 2-aminopurine (2-AP). Indeed, the fluorescence properties of 2-AP are extremely sensitive to local environment (conformation), making this base analog ideal for distinguishing among different quadruplex conformations [32]. This approach has been successfully

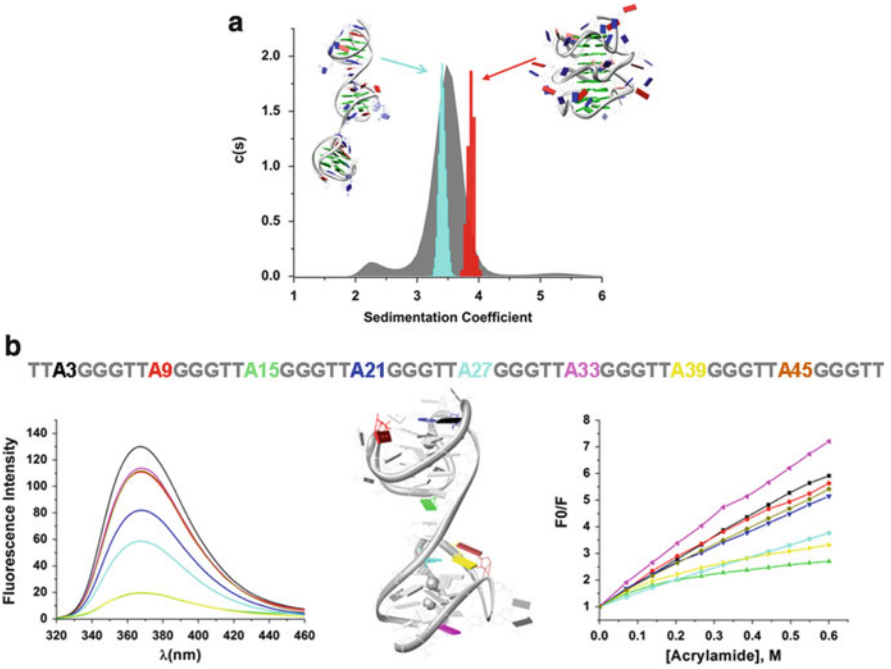

Fig. 4 Comparison between predicted properties of the higher order quadruplex models and their experimental measurement. (**a**) The predicted sedimentation coefficient distributions for the hybrid-based (*cyan*) and parallel-based (*red*) models are compared with the experimentally determined distribution (*gray*). (**b**) The results of fluorescence intensity measurements (plots on the *left*) and fluorescence quenching studies using acrylamide (plots on the *right*) are shown for oligonucleotides containing 2-aminopurine. The structure of the Hybrid-12 model is also shown (in the *middle*). The colors indicate the position of the 2-aminopurine substitution as specified in the sequence written on the *top*. Each experiment was performed in 10 mM potassium phosphate, 100 mM KCl, and 0.1 mM EDTA at pH 7.0 (from [2, 5])

employed to discriminate among the five (Hybrid-12, Hibrid-21, Hybrid-11, Hybrid-22, and all-parallel) two-quadruplex models described above (Sect. 3.2) for the eight-repeat telomeric sequence [19]. The eight-repeat sequence has eight adenine bases, each dA being in a specific loop of the two-quadruplex structure and the analysis of the five models showed that the environment of each adenine base differs depending on the folding adopted by the two-quadruplex structure. In particular, different adenine bases are involved in the junction region between the two quadruplexes depending on the model considered [19]. Both steady-state fluorescence and acrylamide quenching experiments were performed to provide a quantitative measure of the environment of the loop adenines (Fig. 4b). The trend in the fluorescence intensities and 2-AP solvent accessibilities has been shown to be consistent with the adenine environment predicted on the basis of the Hybrid-12 model, indicating that this structure can plausibly exist in solution whereas none of the other models were compatible with all the fluorescence experiments results

[19]. As shown previously, in the Hybrid-12 model the two quadruplex units are not fully independent, and are stabilized by specific interactions at the interface.

5 Energetics of Higher-Order Human Telomeric Quadruplexes Formation

The energetics of multi-quadruplex formation can provide useful information on their whole structure and on the interactions between the quadruplex subunits. Thermal stability of higher order quadruplex structures can be monitored by several spectroscopic techniques involving UV and circular dichroism (CD). In an early studies on stability of long telomeric DNA sequences, UV absorption has been employed to follow the melting behavior of the $(TTAGGG)_{n=1-12}$ series [1, 22]. These authors measured very similar melting temperatures and van't Hoff enthalpy (~190–200 kJ/mol) for the $(TTAGGG)_{n=4,8,12}$ sequences, suggesting that the 8-repeat and 12-repeat sequences are composed of quadruplex subunit similar to the quadruplex formed by the four-repeat sequence. On the other hand, similar melting temperatures are also reported for the $(TTAGGG)_n$TT series [5].

A different result is reported for the $G_3(TTAGGG)_{n=7,11,15}$ series, showing much lower T_m and ΔH° values in comparison with the corresponding $G_3(TTAGGG)_3$ single quadruplex [20]. However, it should be noted that the $G_3(TTAGGG)_3$ sequence is significantly different from the other telomeric sequences as the absence of flanking sequences at 5′-end could induce the form-3 quadruplex [14]. In contrast with the other quadruplex folding (hybrid-1, hybrid-2, and parallel), form-3 has its 5′ and 3′ end pointing in the same direction and could be unable to form linear multi-quadruplex structures. Further, the shape of the CD spectra of the $G_3(TTAGGG)_{n=7,11,15}$ series appears to be length-dependent, suggesting that the conformation of $G_3(TTAGGG)_{n=7,11,15}$ may not result from a simple addition of consecutive $G_3(TTAGGG)_3$ quadruplex units [9]. Interestingly, recent results from temperature-gradient gel electrophoresis (TGGE) experiments suggest that a significant fraction of the $G_3(TTAGGG)_7$ and $G_3(TTAGGG)_{11}$ sequences does not form the expected maximum number of quadruplex subunits [9].

Beside this, it should be noted that in the cited studies the thermodynamic parameters are derived by the van't Hoff analysis of the melting curves. The validity of this analysis is based on the two-state approximation for the melting process and it is questionable for the melting of long DNA telomeric sequences. This assumption has been experimentally tested by means of the singular value decomposition (SVD) analysis of CD spectra, demonstrating that the melting of longer telomeric sequences is clearly not a two-state process [5]. Although the van't Hoff derived parameters can provide some useful information on the average cooperative unit inside a complex structure, an accurate determination of the thermodynamics of higher order quadruplex structures should be achieved by means of model-free techniques such as DSC. This technique has been employed

to determine the thermodynamics parameters for the folding processes of the $(TTAGGG)_{n=4,8,12}$ and the $(TTAGGG)_nTT$ series [5]. Deconvolution of the thermograms by means of a statistical mechanical method unraveled that the complexity of the denaturation processes increases with the number of quadruplex units within the sequence (Fig. 5). Indeed, the DSC data can be adequately represented by two species (folded and unfolded) for the single quadruplex sequence, but there are clearly three species for the two quadruplex assembly and four species for the three quadruplex assembly. This observation suggests that each quadruplex unit in the higher-order structures is not independent and identical, but is thermodynamically unique and is influenced by its neighbors. Table 2 summarized the thermodynamic parameters for each transitions as derived by the deconvolution of the DSC profiles of the $(TTAGGG)_nTT$ series. Interestingly, once the thermodynamic parameters for the single quadruplex and the multi-quadruplex structures are known, it is possible to quantify the apparent coupling free energy [33] for the assembly of more quadruplex units. The difference between the total free energy of the folding of three contiguous quadruplexes and three times the folding free energy of a single quadruplex gives a coupling free energy ($\Delta G_{\text{Coupling}}$) of about +13 kJ/mol. The positive sign indicates an unfavorable coupling free energy in the higher order structure formation, arising from unfavorable interaction between the quadruplex units. Analysis of the energetic contributions to the total free energy change suggests that $\Delta G_{\text{Coupling}}$ contains contributions from both enthalpy and entropy components (Table 2). A similar analysis for the 8-repeat sequences shows small coupling free energies of only 2–3 kJ/mol, indicating lesser destabilizing interactions between two contiguous quadruplexes compared to 3 found in the 12-repeat sequence. It is possible that coupling free energies may become increasingly unfavorable in longer quadruplex assemblies. The ≈200 nt telomeric overhang could potentially fold into a structure with about eight contiguous quadruplex units. Larger unfavorable coupling free energies could, however, limit complete folding of the overhang, leaving single-stranded regions that might facilitate or nucleate protein binding.

Do the thermodynamic data support the presence of QQIs? Thermodynamically, the amount of all the interactions are measured by the enthalpy changes needed to unfold the structure. Theoretically, one could estimate the amount of QQI by subtracting from the total unfolding enthalpy change of the multi-quadruplex structure the sum of the enthalpy changes of the component quadruplex subunits measured separately. Although, practically, this procedure could be imprecise as the enthalpy change can be dependent on the (often unknown) quadruplex subunit conformation within the multi-quadruplex structure, a qualitative comparison of the enthalpy changes for the eight-repeat sequences with the four-repeat sequences reveals that a QQI interaction, if it exists, should be no more than 20–30 kJ/mol [5]. This value seems too small to be consistent with strong QQI as, for example, the extended stacking interactions between the G-tetrad proposed in the parallel-based model discussed previously (~70–80 kJ/mol should be expected for a stacking between two all-anti G-tetrads) [34]. However, weaker QQI as loops-mediated interactions between the consecutive quadruplexes cannot be excluded.

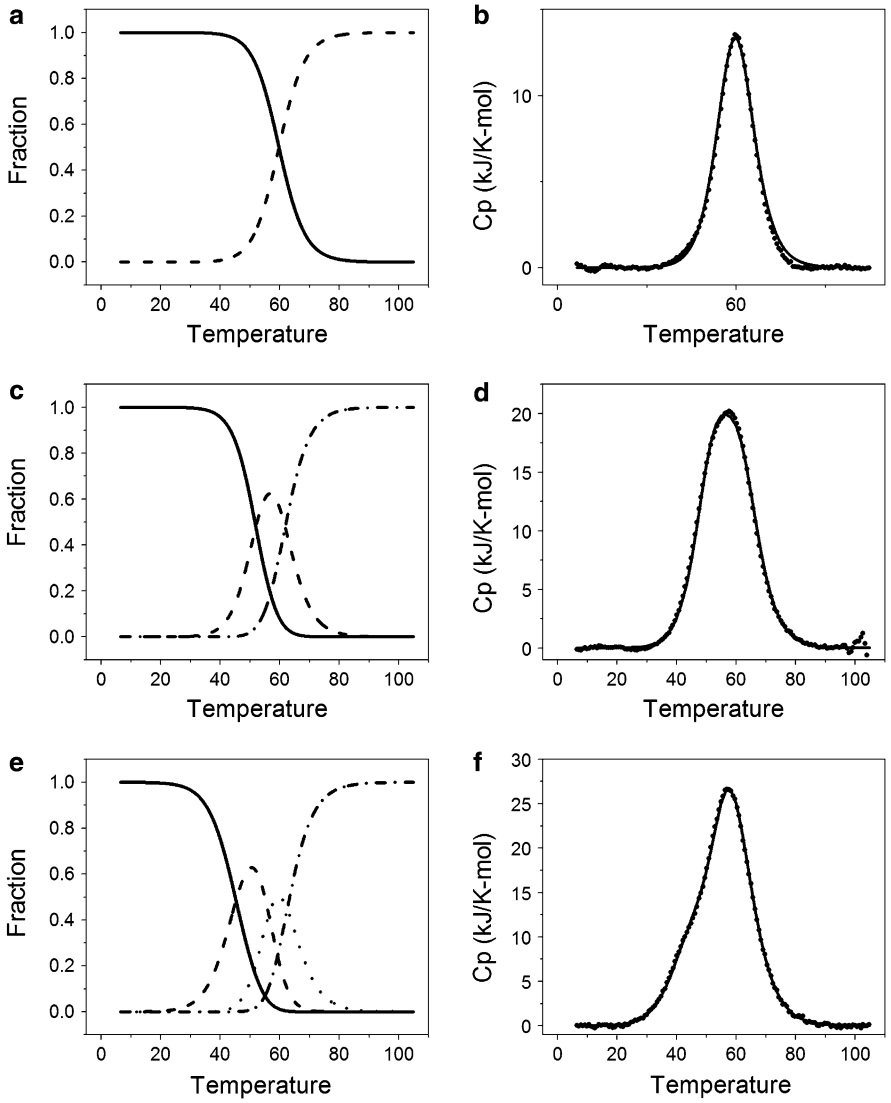

Fig. 5 Deconvolution of DSC profiles for the $(TTAGGG)_nTT$ ($n = 4,8,12$) series. Panels **a**, **c**, and **e** show the species plots for the single quadruplex, two and three quadruplexes structures, respectively. Panels **b**, **d**, and **f** show the corresponding best fits to experimental thermograms (from [5])

Interestingly, fluorescence melting experiment on an eight-repeat sequence containing 2-aminopurine shows that the loops region melts several degrees before the melting of the G-tetrad core of the quadruplexes, thus suggesting that

Table 2 Deconvolution of DSC thermograms for the $(TTAGGG)_nTT$ series[a]

Sequence	T_m (°C)	ΔH (kJ/mol)	ΔS (J/K-mol)	$\Delta G_{folding}$ (20 °C) (kJ/mol)
$(TTAGGG)_4TT$	59.8	213	639	−25.8
$(TTAGGG)_8TT$				
Transition 1	52.0	219	676	−20.9
Transition 2	61.8	222	662	−28.0
$(TTAGGG)_{12}TT$				
Transition 1	45.4	176	553	−14.0
Transition 2	56.3	221	672	−24.1
Transition 3	62.6	204	606	−25.2

[a]Data are from [5]

loops-mediated QQI could be lost before the melting of the whole multi-quadruplex structure [19].

The energetics of the quadruplex folding/unfolding processes for the eight-repeat sequence has also been evaluated in crowding/dehydrating conditions (in the presence of PEG200) by means of kinetic measurements [23]. Under these conditions, on increasing temperature, each quadruplex subunit converts from the hybrid to the parallel conformation before melting. The results suggest that folding properties of each quadruplex subunit are somewhat affected by the presence of the adjacent quadruplex.

6 Binding Properties of Higher Order Quadruplex Structures: New Prospects for Drug Design?

In the past 15 years, numerous research groups have been focused on the design of new ligands trying to optimize the interactions between these small molecules and the G-quadruplex motif [35–40]. However, in most of the G-quadruplex-ligand binding studies, the target DNA sequences are short human telomeric sequences (typically in the range 21–26 nt) able to fold into a single quadruplex structure [6, 35, 41]. Despite the increasing amount of structural information on longer DNA telomeric sequences, very few data are available on the binding properties of these sequences compared with the shorter DNA telomeric sequences. However, the existence of quadruplex–quadruplex interfaces in the longer telomeric sequences could be a predominant factor in determining the recognition properties of the telomeric DNA and the results of binding studies performed on single quadruplex may not necessarily be useful to predict the binding properties of higher order quadruplex structures. Further, the achievement of the specific recognition of the quadruplex–quadruplex interface could become an important step in the design of new quadruplex ligands able to discriminate the telomeric quadruplexes among the large number of quadruplex forming sequences existing in the human genome [42, 43].

The presence of possible binding sites involving two adjacent quadruplex units has been suggested by Neidle and coworkers [3, 44]. On the basis of crystallographic information, they built a stable model of a complex between the drug BRACO-19 and two contiguous parallel G-quadruplexes consisting of the drug sandwiched between two parallel quadruplex units. Shinohara et al., starting from a multi-quadruplex model, designed and characterized a ligand that specifically recognizes the quadruplex–quadruplex interface between two hybrid quadruplexes and that does not bind the single quadruplex unit [45]. In another study the binding of the cationic porphyrin TMPyP4 and of a triazatruxene (azatrux) to a quadruplex higher order structure was compared with the binding of the same ligands to the single quadruplex [46]. The results suggest that the binding properties of TMPyP4 and azatrux to the higher order quadruplex structure cannot be predicted on the basis of binding experiments performed on the single quadruplex. In particular, azatrux binds with a much higher affinity to the multiplex $(TTAGGG)_{12}$ than the single $(AG_3TT)_4$ quadruplex and with a stoichiometry consistent with the binding at the quadruplex–quadruplex interface. A binding study of hemin to multi-quadruplex structures further support the hypothesis of the existence of a new type of binding site (with respect to the isolated quadruplex) located at the quadruplex–quadruplex interfaces (called internal sites). Binding of hemin to the internal sites greatly enhances the hemin-DNA catalytic activity with respect to the hemin binding to an external site (not at the Q–Q interface) [17]. Binding of sanguinarine to the interface between two quadruplex units in an eight-repeat sequence has also been suggested by mass spectroscopy and Taq polymerase stop assay experiments [47].

Although still limited, taken together these preliminary studies suggest that the quadruplex subunits in the higher order structures do not behave independently, at least with respect to their binding properties. Hence, it could be more appropriate to consider the DNA telomeric structure as a higher-order structure with unique binding pockets rather than as simply a collection of single quadruplex units, during the drug-design process. However, further studies are needed to gain reliable insights into the binding behavior of the higher order quadruplex structures.

7 What About Long RNA Telomeric Sequences?

Telomeric DNA can be transcribed by DNA-dependent RNA polymerase II; the resulting RNA transcripts, ranging in size from 100 to 9,000 nucleotides, are composed of the tandem repeats of the sequence r(UUAGGG) [48–50]. All the questions/problems introduced about the long human DNA telomeric sequences arise again when considering the long human RNA sequences (TERRA). Do long RNA telomeric sequences form higher order quadruplex structures (as in DNA)? Which is the folding of the quadruplex subunits? etc. . .?

NMR, ESI–MS, and CD data suggest that RNA quadruplexes are not as polymorphic as DNA and mainly form the parallel structure both in short and long

telomeric sequences [7, 16, 51–53]. There is experimental evidence that long telomeric RNA molecules are compact and form higher-order structures based on parallel quadruplex units [54, 55]. Do the quadruplex units stack on each other or form a "beads-on-a-string" model involving non-interacting blocks? Results from recent RNase T1 cleavage experiments seem to indicate a middle way! Indeed, it was found that quadruplexes comprised of four and eight UUAGGG repeats were most resistant to RNase T1 digestion, suggesting a "beads-on-string"-like model of long human telomeric RNA, whereby each bead is made up of one or two-quadruplexes, respectively. In this model there are both interacting (inside the two-quadruplexes beads) and non-interacting quadruplexes (single quadruplex bead). However, the presence of regions with more than two stacked quadruplexes (longer beads) could not be completely excluded. A structural model involving a cation-mediated stacking of two parallel quadruplex subunits in the eight-repeat sequence r[GGG(UUAGGG)$_7$] has also been suggested by ESI–MS results [16].

Several structural models have been proposed for the RNA two-quadruplexes structure, all being based on the parallel folding and differing only in the stacking mode between the two parallel G-quadruplex blocks. There are four possible stacking modes: (1) 3′–5′, in which the stacking occurs between the 3′-end of the first quadruplex and the 5′-end of the second quadruplex; (2) 5′–3′, in which the stacking occurs between the 5′-end of the first quadruplex and the 3′-end of the second quadruplex; (3) 5′–5′, in which the stacking occurs between the 5′-end of both the quadruplex blocks; and (4) 3′–3′, in which the stacking occurs between the 3′-end of both the quadruplex blocks. In the "alternate-direction stacking" model, successive parallel quadruplexes adopt alternately the 5′–5′ and the 3′–3′ modes. Molecular modeling studies have been carried out on the 8-repeat human telomeric RNA sequence to test the feasibility of the different stacking modes between two parallel quadruplex blocks. The 5′–5′, 3′–3′, and 3′–5′ stacking modes were found to be stable throughout the MD simulations whereas the 5′–3′ stacking mode was found to be unstable, as the UUA linker was too short to span the six G-tetrads layers [55]. Continuous stacking of the loop bases and extensive loop–loop interactions, involving adenine–adenine stacking across the two blocks, were observed for the 5′–5′ stacking mode. This continuous stacking of loop bases was not observed in the simulations of other stacking modes. Experimentally, (intermolecular) 5′–5′ stacking has been observed between two parallel quadruplexes formed by the 10-mer RNA sequence r(GGGUUAGGGU) [52]. However, more stacking modes could coexist in long TERRA structures and additional experiments are needed to validate further a particular structural model.

8 Non-telomeric Higher-Order Quadruplex Structures

In the above paragraphs, computational and experimental studies on intramolecular higher-order human telomeric DNA (and RNA) quadruplex structures have been discussed. However, higher-order quadruplex structures can be found in other

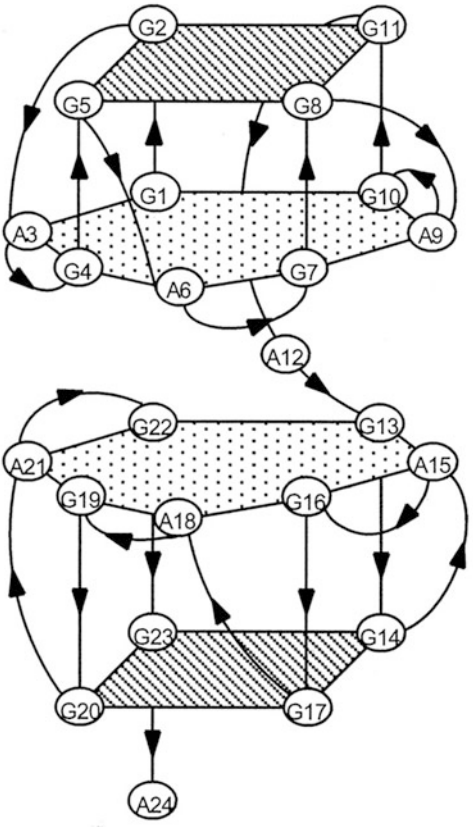

Fig. 6 Schematic representation of the higher order quadruplex structure of the $(GGA)_8$ sequence. There are extensive stacking interactions between the heptads of the quadruplex subunits (from [56])

regions of the genome. Significantly, the first demonstration of higher order packing of quadruplexes has been reported on the non-telomeric $d(GGA)_8$ sequence [56]. The GGA triplet repeat is present in eukaryotic genomes [57] and is frequently located in biologically important regions [58–62]. The four-repeat $d(GGA)_4$ sequence forms an intramolecular quadruplex composed of a G-tetrad and a G(:A):G(:A):G(A:):G heptad [63]. What has later been demonstrated is that two such G-quadruplexes can assemble, forming a two-quadruplex structure; this is, so far, the only NMR structure of a higher order quadruplex structure. The intramolecular packing of the two quadruplexes in $d(GGA)_8$ is achieved through the stacking interaction between the heptads of each quadruplex (Fig. 6). As a result, the two quadruplexes are arranged in a tail-to-tail manner. It should be noted that head-to-tail or head-to-head arrangements should involve the energetically less favorable G-tetrad-heptad or G-tetrad-G-tetrad stacking, respectively. However, sequences with higher numbers of integral multiple of four GGA repeats (i.e., with more than two quadruplex subunits) should necessarily involve these later types of quadruplex–quadruplex junctions. Interestingly, the four G–G segments (in each subunit) are aligned parallel to each other; thus the G-core of this quadruplex

architecture is similar to the G-core of the parallel human telomeric quadruplex and the structure of the $d(GGA)_8$ sequence resembles somewhat the proposed parallel-based model for the human telomeric DNA.

A higher order G-quadruplex structure has also been proposed for the hTERT, full-length, core promoter sequence containing 12 consecutive G-tracts. Taq polymerase stop assay and dimethyl sulfate footprinting experiments revealed a unique end-to-end stacked G-quadruplex structure for this sequence. This structure consists of an all parallel G-quadruplex, linked to another, atypical G-quadruplex, formed by two pairs of consecutive G-tracts separated by a 26-base loop [64].

Another example of a non-telomeric genomic region that can form multi-quadruplex structure is the insulin-linked polymorphic region (ILPR) that contains G-quadruplex forming sequences located at −363 bp upstream of the Insulin coding sequences [65–67]. It is formed by tandem repeats of the most prevalent sequence ACAGGGGTGTGGGG. It has been shown that the sequence containing two ILPR repeats ($ILPR_{n=2}$) forms an intramolecular quadruplex [66]. Although there have been no atomic-level structural studies, DMS footprinting, CD and native gel electrophoresis collectively demonstrated that the sequence containing four ILPR repeats ($ILPR_{n=4}$) forms an intramolecular structure with two G-quadruplexes [68]. Further, DMS footprinting experiments on $ILPR_{n=4}$ identified specific guanines (close to the quadruplex-quadruplex interface) protected only in the presence of the neighboring G-quadruplex, strongly suggesting some kind of QQI. TGGE results and mechanical pulling of $ILPR_{n=4}$ at the single molecule level are also consistent with the presence of QQI, showing that the two quadruplexes are unfolded cooperatively [9, 68].

In contrast with most of the findings on human telomeric DNA, CD of $ILPR_{n=4}$ is different from CD of $ILPR_{n=2}$, demonstrating that the quadruplex conformation in $ILPR_{n=4}$ is different from the quadruplex formed by $ILPR_{n=2}$, perhaps due to the presence of QQI. This result demonstrates that the $ILPR_{n=4}$ structure is far from being described as a simple addition of two $ILPR_{n=2}$ quadruplex units and more detailed structural studies are needed to get more insight into $ILPR_{n=4}$ higher order structure.

9 Concluding Remarks

Intramolecular higher-order quadruplex structures represent a challenge for biophysical and structural studies but at the same time represent a promising target for drug design that demands detailed study. Our knowledge of these higher-order structures is far from being complete and much work needs to be done. However, it is possible to delineate the following tentative conclusions from the available data:

1. Longer DNA and RNA telomeric sequences form intramolecular structures formed by single quadruplex as building blocks.

2. Most of the available data are consistent with higher order structures involving, as building blocks, hybrid-like quadruplexes (both hybrid-1 and hybrid-2) for telomeric DNA and the parallel quadruplex for telomeric RNA.
3. In DNA, the interactions between the quadruplex subunits are relatively weak and most likely based on loops-mediated interactions whereas in long RNA telomeric sequences there are large stacking interactions involving the G-tetrads of adjacent parallel quadruplexes.
4. DNA higher order quadruplex structures (and most likely RNA) have binding properties different from those of the single quadruplex subunit. Particularly, the quadruplex–quadruplex junction provides a new specific binding site.

Regarding this last point, one should emphasize that different higher order structures and quadruplex–quadruplex interfaces could be simultaneously present along the human telomeric overhang. The dynamic equilibrium among these conformations could have a key role in modulating protein recognition and in controlling the biology of the telomeres. This hypothesis awaits experimental verification. Together, the results presented in this chapter represent a good starting point for future studies on quadruplex higher-order structures aimed at a deeper understanding of this fascinating part of the quadruplex world!

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