

Exploring the characterization tools of guanine-quadruplexes

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1. ABSTRACT

Occurrence of guanine-rich sequences throughout the genome at specific locations like chromosomal ends (telomeres), promoters and Untranslated regions (UTR's) is very well documented. Quite recently, visualization of guanine-quadruplex in human and mammalian cells have also provided a very significant evidence for the *in vivo* existence of guanine-quadruplex, reconfirming their biological relevance in cellular processes like replication, transcription, recombination, *etc.* Guanine quadruplexes have enormous potential of exhibiting various topologies which differ, by number/orientation of strands or loop orientations *etc.* Some relatively new polymorphic structures like 3+1 quadruplex, G-triplex, and Tri-G-quadruplex have also been proposed for the guanine-rich sequences. Various biochemical and biophysical techniques have been used to characterize these multistranded DNA structures. An extensive review of the mechanistic models of the already existing and newly emerging techniques is actually required, which may further facilitate our understanding about these structures. This review aims to summarize some of these techniques along with their requirements and limitations, which might further give some insights for the fine tuning of the solution and environmental conditions needed for facilitating guanine-quadruplex formation.

2. INTRODUCTION

Guanine-rich sequences are found throughout the genomes at various locations (1,2) and have been

shown to form Hoogsteen hydrogen bonding between guanine bases. This further result into the formation of G-tetrads which stack on each other to form guanine-quadruplex structures (Figure 1) (2-8). Recently, these quadruplex structures have also been visualized in human cells (9-10) and their *in vivo* existence along with its biological relevance is quite elegantly being reviewed (11). Some advanced structures like 3+1 quadruplex, G-triplex, Tri-G-quadruplex and quadruplex with bulges have also been reported by various laboratories and have recently been reviewed from our own research group (12). The applications of guanine-quadruplexes have shown enormous potential in various fields like nanotechnology, medical field, pharmaceutical industries, biotechnology, cancer biology, epigenetics, supramolecular assembly *etc.* (12).

The most important criteria in the analysis or the structural determination of these multistranded/ alternative or unusual structures of biomolecules is their separation, identification and purity, which is extremely difficult and hence makes the use of the physico-chemical techniques extremely important. This difficulty arises due to the enormous potential of these G-rich sequences of undergoing structural polymorphism, which is actually a result of the presence of the sticky ends (potential hydrogen bonding sites) of guanines. The commonly used biochemical methods are generally based on the physical chemistry principles, like charge, conformation, thermal stability, absorption, diffusion, density *etc.* These techniques should be used in

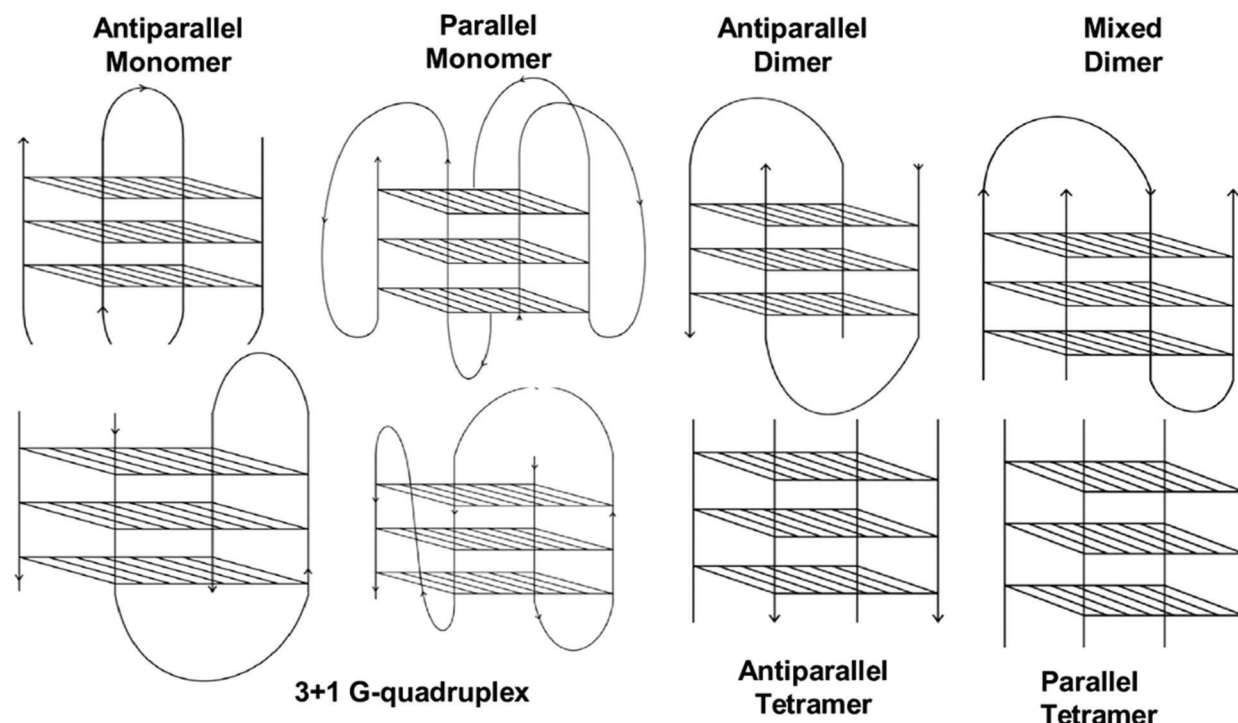


Figure 1. Various topologies of guanine-quadruplexes.

Table 1. Examples of some techniques used for characterizing G-quadruplexes

S. No.	Type of technique	Examples
1.	Biophysical	Single molecule measurements (Atomic force microscopy, AFM; Scanning electron microscopy, SEM; Laser tweezers, Fluorescence resonance energy transfer, FRET), Hydrodynamics (electrophoresis, sedimentation equilibrium analysis), Thermodynamic methods (Melting, Differential scanning calorimeter; DSC), Spectroscopy (Circular dichroism; CD, Vibrational circular dichroism; VCD, Infrared; IR, Raman, Fluorescence, Surface plasmon resonance (SPR))
2.	Biochemical	Enzymatic methods (Polymerase stop assay, Dnase I footprinting, <i>In situ</i> immunoblotting) Chemical methods (Dimethyl sulfate footprinting, ¹²⁵ I-radioprobng, click reaction)
3.	High resolution	Nuclear magnetic resonance (NMR), X-ray crystallography
4.	Computational/Bioinformatics/Databases	Quadparser, Quadfinder, QGRS mapper, QuadBase, QuadDB, Quadpredict

combination to each other in a way, so that they do not cause any harm to the native structure adopted by a DNA oligonucleotide. Keeping this in mind, some of the most commonly and routinely used biochemical and biophysical techniques which are used for the characterization of these guanine-quadruplex structures have been summarized in this review.

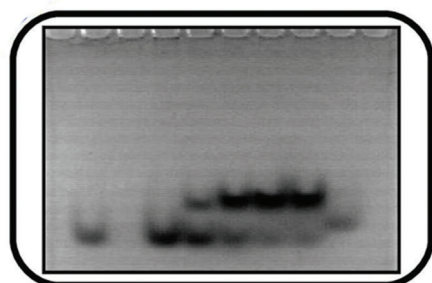
3. TECHNIQUES TO CHARACTERIZE THE G-QUADRUPLEX STRUCTURES

For exploring this interesting area of wide variety of Guanine-rich quadruplex structures which are prevalent throughout the genomes, a wide range of biochemical and biophysical techniques are being used.

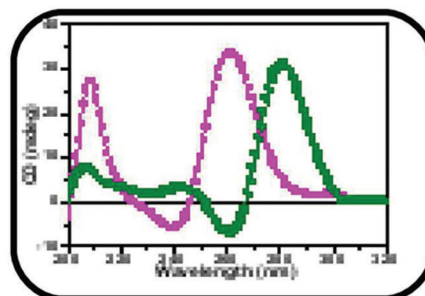
(Figure 2, Table 1) (13-17). Some of these have been discussed in the following section.

3.1. UV- thermal denaturation spectroscopy

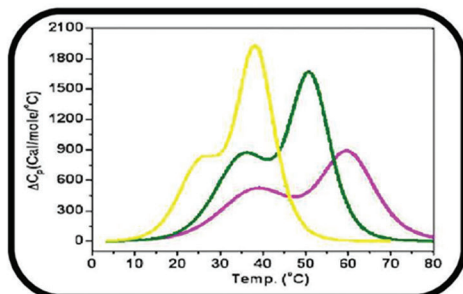
DNA structures are usually characterized by the specific ability of pairing an individual strand with a second strand by hydrogen bonding. The stability of the double helical structure is important for many aspects of nucleic acid metabolism and this stability can be assessed by several physico-chemical techniques like UV-Visible spectroscopy, Fluorescence resonance energy transfer (FRET), Differential scanning calorimetry (DSC) and Isothermal titration calorimetry (ITC) etc. Nucleic acids absorb strongly in the UV region (around 260 nm) and this absorbance increases upon the dissociation of the



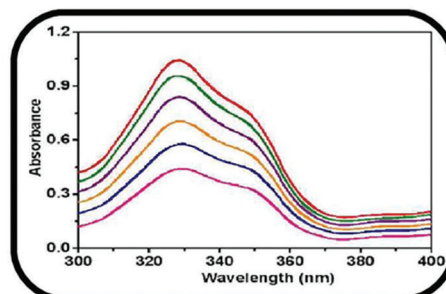
Poly acrylamide Gel electrophoresis



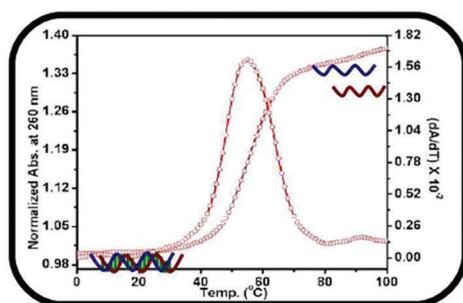
Circular dichroism spectroscopy



Differential scanning calorimetry



UV-absorption spectroscopy



UV-thermal denaturation spectroscopy

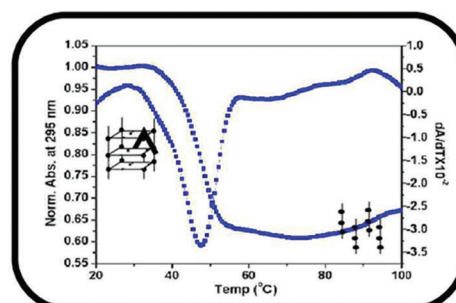


Figure 2. Representative images of various techniques to characterize G-Quadruplex.

strands. This hyperchromicity (increase in absorbance) results from the unstacking or disruption of the nucleotide bases. When a DNA sample is heated, its absorbance increases sigmoidally as a function of temperature. The temperature at which 50% of the DNA sample is melted, is called melting temperature (T_M) and is indicative of the thermal stability of the duplex or structure of interest. It not only provides the stability of any biomolecule but also demonstrates the stabilization or destabilization of a multistranded DNA structure on interaction with a ligand. Such melting experiments can also be analyzed to determine the thermodynamic properties like Van't Hoff enthalpies of DNA or RNA structures. The main factors affecting T_m values are DNA sequence and its length, salt concentration, oligomer concentration, the presence of denaturants (e.g. formamide) and various ligands

etc. Interaction with a ligand may increase or decrease the T_m of DNA structures depicting the stabilization or destabilization of DNA conformation respectively.

Monitoring the temperature variation for studying the stability and kinetics of G-quadruplex structures has been one of the most significant tools in biophysical studies (18-23). On a careful monitoring of absorbance as a function of temperature, an increase in absorbance (hyperchromicity) or a decrease in absorbance (hypochromicity) is generally observed for a guanine-quadruplex formation at various wavelengths. Melting of duplex to single strands is usually performed by measuring the change in absorbance at 260 nm wavelength, which increases by ~25 % on denaturation. In case of quadruplex denaturation, a large variation in

absorbance at 260 nm is generally not observed, hence melting experiments are monitored at 295 nm wavelength because it shows nearly a 40-50 % reduction in signal (hypochromicity) upon quadruplex melting (20). An inverted melting profile on 295 nm wavelength is observed in case of quadruplex melting, which is considered as a significant feature for quadruplex formation (24).

Some other types of melting experiments like thermal difference spectra (TDS), Circular dichroism (CD) melting and fluorescence melting assay have also been used for determining stabilization/destabilization aspects of different unusual/alternative DNA structures. TDS have been used for investigating specific spectroscopic signatures for the structures adopted by various DNA oligonucleotides in which the UV absorbance spectra of the helix and coiled states at temperatures above and below its melting temperature (T_M), is monitored (25). TDS is obtained by simply taking the difference between these two spectra at low and high temperatures (25). In case of CD melting, CD spectra of an oligonucleotide at a constant wavelength (generally maxima of positive peak or negative band) is recorded at different temperatures and then this ellipticity is plotted as a function of temperature, resulting into a CD melting curve. In this case, characteristic sigmoidal curves of the melting of various quadruplex structures are observed, which are usually inverted (at positive peak) and upright (at negative band) curves.

3.2. Differential scanning calorimetry (DSC)

For the quantitative analysis of the DNA and its multistranded structures like G-quadruplexes, DSC is one of the most commonly used physical techniques (26,27). This technique provides all the thermodynamic parameters like change in free energy, enthalpies, entropies etc., which results from the helix to coil transition (folding and unfolding) of various structures in a given solution and environmental conditions. Using DSC, a graph may be plotted showing a change in heat capacity as a function of pH, temperature, oligomer or cation concentrations etc. which might give an idea about the standard solution conditions required for the formation of a multistranded DNA structure. Calculation of various thermodynamic parameters like enthalpy, entropy, change in free energy etc. for the helix to coil transitions usually might give a very clear idea about the stabilization or destabilization aspect of these structures.

Prof. Marky's laboratory had used DSC, to determine the thermodynamic profiles, including the differential binding of ions and water, for the unfolding of the thrombin aptamer, d(GGT₂GGTGTGGT₂GG), along with other complimentary techniques like UV-melting, circular dichroism spectroscopy and fluorescence etc. (28). Contributions of a G-tetrad stack on another in terms of stability and effect of base substitutions in the thrombin aptamer sequence had also been

calculated (28). DSC and ITC have also, recently been used to study the interactions between insulin and guanine quadruplex formed by the DNA sequence of the insulin-linked polymorphic region (ILPR) (29).

3.3. Isothermal titration calorimetry (ITC)

ITC is generally used for the association or dissociation studies of any folded structure like guanine-quadruplex with the ligands (30,31). For thermodynamic calculations and quantitative determination of a protein or DNA complex with certain ligand, especially the calculation of binding affinity (K_a), enthalpy changes (ΔH) and binding stoichiometry (n) of the interactions of two molecules in solution state, isothermal titration calorimetry is applied in biophysical studies. In ITC studies, usually DNA or protein is taken into the sample cell and a drug or ligand is added in small increments and a titration analysis is performed. In almost all reactions, absorption or evolution of heat is involved, leading to changes in enthalpy, which might further be utilized for calculating the complete thermodynamic profile of the reactions involved. With recent most studies, role of G-quadruplexes *in vivo* has already been proven and hence the drugs which might bind to the same, are the future targets of interest. ITC is actually the only technique, which provides the most precise enthalpic changes which occur during the interaction studies of ligand with multistranded structures like G-quadruplexes and hence giving a complete detail of the thermodynamics of such complexes. These complete energy profile studies of binding isotherms along with enthalpic and entropic contributions of quadruplex-ligand interactions might further facilitate the structure and sequence specific drug designing to target these DNA structures.

3.4. Fluorescence resonance energy transfer (FRET)

Usually, the melting temperature (T_M) which are obtained using UV or CD-melting are not very accurate, hence fluorescence is also used as one of the spectroscopic techniques to find out the affinity or the stability of a DNA structure. For the studies of G-quadruplex formation and its stability, fluorescence resonance energy transfer (FRET) technique has been recognized as a very powerful technique (32-34). FRET based melting assays have also been standardized and used for screening various G-quadruplex binding ligands (35). FRET is also used for design of nanomolecular machines and a molecular aptamer beacon for protein recognition. In FRET studies, one or both ends (3' and 5') of the DNA oligonucleotides are being attached with a fluorophore and a quencher like 5'-fluorescein (FAM) and 3'- tetramethyl-rhodamine (TAMRA) and then the formation of a particular topology of G-quadruplex is monitored. These fluorophore and quencher molecules are generally a set of donor and acceptor molecules, in which the donor is excited at a particular wavelength and acceptor receives the signal, which develops the quenching. This will only happen, if

these donor and acceptor molecules are present at a small distance, eventually giving an idea of the structure of the molecule. With a fluorescent ligand, the interaction studies of guanine-quadruplex also have been used for providing the details about the binding affinities, constants and preference etc.

Reporter groups have been quite extensively used for determining the changes in conformation of multistranded DNA structures, using fluorescence. For loop structures in quadruplexes, a reporter group 2-amino purine (2-AP), (a highly fluorescent analogue of adenine) is quite extensively used because it can be incorporated at any position of the quadruplex, without affecting its structure (36). 2-AP is generally incorporated in a DNA oligonucleotide synthesis only. For gathering the relevant information about the biological relevance and *in vivo* existence of G-quadruplex structures, a large number of fluorescent probes (like propidium iodide, 9-amino acridine, thiazole orange, TmPyP4, crystal violet, ethidium derivatives etc.) have been used in structural studies of the multistranded DNA structures (37). In a quite recent FRET (fluorescence resonance energy transfer) study, direct visualization of the quadruplex in human chromosomes have been reported in which G-quadruplex targeting fluorophore BMVC (3,6-bis-(1-methyl-4-vinyl pyridinium)-carbazole diiodide) was used as a donor and duplex-binding fluorophores, Hoechst or propidium iodide acted as acceptors (38). In a recent study, guanine quadruplex formation is shown to be dependent on the sequence and loop length of G-rich sequences, using single molecule fluorescence. Also, the selective binding of parallel quadruplexes with N-methyl mesoporphyrin IX (NMM), its analog, NMP (GQ-stabilizing small molecules) and the G4R1 protein have recently been reported (39).

G4-FID (Guanine-quadruplex fluorescent intercalator displacement) assay is a recent test which is used to screen G4-ligands and is based on the displacement of thiazole orange (TO), from quadruplex or duplex DNA matrices by increasing amounts of a putative ligand (40-42).

3.5. Circular dichroism spectroscopy

Circular Dichroism (CD) is a powerful spectroscopic technique that has been widely used in biochemistry to detect conformational alterations and secondary structures of nucleic acids or proteins and to study their interaction with ligands. For example, in proteins, the CD spectrum can be used to determine an alpha helix or beta sheet conformation, and in nucleic acid structure, it has been used to differentiate between A-, B- or Z- forms of DNA. CD monitors the changes in global conformations of DNA structure which are generally induced by modifications in the environment, such as pH, temperature, the nature and/or concentration of counterions, or the addition of molecular crowding agents etc. Chiral or asymmetric molecules produce a

CD spectrum because they absorb left and right handed polarized light to different extents and thus are considered to be "optically active". The difference between the signals of left and right circularly polarized light is termed as ellipticity and is expressed in degrees and is generally plotted as a function of wavelength giving rise to a CD spectrum. Non-chiral molecules exhibit no CD signal in solution, however, when they bind to a chiral host, such as DNA, a CD signal is induced in the wavelength region corresponding to the maximum absorbance of the bound compound. Likewise, when a ligand binds to DNA, an induced CD (ICD) spectrum is observed because of its interaction with DNA. This may result from either a geometric change in the ligand or from coupling between its electronic transitions and those of the DNA.

Structural transitions of various topologies adopted by guanine-quadruplexes, like conversion from antiparallel to parallel or vice versa can be very easily studied using CD spectroscopy, which might be induced due to the change in solution conditions. As, CD alone cannot reveal a detailed atomic structure, it is always used in combination of other techniques like UV-absorption spectroscopy, Fluorescence or Gel-electrophoresis etc. The G-quadruplex conformations (parallel, antiparallel or mixed) adopted by various short and long guanine-rich sequences can be determined from the position and magnitude of the CD peaks (43). CD spectra of parallel G-Quadruplex is characterized by a positive CD peak at 260 nm and a negative band at 240 nm, whereas a positive peak at around 295 nm and a negative band at 260 nm are indicative of an antiparallel G-quadruplex conformation (44-46). CD spectroscopy is extremely sensitive, therefore, it requires small amount of sample, but a wide range from very dilute (1 micromole oligomer) to highly concentrated (NMR conc.) can also be used for obtaining a CD spectra.

Vibrational Circular Dichroism (VCD) is the extension of circular dichroism spectroscopy into the infrared and near infrared ranges. It is a spectroscopic technique which detects differences in attenuation of left and right circularly polarized light passing through a sample. This technique has been employed for evaluating conformational changes in biopolymers (47,48). As, VCD is sensitive to the mutual orientation of distinct groups in a molecule, it provides three-dimensional structural information. In case of nucleic acids, it is possible to probe the in-plane base deformation modes to reveal the interbase stereochemistry and stacking interactions, the phosphate stretches to monitor backbone stereochemistry, and the various sugar modes to analyze the ribose conformation (49). The characteristic C=O, C=N and C=C vibrations of nucleobases are observed in the spectral region 1800-1300 cm^{-1} and therefore conformational changes in nucleotides can be observed by monitoring this spectral region (50).

3.6. Nuclear magnetic resonance spectroscopy (NMR)

NMR is a well-established biophysical tool that exploits the magnetic properties of certain atomic nuclei and allows high-resolution structural determination of a complex at an atomic level. NMR spectroscopy is employed to obtain information about the structure and dynamics of nucleic acid molecules (DNA or RNA). This is evident by the fact that nearly half of all known RNA structures had been determined by NMR spectroscopy (51). The main advantage of using NMR over X-ray crystallography is that, the molecules are being observed in their natural solution state rather than in a crystal lattice which may affect the molecule's structural properties. Acquiring knowledge about the molecular structures in solution state is highly significant because the body fluids, such as blood, saliva and stomach liquid are solutions where they perform their physiological functions.

NMR spectroscopy is also widely used for studying G-Quadruplex structures (52,53). In one dimensional proton NMR ($1D^1H$ -NMR), the formation of a G-tetrad gives rise to characteristic guanine (NH1) imino protons, which exhibit their chemical shifts within the range of 10-12 ppm (54), as compared to 13-14 ppm for those involved in Watson-Crick base pairing (55). This method is not restricted only for providing structural data but can also be employed for obtaining information on dynamics, reaction state, folding, conformational equilibria, kinetic and thermodynamic aspects of biomacromolecules etc. Apart from this, it can also be utilized to monitor the interactions of oligonucleotides with other molecules, such as proteins or ligands, by analyzing the shifting in the peaks upon binding (56). For structural studies using NMR, highly concentrated oligonucleotide samples with at least 1 milliMolar (mM) concentration in 300-600 microlitres volume is required (57). The purified sample is usually dissolved in a buffer solution and adjusted to the desired solvent conditions. The major limitation of NMR is not only the requirement of high oligonucleotide concentration but also the necessity of base substitutions and isotope labeling of the DNA oligonucleotides. Also, in case of polymorphic sequences, the alternate structures change their conformation with a change in oligonucleotide concentration, which will also affect the structural studies with concentrated NMR samples. DNA sequence d-TGGGGGCCCA (11-mer) had been shown to exhibit equilibrium between hairpin and duplex forms, with an increase in oligonucleotide concentration from our own laboratory (58). Such conformational equilibrium between different polymorphic forms of a DNA sequence is tedious to study because of the requirement of high NMR concentrations.

The structural studies using NMR have been modified and upgraded with the use of new and more sophisticated instruments, giving more information's like about H5-H6 resonance assignments (TOCSY), $3J_{56}$

scalar coupling in U and C, ($13C$ HSQC or HMQC for H2), for sequential assignments (NOESY) etc. (53). Diffusion ordered NMR spectroscopy (DOSY) has also been used for the optimization and analysis of guanine-quadruplexes adopted by repeats of –TTAGGG sequence (human telomere) and some promoter regions of *bcl-2* and *c-myc* genes (59).

3.7. X-ray crystallography

The high-resolution structures obtained by the X-ray crystallography have always been very important asset for the biophysical and structural biology related studies of nucleic acids and proteins. This technique is very tedious in itself, as it needs crystal lattice of DNA structures which is very difficult to obtain. Also, because of its limitations of not giving information of the solution state of the structures and their complexes, this technique mostly does not provide consistent results with other high resolution structures like NMR spectroscopy. The major limitation of X-ray crystallography exists especially for highly polymorphic DNA sequences (for exa. most of the guanine-rich sequences), which adopt more than one alternate structure in solution. X-ray crystallography will only provide information about the structure which is preferred in crystal form, rather than the favored structure in physiological solution conditions. X-ray crystallography should be used as a complementary technique and not the alternative to any other technique. This technique provides the information related to the type of guanine-tetrad arrangement, orientation of different strand, ordered water structure and the structural arrangement of all atoms at atomic level (60). The availability of high brightness and intensity of modern X-ray sources along with highly sophisticated computer softwares for data processing have been used nowadays to increase the database of oligonucleotide structures. The most widely studied guanine-rich sequence is of human telomere having repeats of TTAGGG sequence. This sequence has shown enormous potential of showing structural polymorphism, in which it had shown a large number of topologies, depending upon even a small change in solution condition. This structural polymorphism has also resulted in different type of structures, which are studied by various techniques. In X-ray crystallography and NMR studies, four-repeat 22-mer sequence d(AGGG(TTAGGG)₃) had shown contradictory results in Na^+ and K^+ ions, which might be attributed to the difference in crystalline and solution state of the studied DNA oligonucleotide (61).

3.8. Gel electrophoresis

Gel electrophoresis is one of the biochemical methods used for characterizing macromolecular sizes and their distribution. Gel electrophoresis experiments enable the separation of macromolecules, especially nucleic acids and proteins using an electric current applied to gel-matrix. Different types of gel electrophoresis experiments are being performed, which

may differ in shape, size, porosity, gel-type, and the type of apparatus used to run the gel. Denaturing and Non-denaturing (native) are the two different types of gel- electrophoresis experiments, where earlier is used to determine the size and purity of an oligonucleotide, while the later is used for determining the stoichiometry of different types of structures adopted by the same oligonucleotide in the native solution conditions. Gels which are typically used for the characterization of biomolecules are agarose and polyacrylamide gels. Agarose is an alternating copolymer of 1, 3-linked β -D-galactose and 1, 4-linked 3, 6-anhydro- α -L-galactose, which is used for the separation of a mixed population of DNA or proteins. Polyacrylamide gels are chemically cross-linked gels formed by the reaction of monomer molecule, acrylamide with a bifunctional cross linking agent such as N, N'-methylene-bis-acrylamide (normally referred to as 'bis-acrylamide'). The polymerization of acrylamide is an example of free radical catalysis, and is initiated by the addition of ammonium persulphate and the base N, N, N', N'-tetramethylethylenediamine (TEMED).

In an electrophoretic apparatus, when the electric current is applied, molecules which are placed in wells of the gel, start moving through the matrix at different rates, based on their size and charge. In case of nucleic acids, due to the naturally occurring negative charge carried by their sugar-phosphate backbone, the direction of migration is from negative to positive electrodes (62). When an electric current is applied, the larger molecules move slowly through the gel, while the smaller molecules move faster. The basic principle is that, the folded species would always move faster than its unfolded form, due to the difference in size. For instance, a G-quadruplex structure is more compact than its single stranded form and hence, will show higher mobility in a gel, with sufficient pore size. However, it is difficult to determine the size and topology of G-quadruplex in comparison to duplex DNA (63). Double-stranded DNA fragments naturally behave as long rods, so their migration through the gel is relative to their size or, for cyclic fragments, their radius of gyration. Nevertheless, this method is commonly used for the detection of multiple species (64-66) and for studying the DNA: ligand interactions also, since the ligand bound form of oligonucleotide structure would show a retarded mobility in comparison to the free structure in native gel.

4. OUTLOOK AND FUTURE DIRECTIONS

Detailed understanding of the guanine-rich DNA sequences with enormous potential of quadruplex formation in varied topologies, is one of the prime concerns for research laboratories in last almost more than five decades. With an increasing evidence of *in vivo* existence and the role of these guanine quadruplexes in biological processes, an urgent requirement of upgrading and developing new biophysical and biochemical techniques for their characterization is being taken

quite seriously by researchers working in this field. Any of the physicochemical, biochemical or biophysical techniques does not provide complete information about each and every aspect of different structures adopted by any DNA or RNA oligonucleotide sequence or even of their complexes formed with various ligands. Most of these techniques are complementary to each other and can be more useful, when used in combination. More sophisticated modern techniques which might provide a larger database of the alternative structures of nucleic acids are required, which might also provide essential understanding about developing new ligands using rational drug-designing in sequence and structure-specific manner.

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