

How to study G-quadruplex structures

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Abstract

All guanine-rich DNA and RNA molecules tend to fold into inter- and intra-molecular structures called G-quadruplexes. The core of a G-quadruplex is composed of at least two adjacent G-tetrads stabilized by monovalent cations. While it has been suggested that there could potentially be over 375 000 quadruplex forming sequences in the human genome alone, only a small number of three-dimensional G-quadruplex structures are known. More structural data are needed to explain their presence and function *in vivo*. We offer here a short review of experimental methods commonly used to determine G-quadruplex topologies.

Key words: G-quadruplex, UV spectroscopy, CD spectroscopy, mass spectrometry, NMR spectroscopy

Introduction

In 1910 Bang (Bang, 1910) reported that concentrated solutions of guanosine easily self-associate and form polycrystalline gels. This unusual phenomenon puzzled researchers for the next five decades until 1962 (Gellert et al., 1962) when the association of guanine bases into a tetrameric arrangement was first determined by crystallographic methods, revealing the assembly of tetrameric units into large helical structures. For the next 25 years little attention was paid to the possible role of G-quadruplexes in biological systems until the observation that oligonucleotides corresponding to the G-rich strand of telomeric DNA displayed unexpectedly high electrophoretic mobility on nondenaturing polyacrylamide gels (Williamson et al., 1989). Two laboratories independently proposed that G-rich sequences in the chromosomes of eukaryotic cells, including the single-stranded 3'-overhang of telomeres, could form four stranded DNA structures (Williamson et al., 1989; Sen et al., 1988).

Currently, there is considerable interest in quadruplexes because G-rich sequences with the potential to form this type of structure have been found to exist in several biologically important DNA regions, such as telomeres and the promoter regions of human oncogenes. Bioinformatic analysis has identified 375 000 candidate sequences within the human genome that could adopt G-quadruplex structures, although many are likely to be due to chance and may be relatively unstable (Huppert

et al., 2005; Todd et al., 2005). Another bioinformatic study has revealed that not only DNA but also numerous mRNA that contain G-rich sequences may be able to fold into G-quadruplex structures (Huppert et al., 2008a) suggesting that the RNA quadruplexes may be involved in biological processes.

G-quadruplex structures are important not only because they are involved in the regulation of transcription and translation (Di Antonio et al., 2012, Huppert, 2008b). There is increasing pharmaceutical interest in the potential possibility of manipulating these processes to develop novel therapeutics (Bugaut et al., 2012; Balasubramanian et al., 2009; Collie et al., 2011). Knowledge of G-quadruplex structures is essential for the rational design of small molecules that could selectively bind to these structures to specifically modulate gene transcription, or to inhibit telomerase, as well as for the development of aptamers that can selectively bind to particular proteins (Collie et al., 2011; Gatto et al., 2009). Recently, experimental evidence for the presence and function of G-quadruplexes *in vivo* has started to emerge (Lipps et al., 2009).

The number of potential applications of quadruplexes in supramolecular chemistry and material science is enormous, ranging from the formation of biosensors (Guo et al., 2012) and nanomachines (Alberti et al., 2006) to G-wires (Miyoshi et al., 2011) and they may serve as a basis for developing advanced biomaterials.

The high potential of guanosine and its derivatives for self-recognition and self-assembly as well as their recognition ability for other biologically important molecules has been noted by the scientific community. The design of novel guanosine-based molecular architectures and their exploration as possible building blocks for new hybrid biomolecular electronics and biosensing was one of the objectives of the COST (European Cooperation in Science and Technology) Action MP0802 (2008-2012).

The fundamental unit of the quadruplex is a G-tetrad composed of a planar array of four guanine bases associated through a network of hydrogen bonds. Each guanine base is both a donor and acceptor of two hydrogen bonds, respectively (Fig. 1A). Quadruplex formation requires the presence of monovalent cations, in particular K^+ and, to a lesser extent, Na^+ or NH_4^+ . The precise location of the cations between the tetrads depends on the nature of the ion. The Na^+ ions lie on a plane with a G-tetrad, whereas the K^+ and NH_4^+ ions exist between two successive G-tetrads. Quadruplexes can be formed by the association of four separate DNA or RNA strands, the dimerization of two strands that each contain two G-tracts, or the intramolecular folding of a single strand that contains four (or more) tracks of two or more guanine residues. Quadruplex structures can be parallel when all the strands are parallel or anti-parallel when at least one of four strands is anti-parallel to the others (Fig. 1B). In particular, a parallel quadruplex conformation implies that all nucleosides in individual G-tetrads have the same conformation of the glycosidic bond (*anti*•*anti*•*anti*•*anti* or *syn*•*syn*•*syn*•*syn*). G-tetrads in anti-parallel quadruplexes comprise two guanines in *syn* conformation and another two in *anti* conformation (*syn*•*syn*•*anti*•*anti* and *syn*•*anti*•*syn*•*anti*). In the hybrid type, parallel-antiparallel (3+1) G-quadruplexes, three guanines have the same conformation, while the fourth residue has an opposite one (*syn*•*anti*•*anti*•*anti* and *anti*•*syn*•*syn*•*syn*). The guanine tracts are connected by loops of variable size and sequence. There are three different types of loops (Fig. 2). Edgewise loops (also known as lateral loops) connect two adjacent anti-parallel strands. Diagonal loops connect two opposing anti-parallel strands, while the double chain reversal loops (also known as propeller loops) connect the adjacent parallel strands. Both the length and the composition of the loops connecting the G-tetrads can influence the topology of a quadruplex and its thermodynamic sta-

bility. All quadruplex structures have four grooves, the dimensions of which (narrow, medium and wide) are defined by the glycosidic conformation of the adjacent bases in the tetrad. For a more comprehensive description concerning the fundamentals of a quadruplex reference structure Burge et al. (2006) provides an excellent overview.

G-quadruplex structures reveal a high degree of structural polymorphism in terms of nucleotide sequence, the orientation of the strands, the *syn/anti* glycosidic conformation of guanines, groove widths, the sequence and length of the loops or the nature of counter ions. Understanding the molecular forces that govern quadruplex folding is of particular interest. Many experimental techniques have been used to study G-quadruplex formation, each examining different aspects of the structure. Although the combined use of a few methods provides invaluable structural information on the conformational changes which occur under various conditions (concentration, pH, temperature, salt type and its concentration, type of buffer, etc.). However, only X-ray crystallography and NMR spectroscopy allow complete structure determination with atomic resolution. Here, we describe the most common techniques which, in our opinion, are essential in G-quadruplex structural studies.

Circular Dichroism Spectroscopy (CD)

Circular dichroism spectroscopy (CD) is one of the simplest methods that can be used to assess the nature of the quadruplex fold. This technique relies on the differential absorption by chiral molecules of left and right-handed circularly polarized light (Boczkowska, 2002). CD spectra of nucleic acids are very sensitive to stacking interactions between the bases. In G-quadruplexes, three different types of topology (parallel, antiparallel, hybrid) are correlated with the *syn/anti* conformation of guanosine involved in G-tetrad formation. As a result, different stacking is observed between adjacent G-tetrads and therefore a unique CD signature for a given topology occurs (Fig. 3) (Karsisiotis et al., 2011; Randazzo et al., 2012; Vorlickova et al., 2012). In general, CD spectra of typical four stranded parallel DNA quadruplexes exhibit absorption maxima at 264 nm and minima at 240 nm. They can be easily distinguished from single or double stranded parallel quadruplexes with external loops, because an additional positive peak near 290 nm

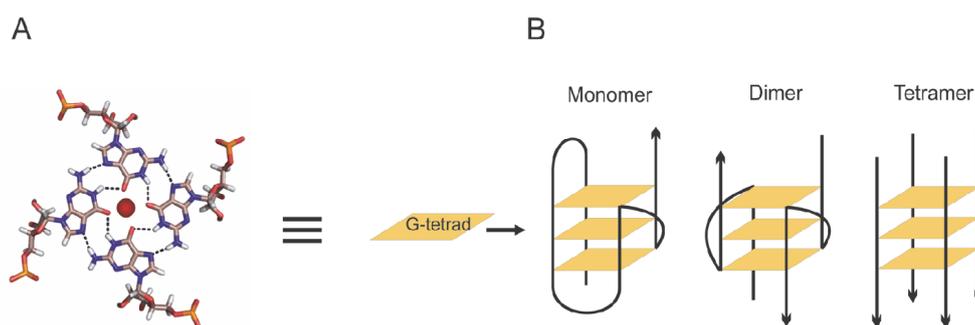


Fig. 1. Schematic representation of G-tetrad (A) and examples of possible quadruplex folds: anti-parallel single stranded, bimolecular anti-parallel and tetramolecular parallel

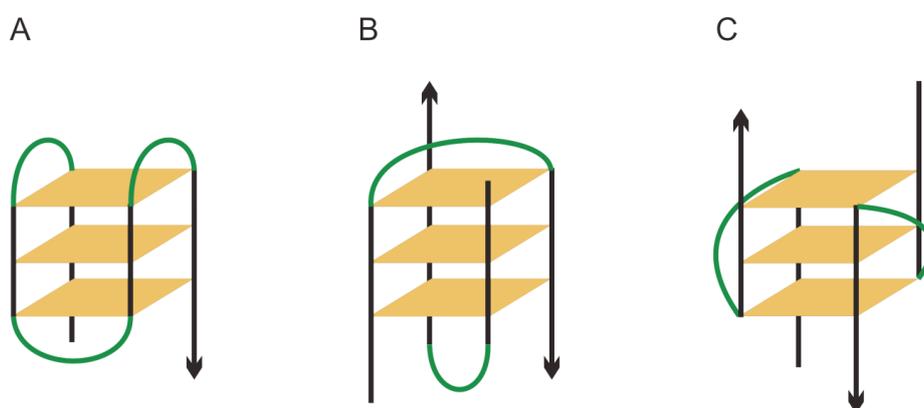


Fig. 2. Schematic diagrams showing potential linking loop arrangements: edgewise (A), diagonal (B) and double chain reversal loops (C)

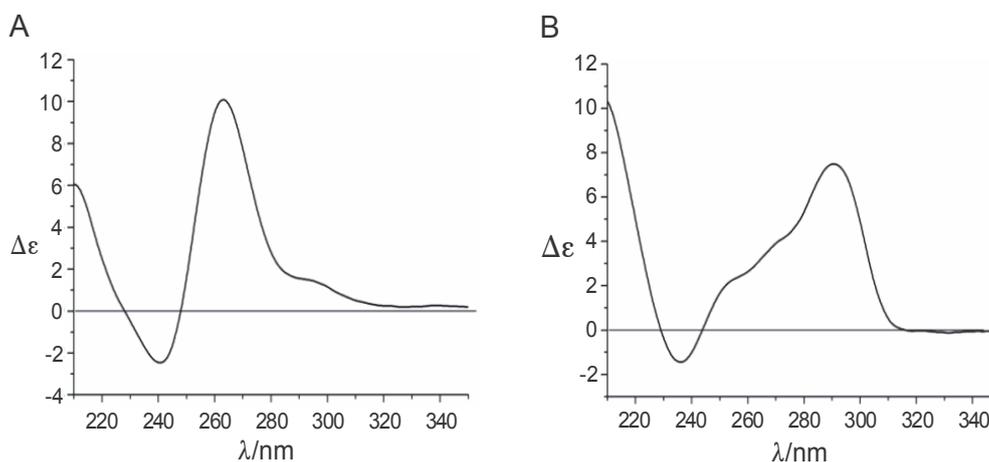


Fig. 3. The CD spectrum of parallel (A) and anti-parallel (B) DNA quadruplexes

appears in their CD spectra (Paramasivan et al., 2007). The CD spectra of DNA and RNA parallel quadruplexes are also very similar to those of A-form duplexes (Kyrp et al., 2009). However, these two types of structural motifs can usually be distinguished by the positive or negative sign of a peak present at 210 nm. A-form duplexes display

a characteristic negative band, whereas parallel quadruplexes have a positive band at this wavelength. Thus, only the presence of two positive peaks, at 210 nm and 260 nm, is indicative of the quadruplex structure. The appearance of an absorption maximum at 295 nm and minimum at 265 nm is characteristic of anti-parallel DNA quadruplexes.

Table 1. The characteristic peaks observed in CD spectra for different topologies of quadruplexes

Type of the quadruplex	CD profile at λ [nm]			
	210	240	265	295
Tetramolecular parallel	+	–	+	
Bimolecular or unimolecular parallel with external loops	+	–	+	+
Antiparallel single stranded	+	+	–	+
Hybrid (3+1)	+	–	+	+

plexes. Finally, the spectrum of hybrid quadruplexes is characterized not only by dominant positive bands at 295 nm and 265 nm but also a negative band at 240 nm (Table 1).

CD spectroscopy can also provide information on conformational changes induced by altered solution conditions. Using this technique one is able to follow the kinetics of quadruplex formation (Liu et al., 2012). It is also possible to analyze the map of conformational changes because the same molecule can isomerize due to many factors. For example, the addition of a molecular crowding agent such as poly(ethylene glycol) or co-solvent (methanol, ethanol, acetonitrile) induces structural changes in quadruplexes which can be observed by CD spectroscopy (Wei et al., 2009). Furthermore, analysis of the temperature dependence of the CD spectra allows the monitoring of the stability of quadruplex structure and a determination of the melting temperature. By minor modifications of the oligonucleotide sequence and careful optimization of solution conditions it is possible to obtain a single conformer in solution, which is crucial for the study of quadruplex structures by NMR spectroscopy and X-ray crystallography.

A better understanding of the various factors that contribute to the specific patterns of CD spectra is required before CD data alone can be used to determine quadruplex topology unambiguously (Gray et al., 2008). Until such data are available, the results obtained from CD spectra must be analyzed with caution and other biophysical methods should be used to confirm quadruplex formation.

UV Spectroscopy

Profile of the melting curve

UV spectroscopy is a fast method to confirm quadruplex structure formation and evaluate its structural sta-

bility. Typically, measurements of the melting temperatures of nucleic acids are performed at a wavelength of 260 nm, at which the maximum of their absorption is observed. For DNA and RNA duplexes approximately a 25% difference in absorption at 260 nm between the completely folded and unfolded (melted) states are detected. However, for DNA and RNA quadruplexes those differences are not that evident as they barely amount to 4%. Optionally, it is possible to record the melting profile at different wavelengths, preferably at 295 nm, where the absorbance is significantly lower, but denaturation of the sample is exhibited in an over 50% change in absorbance amplitude (Mergny et al., 1998, Rachwal et al., 2007). A detailed protocol for UV melting studies of quadruplexes was prepared by J.L. Mergny and L. Lacroix (2009).

Confirmation of quadruplex structure formation is possible by simple analysis of the UV curve. This is because at 295 nm the melting profile of a quadruplex is inverted in relation to the profile of duplex structure. G-quadruplexes undergo a hypochromic shift (i.e. lower absorbance) at 295 nm upon melting (Fig. 4). Measurements of melting profiles in varying conditions enable a characterization of the dependence of the stability and kinetics of the quadruplex association and dissociation processes on various factors, including strand concentration, type and concentration of various ions. In addition, the thermodynamic parameters of this structure formation may be determined (Mergny et al., 1998 and 2005a; Gros et al., 2007; Smargiasso et al., 2008; Pasternak et al., 2011; Avino et al., 2012).

Thermal Difference Spectra (TDS)

A thermal difference spectrum (TDS) is obtained by subtracting the UV spectra recorded above and below the melting temperature (usually at 20 °C and 90 °C, respectively). It has a shape that is unique for each type of

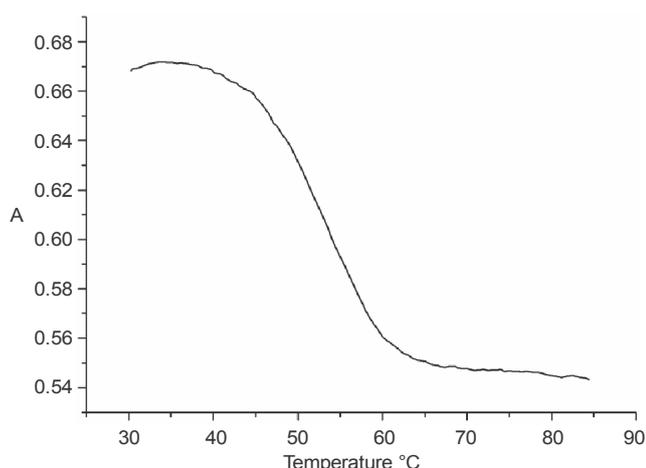


Fig. 4. UV denaturation profile of RNA quadruplex at 295 nm

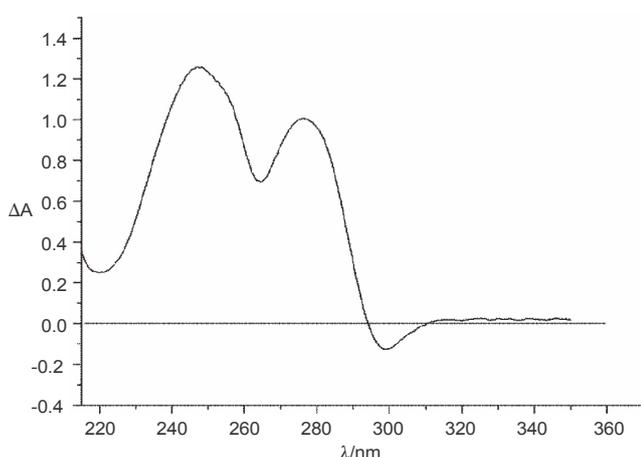


Fig. 5. An example of the thermal difference spectrum of RNA quadruplex

nucleic acid structure. A detailed description of the TDS profiles of nucleic acid structural motifs can be found in the literature (Mergny et al., 2005b). Over 900 TDS spectra were recorded for 200 different oligonucleotides that were representative for each type of possible folding. It is assumed that every structural motif is characterized by unique base stacking interactions, which is reflected in the TDS profile.

The TDS “fingerprint” of G-quadruplexes is characterized by a negative band at 295 nm and positive bands centered at about 240 nm and 270 nm (Fig. 5). The most characteristic is the band at 295 nm, because it corresponds to the stacking interactions between G-tetrads that form the spine of the quadruplex structure. In the case of parallel quadruplexes the negative band is less intense than the band occurring for anti-parallel quadruplexes. A.T. Karsisiotis and colleagues (2011) proposed

another TDS strategy to distinguish quadruplex topology. They determined TDS ratios as the absolute values of $\Delta A_{240\text{nm}}/\Delta A_{295\text{nm}}$, $\Delta A_{255\text{nm}}/\Delta A_{295\text{nm}}$ and $\Delta A_{275\text{nm}}/\Delta A_{295\text{nm}}$, where ΔA_{λ} is the difference between the absorbance above and below the melting point at a given λ , and it transpired that the values of the $\Delta A_{240\text{nm}}/\Delta A_{295\text{nm}}$ ratio for parallel quadruplexes exceeded 4, and for anti-parallel folding it was below 2.

Unfortunately, the shape of the TDS spectrum alone is not sufficient to prove quadruplex formation and it is always required to interpret this together with the data obtained from other methods such as CD or NMR spectroscopy. It is because the negative band which is characteristic of quadruplex structures can also be observed for Z-DNA, i-motif, Hoogsteen duplexes and pyrimidine triplex structures (Mergny et al., 2005b). However, the absence of this negative band provides evidence that the molecule does not fold into the quadruplex structure.

Electrospray Ionization Mass Spectrometry (ESI-MS)

Electrospray Ionization Mass Spectrometry (ESI-MS) was successfully employed for detection a G-quadruplex structure formation in 1993 (Goodlett et al., 1993). Since then the technique has been a useful tool in structural studies of G-quadruplexes, including strand stoichiometry (Joly et al., 2012; Borbone et al., 2011; Cui and Yuan, 2011; Rosu et al., 2002 and 2010; Zhou et al., 2007), quadruplex-ligand interactions (Rosu et al., 2008; Li et al., 2008; Gabelica et al., 2007), structural transitions (Ferreira et al., 2012; Amato et al., 2009 and 2011; Li et al., 2009), the kinetics of structure formation (Rosu et al., 2010; Gabelica et al., 2009) and many others (for a review, see Yuan et al., 2011).

Mass spectrometry relies on ionization of the compounds to generate charged molecules or molecule fragments in order to measure their mass-to-charge ratios (m/z). For structural studies of nucleic acids prevention of molecule fragmentation is often required. The electrospray ionization method allows structure destruction to be avoided during the measurement by keeping both the temperature of the capillary or Z-spray source and acceleration voltages in the transfer optics as low as possible. A very helpful experimental report containing sample preparation hints, preferred instrument settings

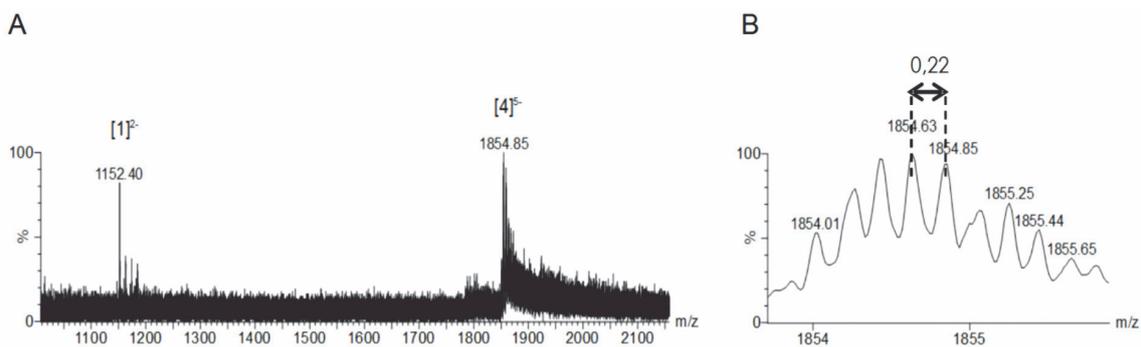


Fig. 6. Electrospray mass spectrum of r(AGGAGGA)₄ quadruplex (A) and the distance between isotopic peaks (B)

and recording procedures was prepared by V. Gabelica (Gabelica, 2010).

ESI-MS appears to be the method of choice for determination of the number of nucleic acid strands involved in a G-quadruplex formation and the number of ions adducted between its G-tetrad layers. Importantly, the negative ion mode is preferably used for recording the ESI-MS spectra of quadruplexes, because nucleic acid backbones are negatively charged in solution (fully deprotonated) (Gabelica, 2010).

In order to determine the number of nucleic acid strands involved in the formation of a particular structure it is necessary to determine the molecular weight (MW) of the ionized quadruplex, based on the measured m/z value. This value may also be used to calculate the number of cations located inside the G-quadruplex structure. Next, the information on the exact number of ions that are trapped inside the structure helps to predict the number of G-tetrad planes existing in the G-quadruplex structure. Sodium and potassium ions should be avoided in ESI-MS structural studies of G-quadruplexes. Although their presence between adjacent G-tetrads stabilizes the structure, they also tend to condense around negatively charged nucleic acid backbones during solvent droplet evaporation. This results in the occurrence of additional $[\text{DNA/RNA} + n \text{Na}^+/\text{K}^+]$ adducts that are heterogeneous in mass, which often leads to the loss of sensitivity and mass accuracy. To overcome this problem, NH_4^+ ions are usually used, in a typical concentration of 150 mM and with CH_3COO^- as the counter-ions. Ammonium ions allow salt adducts to be avoided because complete solvent evaporation is followed by proton transfer between NH_4^+ and PO^- from the negatively charged phosphodiester backbone, and in consequence

ions that are not coordinated between G-tetrads evaporate as NH_3 (Gabelica, 2010; Rosu et al., 2008). Usually the detected number of ammonium ions is one less than the number of G-tetrad planes involved in the structure formation, as only one ion is typically found between each pair of adjacent G-tetrads. This rule was clearly shown by F. Rosu et al., 2008. Determination of the number of adjacent G-tetrads may help in the understanding of the topology of the analyzed G-quadruplex structure.

To calculate the structural parameters the equation: $m/z = (n \times m_{\text{NA}} + (t-1) \times 17 - z)/z$ can be used (n – the number of strands, m_{NA} – the molecular weight of neutral nucleic acid in Daltons, t – the number of consecutive G-tetrads, z – the charge of the ion). If the resolution of the mass spectra is sufficient and the isotopic distribution can be resolved, the charge can be easily calculated based on the rule that the distance between isotopic peaks is $1/z$ (Rosu et al., 2008). For example, if the molecular weight of the r(AGGAGGA) molecule is 2306.50 Da, the m/z value is 1854.85 (Fig. 6A), and the distance between isotopic peaks is 0.2 (Fig. 6B), then the charge is 5, the number of nucleic acid strands is 4 ($4 \times 2306.50 = 9226$) and the number of ammonium ions sited between G-tetrads is equal to 3 ($(1854.85 \times 5 - 9226)/17 = 2.8$). So, the analyzed quadruplex structure contains four adjacent G-tetrads.

ESI-MS spectra recorded for the samples prepared in aqueous solutions may exhibit quite low signal intensities. This happens mainly because of the high surface tension of the formed droplets, which slows down the evaporation processes and allows fewer ions to reach the detector. To obtain a higher signal intensity, methanol with a 10-20% (v/v) final concentration is added to the sample

immediately before injection into the mass spectrometer. Unfortunately, in some cases methanol was observed to influence the structure formation kinetics (Rosu et al., 2010). To be sure that the obtained results are reliable it is advisable to compare them with the spectra recorded for molecules in 100% aqueous solutions (Ferreira et al., 2012).

Nuclear Magnetic Resonance Spectroscopy (NMR)

As already mentioned, high-resolution NMR spectroscopy and X-ray crystallography can both be used to determine G-quadruplex structure with atomic resolution. However, only NMR spectroscopy allows determination of the structure in solution, a natural condition for molecules found in living cells.

G-quadruplexes give characteristic NMR spectra (Webba da Silva, 2007). The resonances corresponding to imino protons of guanine residues involved in G-tetrad formation appear at characteristic chemical shifts around 10.5–12 ppm (Fig. 7A). Usually, the number of the imino proton resonances is correlated with the number of guanosine residues involved in G-tetrad formation. This spectral region is separated from imino regions of other forms of nucleic acids, such as duplexes, single-strands, or other secondary structures. Therefore, this region of the NMR spectra provides a convenient method for monitoring not only the formation of a G-quadruplex structure but also its ligand binding interactions (Phan et al., 2005).

Another characteristic feature of the NMR spectra of quadruplexes is the very slow exchange of guanine imino protons with deuterated solvents. For example, the exchange of the solvent from H₂O to D₂O causes an immediate loss of exchangeable imino- and amino proton signals for duplex forms, while in quadruplexes a complete replacement of the hydrogen bonded atoms with deuterium generally occurs after many hours, sometimes even after a few weeks or months (Fig. 7B). This observation is often used to prove G-quadruplex formation. G-quadruplex structure depends not only on the nucleotide sequence but also on sample preparation and solvent conditions. Such factors as sample annealing and quenching, nucleic acid concentration (Trajtkovski et al., 2012), temperature (Phan et al., 2003), salt type and concentration (Phan, 2010), the type of the buffer used and the presence of a co-solvent (Vorlickova et al., 2007)

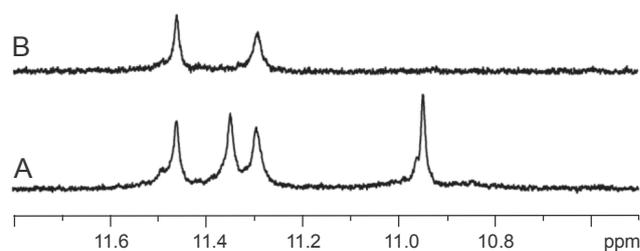


Fig. 7. Imino region of r(AGGAGGA)₄ quadruplex in 90% H₂O/10% D₂O (A) and after 12 h in D₂O (B)

can also affect the relative population and NMR spectra of G-quadruplexes.

In order to determine the three-dimensional structure of G-quadruplexes by NMR spectroscopy, the application of multidimensional methods is needed to allow the assignment of ¹H, ¹³C, ¹⁵N and ³¹P resonances. After the complete spectral assignment, the folding topology of a G-quadruplex is determined, followed by three-dimensional structure determination using NMR-derived distance restraints in combination with molecular dynamics calculations. However, successful structure determination by NMR spectroscopy is possible only for molecules which form a major stable structure with good NMR spectral properties in solution, i.e., sharp, well resolved lines. The coexistence of different forms in solution not only makes structural studies harder by significantly reducing the amount of available information, but usually makes it impossible to draw any conclusions. To improve the quality of NMR spectra and facilitate structural analysis, different approaches to obtain a single G-quadruplex conformation in solution have been established. It has been found that even minor changes of the oligonucleotide sequence at either the 5' or 3' ends may change the quality of the spectrum (Fig. 8) (Phan et al., 2007). In quadruplex studies a common practice is to screen many different oligonucleotide sequences until one is found which gives a good NMR spectrum and allows a structural analysis to be initiated (Phan et al., 2004, Ambrus et al., 2005, Phan et al., 2005). However, the introduction of such modification may result in a change of the quadruplex topology (Fig. 9). Alternatively, in order to force a single conformation in solution some guanosine residues can be substituted by 8-bromoguanosine or inosine (Lim et al., 2009, Lim et al., 2010). In fact, even if the methods above are used, about 10% of higher-order structures as

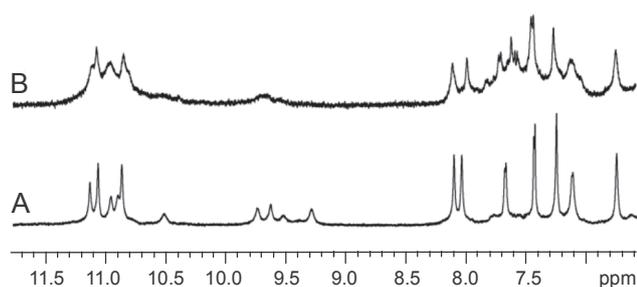


Fig. 8. The influence of the addition of 5'-phosphate group to r(UGGUGGU)₄ quadruplex. The ¹H NMR spectrum of r(UGGUGGU)₄ (A) and r(pUGGUGGU)₄ (B)

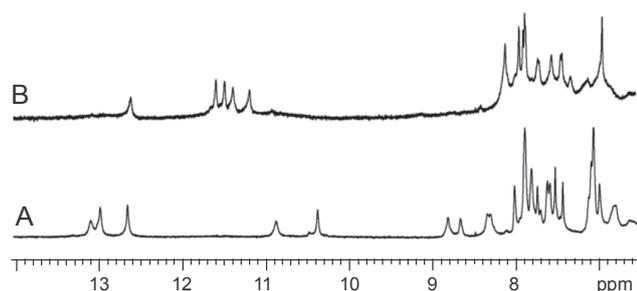


Fig. 9. The change of quadruplex topology after the substitution one guanosine residue by 8-bromoguanosine, ^{Br}G. The ¹H NMR spectrum of r(GCGGCGGC)₄ (A) and r(GC^{Br}GGCGGC)₄ (B)

well as an alternative conformation background is usually observed at ¹H NMR spectra.

Two reviews on the application of NMR spectroscopy to study the structure, dynamics and interaction of G-quadruplexes have been recently published and many details concerning spectral assignment strategy, fold determination, structure calculation can be found there (Webba da Silva 2007, Adrian et al., 2012).

Conclusions

In addition to the methods presented above there is a wide range of other techniques that have been used to study G-quadruplex structures. These include biophysical methods such as FRET (*Fluorescence Resonance Energy Transfer*), DLS (*Dynamic Light Scattering*), DCS (*Differential Scattering Calorimetry*), biochemical techniques, such as gel electrophoresis or enzymatic probing and computational methods such as molecular dynamics. Nevertheless, in our opinion, the combined use of the methods described in this review have many advantages. UV and CD spectroscopies as well as mass spectrometry are convenient, fast, sensitive and informative experimental techniques, whereas NMR spectro-

scopy provides the G-quadruplex structures with atomic resolution.

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