Chapter-II

MATERIALS AND METHODS
2.0 Materials and Methods

This chapter gives the details of materials and methods used for carrying out the work included in the thesis. Oligonucleotides are synthesized in the laboratory using DNA synthesizers by following phosphoramidite chemistry. After forming G quadruplex DNA, its conformation stability and interaction with different drugs are studied with circular dichroism spectropolarimetry. G quadruplex DNA interaction and its stability at different concentrations, nature of interaction of quadruplex DNA with metal ions, drugs are studied using sensitive and accurate technique like fluorescence spectrophotometer. Quantitative and qualitative analysis of quadruplex DNA is done by using UV Visible spectrophotometer and nano ESI MS instruments respectively. G quadruplex DNA and its different forms are identified and analyzed using different gel shift assays. To perform all the experiments, fine quality chemicals are used and they are purified by conventional methods where necessary. Details of the techniques used to accomplish the work presented in the thesis are given hereunder.

2.1 Oligonucleotide Synthesis

Oligonucleotides are synthesized using ABI 394 DNA synthesizers. G-rich oligonucleotides which are used for the formation of G quadruplex structure are synthesized using solid phase phosphoramidite chemistry. Solid phase synthesis is fast, suitable for synthesis of long oligonucleotides, and for getting high yield. Three different types of chemical units form an oligonucleotide: namely base
sugar and phosphate. The backbone of the molecule is constructed with alternating 2-deoxyribose units and phosphodiester bridges where the diester connects position 5 (5') of the first sugar with position 3 (3') of the second one [146]. A base (purine or pyrimidine type) bound to position 1 of each 2-deoxyribose sugar unit. The four bases are thymine (T), adenine (A), cytosine (C), and guanine (G).

The basic units of oligonucleotides consist of three parts:
1. the central 2-deoxyribose sugar
2. the base attached to position 1 of the sugar
3. phosphoric acid monoester attached to position 3 of the sugar

To form a deoxyribonucleotide dimer, the hydroxyl group in position 5 of the first 2-deoxyribose sugar is condensed with the 3-phosphate group of the second nucleotide. Many such condensation reactions carried out on the growing molecule result in an oligonucleotide. The condensation reaction (coupling) takes place under absolutely anhydrous conditions in organic solvents like acetonitrile in order to avoid competition of the water hydroxyl groups with the sugar hydroxyl group. Oligonucleotides are extremely hydrophilic (they are short fragments of DNA) hence they are insoluble in organic solvents. Furthermore, there are a number of functional groups in nucleotides which may cause ambiguous reactions during coupling. In order to protect the active reaction sites hydrophobic groups are used during the synthesis as protection (protecting groups) for the reactive sites.
A protecting group should have certain important characteristics like

1. easy to attach to an active site
2. stable under coupling conditions
3. easy to cleave without degradation of the reaction product

Using solid phase technique, oligonucleotides can be rapidly assembled by repetitive addition of deoxyribonucleotide monomers with coupling efficiencies exceeding 98%. By performing the synthesis on a solid support, the need to purify the product after each coupling is eliminated since excess reagents are simply washed off with an appropriate solvent.

When the first deoxynucleoside in its protected form has been attached to the solid support through its 3'-hydroxyl group, the reaction cycle consists of three distinct steps. Synthesis of oligonucleotides proceeds from 3' to 5' direction.

1. the 5' protecting group of the terminal 2 deoxyribose unit is cleaved, thus step is called detritylation or 5' deprotection
2. together with the phosphorous containing group of an incoming protected deoxyribonucleotide, a 3'→5' internucleotide linkage is formed. This is called coupling or condensation reaction
3. 5' hydroxyl groups which have failed to react during the coupling reactions are blocked (capped) by means of acetylation. This step of synthesis is called capping reaction
When the reaction cycle has been repeated several times according to the desired sequence, the support carries the fully protected oligonucleotide. After synthesis to get the final product, the synthesized oligonucleotide is incubated with 25–30% ammonium hydroxide solution at 55°C for 16 hours. This will cleave all the protecting groups present on the oligonucleotide at active sites. Hence, this step is called deprotection. After deprotection, the synthesized oligonucleotide is dried in a Savant speedvac. During drying, traces of ammonia present in the oligonucleotide solution will be removed. Oligonucleotide is then purified using Sephadex G25 spin column. Further purification is done either by polyacrylamide gel electrophoresis (PAGE) or by HPLC techniques.

The main types of support used are silica glass beads (CPG), polystyrene cellulose paper or monodisperse plastic beads [147], etc. The solid support has to be derivatized in order to enable coupling of the first deoxyribonucleoside to it. The functional group introduced for that purpose is a primary amino group. In general, it is situated at the end of a spacer arm in order to avoid steric crowding, which might occur if the first deoxyribonucleoside is attached close to the backbone of the support. Coupling efficiencies are normally higher (approximately 98%) if a spacer is used.

A deoxyribonucleoside 3' succinate is then condensed with the primary amino group forming an amide linkage. The primary amino group used to attach the first deoxyribonucleoside is linked to the support via a dodecyl spacer as shown in Figure 1. The deoxyribonucleoside 3' O succinates are attached to the support by means of an active ester.
Figure 1  Solid support (S) and attachment of first deoxyribonucleoside to the solid support

B = Base,  S = Solid support and DMTrO = 5 Protecting group
There are two widely used methods of chemical oligonucleotide chain assembly on solid support. They are the phosphotriester [148 149] and phosphotetriester methods [150]. A more recent and modern method of phosphite triester synthesis also called phosphoramidite synthesis [151 152]. Instead of chlorine, a highly reactive group, a secondary amine (preferably diisopropyl amine) is bound to the phosphate group to give a phosphoramidite. The structures of different phosphoramidites are shown in Figure 2. Various steps involved in oligonucleotide synthesis by phosphoramidite method are given below.

1 Detritylation

A 3' protecting group usually 4,4-dimethoxytrityl group is removed with dichloro or trichloro acetic acid in ethylene dichloride. Then the support is washed with an appropriate solvent mostly by acetonitrile or pyridine.

2 Coupling

The 5' hydroxyl group of the terminal 2 deoxyribose unit is condensed or coupled with a protected phosphodiester monomer (triethylammonium salt) in the presence of a coupling reagent called tetrazole in acetonitrile. After the coupling reaction, the excess of the reagents are removed from the support by washing with acetonitrile or pyridine.

3 Capping

To block the uncoupled sites by acetylation, the support is then treated with a mixture of acetic anhydride \( (\text{AC}_2\text{O}) \) 2,4,6-collidine 4-dimethylaminopyridine (DMAP) in acetonitrile. This reaction makes sure that only chains with the right
Figure 2 Structure of phosphoramidites used for DNA synthesis
sequence can grow. Then the support is washed with acetonitrile in order to remove excess reagents.

4 Oxidation

The phosphite triester is then oxidized to the more stable phosphate triester. Oxidation is performed by treatment of the phosphite with a mixture of iodine, collidine, and water in acetonitrile. Then the solid support is washed with acetonitrile.

This process is repeated until the required oligonucleotide sequence has been synthesized. The entire oligonucleotide synthesis cycle is shown in Figure 3. In the present study, different oligonucleotides rich in dG and few dT bases in various combinations are synthesized and used for the formation of G quadruplex structure. Oligonucleotides synthesized for different experiments in the thesis are \( d(T_2G_4) \), \( d(T_3G_4) \), \( d(T_4G_4) \), and \( d(T_5G_5) \). These oligonucleotides are designed based on the data available in the literature on telomeric sequences from various organisms like Tetrahymena [153], Arabidopsis [154], Oxytricha [155], etc.

All the chemicals used for synthesis are of high quality and tested for their purity. Chemicals required for DNA synthesis like acetonitrile, dichloro, or trichloro acetic acid, phosphoramidites, tetrazole solution, capping solutions, oxidation solution are purchased from Applied Biosystems, USA. 30% ammonia solution is procured from Qualigens, India. Sephadex G25 is purchased from Amersham Biosciences, England. After deprotection, oligonucleotides are dried in Savant Speedvac. It is essential to dry the oligonucleotides in Speedvac after purification because even traces of ammonia in the deprotected oligonucleotide
Figure 3  DNA Synthesis using phosphoramidite chemistry

1 Detritylation 2 Coupling 3 Capping
solution may interfere with the formation of G quadruplex structure with NH₄(I) ion. Formation of NH₄(I) quadruplex during deprotection does not take place because incubation is performed at 55°C instead of 4°C.

DNA synthesis is performed in anhydrous conditions only. Traces of moisture in the solvent (which contain -OH group in them) may cause interference in the coupling reaction because oligonucleotide chain grows over -OH group. In case of need, the raw chemicals are purified and used for DNA synthesis. DNA synthesis grade acetonitrile is purchased from J T Baker USA. In order to remove traces of -OH group from solvents, mainly from acetonitrile, it is refluxed with fresh calcium hydride. After about 16-24 hours of refluxing, the solvent is collected carefully in a round bottom flask. Blue silica trap is fixed while collecting the solvent to remove traces of moisture entering from the atmosphere. After collecting anhydrous acetonitrile in a round bottom flask, they are stored over 4 Å molecular sieves to remove any traces of hydroxyl group entered into the flask while transferring the solvent. The vacant space above the solvent is filled with dry nitrogen gas thoroughly sealed and stored in a dry place. All the solvents are stored under argon or dry nitrogen and kept over 4 Å molecular sieves activated at 350°C so as to maintain water content less than 20 ppm.

Another important hygroscopic solvent namely acetic anhydride used for making capping solution is refluxed with fused sodium acetate to make it fully anhydrous. About 100 gm of sodium acetate is fused and cooled to room temperature in a desiccator then it is added to about 50 ml of anhydrous acetic acid solution and refluxed for 4-6 hours. As most of the solvents used in synthesis are highly hygroscopic, molecular sieves are added to all the solvents in order to
remove the hydroxyl groups through absorption. Calcium hydride and molecular sieves are obtained from Fluka Chimica or Aldrich Chimica, Italy. Throughout the study, Milli Q ultra pure water is used wherever necessary. Milli Q ultra pure water is obtained from Milli Q Biocel Water Purification system supplied by Millipore SA, France. The source for Tb(III) ion namely Terbium chloride acridine hoechst 33258 and actinomycin D are procured from Sigma. Where as bis Acridine is chemically synthesized in the laboratory (a gift from Dr. Darshan Ranganadhan).

The chemicals used for performing polyacrylamide gel electrophoresis namely acrylamide bis acrylamide ammonium persulfate (APS) TEMED Tris Boric acid EDTA etc. are purchased from Sigma. Fine quality chemicals are used for preparing the non denaturing polyacrylamide gel. 5 end of the oligonucleotide is labeled with \( ^{32}P \) using T4 polynucleotide kinase enzyme. The T4 polynucleotide kinase enzyme and the label \( ^{32}P \) are purchased from Pharmacia and BRIT, Hyderabad respectively. The details of chemicals purchased its source and assay performed are given in the Table 1.

2.2 Formation of G quadruplex DNA

After deprotecting the oligonucleotide with ammonium hydroxide solution oligonucleotides are purified by polyacrylamide gel electrophoresis or HPLC. Oligonucleotides which are used in this work are incubated with certain cations like K(I), Na(I), Sr(II) or NH4(I) etc. in appropriate concentrations for about 48 hours at 4°C.

After the incubation with appropriate cations formation of G quadruplex is tested by loading the complex on a non denaturing polyacrylamide gel which is
Table 1 Details of various chemicals used in the study

<table>
<thead>
<tr>
<th>S No</th>
<th>Name of the chemical</th>
<th>Source</th>
<th>Assay (if any)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DNA synthesis chemicals</td>
<td>Applied Biosystems USA</td>
<td>&gt; 99 0 %</td>
</tr>
<tr>
<td>2</td>
<td>30 % Ammonia solution Sodium Acetate Sodium Hydroxide Potassium/ Sodium Phosphate Ammonium Acetate Acetic Anhydride etc</td>
<td>Qualigens India</td>
<td>&gt; 98 0%</td>
</tr>
<tr>
<td>3</td>
<td>Acetonitrile (Raw)</td>
<td>J T Baker USA</td>
<td>&gt; 98 0 %</td>
</tr>
<tr>
<td>4</td>
<td>Calcium Hydride &amp; 4 Å Molecular Sieves</td>
<td>Aldrich Chemica Italy</td>
<td>&gt; 99 0 %</td>
</tr>
<tr>
<td>5</td>
<td>Terbium Chloride Acridine Hoechst 33258 Acrylamide Actinomycin D Bis acrylamide TEMED Tris Boric Acid EDT Tris Fluoro acetate (TFA) etc</td>
<td>Sigma USA</td>
<td>&gt; 99 0 %</td>
</tr>
</tbody>
</table>
Table 1  Details of various chemicals used in the study (continued)

<table>
<thead>
<tr>
<th>S No</th>
<th>Name of the chemical</th>
<th>Source</th>
<th>Assay (if any)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6</td>
<td>Bis Acridine</td>
<td>Synthesised in laboratory (A gift from Dr. Darshan Ranganathan)</td>
<td>99.0%</td>
</tr>
<tr>
<td>7</td>
<td>Highly pure KCl NaCl</td>
<td>Qualigens India</td>
<td>99.0%</td>
</tr>
<tr>
<td></td>
<td>SrCl₂ and NH₄Cl</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Milli Q Water</td>
<td>Millipore S A France</td>
<td>&gt;99.0%</td>
</tr>
<tr>
<td>9</td>
<td>Sephadex G 25 (Medium)</td>
<td>Amersham Biosciences England</td>
<td>99.0%</td>
</tr>
<tr>
<td>10</td>
<td>Radioactive γ³²P ATP</td>
<td>BRIT Hyderabad</td>
<td>98.0%</td>
</tr>
<tr>
<td>11</td>
<td>T₄ Polynucleotide kinase enzyme</td>
<td>Pharmacia</td>
<td>99.0%</td>
</tr>
</tbody>
</table>
pre cooled to 4°C. Mobility of the complex is compared with a control (usually same oligonucleotide which is incubated at 4°C without any cations in milliQ water). If the complex formation has taken place, the oligonucleotide complex will move faster than the control oligonucleotide due to the formation of folded secondary structure. A nondenaturing gel picture showing G quadruplex DNA after incubation with appropriate cations is shown in the chapter III (section I and section II). The salts used for incubating the quadruplex DNA like NaCl, KCl, SrCl₂, and NH₄Cl are purchased from Qualigens. Highly pure quality salts are used for incubating DNA with quadruplex forming ions to avoid contamination with other metal ions (Analytical Reagent Grade: e AR grade).

In order to see the effect of temperature on the G quadruplex structure, oligonucleotide is heated to 100°C in a water bath and then the samples are incubated at 4°C for 48 hours. After 48 hours of incubation, the samples are again kept in water bath at 100°C for 5 minutes. Before loading them on a nondenaturing PAGE, samples are allowed to come back to room temperature by keeping them in a beaker containing water. This process of heating the samples to higher temperature and then bringing them back to lower temperature is called thermal treatment. Thermal treatment is required for identifying secondary structures usually known as inter and intramolecular G quadruplex structures. Effect of thermal treatment on G quadruplex DNA is discussed in chapter III (section II).

2.3 UV Visible Spectrophotometric Studies

Ultraviolet absorption spectra arise from transition of electron or electrons from a lower to a higher electronic energy level. When a molecule absorbs ultraviolet
radiation of frequency $v$ sec$^{-1}$ the electron in that energy level undergoes transition from a lower to a higher energy level. The difference is given by $E = hv$ erg. The actual amount of energy required depends upon the difference in the energy between the ground state $E_0$ and the excited state $E_1$ of the electrons. Therefore, the energy absorbed by the electron for the transition from lower energy state to higher energy state is shown as

$$E_1 - E_0 = hv$$

Ultraviolet absorption spectroscopy is generally used for the quantitative determination of compounds that absorb ultraviolet light. The extent of absorption at appropriate wavelength of UV light is directly proportional to the quantity of UV absorbing compound present in the system. This determination is based on Beer's law, which is represented as

$$A = \log T = \log \frac{I_0}{I_t} = \varepsilon C l$$

Where $\varepsilon$ is the extinction coefficient, $C$ is the concentration and $l$ the length of the cell used in UV spectrophotometer. The extinction coefficient $\varepsilon$ is a constant at any given wavelength for a given species and is independent of the concentration of the absorbing species. After synthesis, oligonucleotides are deprotected, purified by Sephadex G 25 spun column or polyacrylamide gel electrophoresis, then optical density of oligonucleotides are measured at 260nm.
(OD\textsubscript{280}) after making appropriate dilutions with milli Q water. Concentration of oligonucleotides is calculated by using corrected optical density and by considering 1 OD\textsubscript{280} of oligonucleotide is equal to 37 \( \mu \)g/ml [170].

Fluorescent drugs like acridine, \textit{bis} acridine, actinomycin D and hoechst 33258 are quantitated using UV Visual spectrophotometric analysis. UV Visual spectroscopy is a very sensitive and accurate means to calculate the concentration of UV absorbing molecules. Concentration of DNA intercalating drugs is found out using their respective extension coefficients. Optical densities are taken using a double beam spectrophotometer from Hitachi (model 200 20) attached with Hitachi 611 digital recorder. UV spectra of the oligonucleotide \( d(T\textsubscript{6}G\textsubscript{18}) \) synthesized in the laboratory is shown in the Figure 4.

Concentration of actinomycin D is determined by measuring the optical density at 440 nm and by using extinction coefficient 24 500 M\(^{-1}\) cm\(^{-1}\) [156]. Similarly, hoechst 33258 concentration is obtained by finding optical density at 340 nm and using extinction coefficient 39 200 M\(^{-1}\) cm\(^{-1}\) [157]. Structure of actinomycin D and hoechst 33258 are shown in Figure 5. Concentration of acridine and \textit{bis} acridine are calculated by measuring optical density at 430 nm and using the extinction coefficient 27 000 M\(^{-1}\) cm\(^{-1}\) [158]. The structures of acridine and \textit{bis} acridine are shown in Figure 6.

2.4 Circular Dichroism Spectrophotometric Studies

Circular dichroism spectrophotometry is a very sensitive and powerful technique for finding the conformation existence of different forms of DNA (inter or intramolecular DNA) stability of molecules etc. The optical activity of a molecule is its ability to rotate the plane of polarized light. The rotatory power of
Figure 4  UV Spectra of synthetic oligonucleotide d(T₆G₁₈)
Figure 5  Structure of Actinomycin D and Hoechst 33258
A – Actinomycin D  B – Hoechst 33258
Figure 6  Structure of acridine and *bis* acridine
circularly polarized light by a given molecule is expressed by molar ellipticity. Molar ellipticity ($\theta_m$) is calculated by using the following equation:

$$\text{Molar ellipticity } (\theta_m) = \left( \frac{\theta_{\text{observed}} \times \text{MRW} \times 100}{\text{path length} \times \text{concentration}} \right)$$

Or

$$\theta_m = \left( \frac{\theta_{\text{observed}} \times \text{MRW} \times 100}{l \times C} \right)$$

Where $\theta_m$ is molar ellipticity, $\theta_{\text{observed}}$ is the observed angle of rotation, $l$ is the length of the polarimeter tube in centimeter, and $C$ is the concentration of the substance in mg/ml and MRW is mean residual mass. When a circularly polarized light is passed through the medium containing optically active substance, the two circularly polarized components are differentially absorbed by the optically active substance. Thus leads to the imbalance between the strengths of the two circularly polarized beams emerging out of the cuvette. So the emerging beams are not truly plane polarized but elliptically polarized. This phenomenon is called circular dichroism (CD).

In this work, this technique is used to identify inter and intramolecular quadruplexes and to find the interaction between inter or intramolecular species with drugs. Circular dichroism has been reported earlier for studying the structure conformation interaction of G quadruplex DNA with other molecules etc. [3 19 192] Circular dichroism studies are performed with Jasco J 715 spectropolarimeter. Circular dichroism experiments are conducted in sodium phosphate or potassium phosphate buffer in presence of appropriate cation at a specified concentration. The composition of sodium or potassium phosphate buffer
used in the study is 10 mM sodium/potassium phosphate (pH 7 0) 0 1mM EDTA. Potassium/sodium phosphate buffer is made in milliQ water. All the experiments are performed with CD cuvette which has 1 cm path length. Each spectrum corresponds to an average of three scans from which the buffer background is subtracted.

2.5 Fluorescence Spectroscopic Studies

A large number of substances are known to absorb ultraviolet or visible light energy. But a number of important substances are also known to lose part of the excess energy as heat and emit the remaining energy as electromagnetic radiation of wavelength longer than that absorbed. This process of emitting radiation is collectively known as luminescence. Luminescence is of two types: fluorescence and phosphorescence. The absorption of light (hv_A) by the ground state molecule leads to promotion of electrons from a ground state (S0) to one of the vibrational sublevels of the first excited state (S1). This rapidly deactivates in solution by a radiationless process to the lowest vibrational sublevels of S1. The electron when return from S1 to the ground state it must emit radiation (hv_E). The energy of emitted radiation is lower than the absorbed radiation and thus of longer wavelength than the wavelength of light absorbed by the substance.

Among the two types of luminescence, fluorescence is used extensively for many analytical purposes. Using this technique analysis can be carried out even at very low concentration without destructing the substance. DNA interactions with drugs, metals and other macromolecules can be studied at low concentrations. Many reports are available in literature where fluorescence spectroscopic analysis is used for studying
the structure and interaction of proteins and nucleic acids with other molecules [162, 166]

The stability of G quadruplex DNA complex is monitored by observing the fluorescence of interacting moieties like Tb(III) ion and drugs like acridine bis acridine actinomycin D hoehst 33258 etc. Fluorescence titration experiments give the nature and stability of G-quadruplex DNA at different concentrations of interacting drugs or metal ions. Fluorescence studies are performed with J 4500 spectrofluorimeter. In fluorescence titration studies also G quadruplex DNA is taken in sodium/potassium phosphate buffer (10 mM sodium/potassium phosphate (pH 7 0) 0 1mM EDTA) along with the respective ion in appropriate concentration.

In the present study Tb (III) induced fluorescence is used to find out the nature of interaction and stability of quadruplex DNA at different concentrations. The Tb(III) - DNA complex is excited with appropriate light (λ 295 nm). Then the Tb(III) - DNA complex shows two emission signals at 488 nm and 545 nm generating from the 5 D 4 to 7 F 5 and 5 D 4 to 7 F 0 transitions of the metal ion[167]. These enhanced emission signals from quadruplex DNA - Tb(III) complex in comparison to those from Tb(III) alone have been utilized for the determination of structure and stability of quadruplex DNA.

2.6 Polyacrylamide Gel Electrophoresis Studies

Molecules of linear double stranded DNA which tend to orient themselves in an electric field in an end on position migrate through gel matrices at rates that are inversely proportional to the log10 of the number of base pairs. Larger molecules migrate more slowly because of greater frictional drag and because they find their
way through the pores of the gel less efficiently than smaller molecules. The main component in preparing the polyacrylamide gel is acrylamide, which is a monomer and its structure is

\[
\text{CH}_2 = \text{CH} - \text{C} = \text{NH}_2 \\
\| \\
\text{O}
\]

In the presence of free radicals, which are usually supplied by ammonium persulfate and stabilized by TEMED (N,N,N,N tetramethylethylenediamine), a chain reaction is initiated in which monomers of acrylamide are polymerized into long chains. When the bifunctional agent N,N methylenebisacrylamide is included in the polymerization reaction, the chains become cross-linked to form a gel whose porosity is determined by the length of the chains and the degree of cross-linking. The length of the chains is determined by the concentration of acrylamide in the polymerization reaction (between 3.5% to 20%). Lesser the percentage of acrylamide (w/v) more effective the range of separation (in terms of number of base pairs) in case of polyacrylamide gels. There is a linear relationship between the logarithm of the electrophoretic mobility of DNA (\(\mu\)) and the gel concentration (C) which is described by the equation

\[
\log \mu = \log \mu_0 + K_c C
\]

Where \(\mu_0\) is the free electrophoretic mobility of DNA and \(K_c\) is the retardation coefficient, a constant that is related to the properties of the gel and the size and the shape of the migrating molecule. Thus, by using gels of different concentrations, it is possible to resolve a wide size range of DNA molecules.
Basically two varieties of acrylamide gels are commonly used. They are

a) Nondenaturing polyacrylamide gels are used for the separation and purification of fragments of double-stranded DNA. Low voltages are usually applied (1-8 V/cm).

b) Denaturing polyacrylamide gels are used for the separation and purification of single-stranded fragments of DNA. Gels are polymerized in the presence of agents like urea or less frequently formamide, which suppresses base pairing in nucleic acids. Higher voltage runs are allowed.

In this study, mostly 12% gels are used. For preparing 12% of polyacrylamide gel, the chemicals are mixed in the following proportion:

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% Acrylamide</td>
<td>400 ml</td>
</tr>
<tr>
<td>Water</td>
<td>493 ml</td>
</tr>
<tr>
<td>10X Tris-Borate solution (TBE)</td>
<td>100 ml</td>
</tr>
<tr>
<td>10% Ammonium persulfate</td>
<td>0.7 ml</td>
</tr>
</tbody>
</table>

After mixing the chemicals, the solution is degassed and TEMED is added prior to casting the gel. If the gel is a denaturing gel, 7.8 M Urea is added to the solution.

DNA is a charged molecule and hence the DNA molecules can be separated on the basis of charge and pore size of the gel by applying high voltage across the acrylamide gel. In the present study, gel electrophoresis is primarily used to purify...
oligonucleotides and to identify the higher order structures. Polyacrylamide gel electrophoresis is a useful and efficient technique for finding the secondary structures formed during G-quadruplex formation. Structure of G-quadruplex DNA molecule becomes compact due to folding of DNA strands. Hence it moves faster on a non-denaturing gel compared to unfolded molecules. G-quadruplex DNA is purified using the mobility difference between the folded and unfolded DNA structures. As described earlier, there are two varieties of G-quadruplex structures: inter or intramolecular G-quadruplex structures, as shown in Figure 7. Using gel shift assays, it is possible to resolve them as the mobility of inter and intramolecular structures differs on a native polyacrylamide gel. The mobilities differ on the gel due to the difference in their structure and conformation. In this work, 12% non-denaturing gel is used for resolving the two secondary structures and to identify the formation of G-quadruplex structure. But for purification of oligonucleotides, 12% denaturing polyacrylamide gel is used. The slowest moving band is cut into small pieces and the oligonucleotide is eluted using elution buffer. Elution buffer is made up of 1 M ammonium acetate and 1 mM EDTA. Oligonucleotides are checked for its homogeneity by 5' end labeling them with the radioactive $^{32}$P molecule and loading them on a 12% denaturing polyacrylamide gel. The typical denaturing gel picture of the oligonucleotide d(T8G18) is shown in Figure 8 after end labeling the 5' end with radioactive label namely $^{32}$P molecule.

To extract the DNA, which is utilized for forming $NH_4(I)$ G quadruplex stem, milliQ water is used instead of conventional elution buffer to avoid contamination of $NH_4(I)$ ions. In order to keep the structure of G quadruplex DNA intact while running the samples on polyacrylamide gel, certain precautions are taken. Few among them
Figure 7  Structure of inter and intramolecular G quadruplex complex

A and B are Parallel and Dimer forms of intermolecular quadruplex

C and D are two different forms of intramolecular quadruplex
Figure 8  Denaturing Gel Electrophoresis of d(T₆G₁₆)

Lane 1  Molecular Weight Marker, Lane 2 – Oligonucleotide d(T₆G₁₆)
are while preparing the nondenaturing gel the respective ion concentration is maintained in the gel. To keep the structure of the G-quadruplex intact low voltage is applied across the gel (4.8 V/cm) The gel is allowed to attain 4 °C before starting the electrophoresis and the temperature is maintained throughout the run.

The radioactively labeled oligonucleotides are loaded on an appropriate polyacrylamide gels and the autoradiogram of the gel is obtained by exposing the gel to a photographic film obtained from Fuji. The intensity of radioactive bands are measured by using Fuji Phosphor Imager (model BAS-1800).

2.7 Electrospray Ionisation Mass Spectroscopy (ESI MS)

Matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) time-of-flight mass spectrometry (TOFMS) are very interesting and modern mass spectroscopic techniques which play crucial role in the analysis of biological molecules. They play vital role not only in the study of peptides and proteins but also to understand nucleic acid interactions with other macromolecules. The main advantage of ESI MS technique is accurate measurement of the mass to charge ratio (m/z) of the intact analyte for molecular mass determination and high resolution separation of the analyte ions. After the introduction of ESI by Yamashita and Fenn [189] it has been used extensively for the analysis of complex biological molecules. Russell et al. [160] have used mass spectroscopic technique extensively to predict the accurate mass of DNA oligonucleotides Mergny et al. [133] have studied interaction of cryptolepine and neocryptolepine with G-quadruplex DNA using dialysis competition assay and mass spectrometric experiments.
Chapter II

In this investigation nano ESI MS is used for the analysis and characterization of oligonucleotides. Nano ESI MS is a very sensitive and accurate instrument for analysis. The experiments are performed in negative ion mode. ABI QSTAR PULSAR is used to characterize the oligonucleotides. Each time about 2 µl (containing about 40 picomoles) of sample dissolved in 1:1 acetonitrile-water and 5 mM ammonium acetate is injected into the instrument to record the spectra. ESI MS is a suitable technique for characterizing the G-rich oligonucleotides as these oligonucleotides have the tendency of forming folded structures in solution. ESI MS is an accurate and fast technique for finding the purity and quality of the G-rich oligonucleotides. Before injecting the oligonucleotide into the instrument, the instrument requires a calibration. The calibration is performed by following the procedure given below.

2.7 (a) Calibration of ESI MS for the analysis of G quadruplex DNA in negative ion mode

Sodium trifluoroacetate (TFA) is used to tune/calibrate for negative ion electrospray ionization mass spectrometry [161]. To start with, 0.1% by volume stock solution of TFA in water and 10 mM solution of sodium hydroxide are prepared. Then, the 0.1% TFA solution is titrated to pH 3.5 with sodium hydroxide solution and thus solution is diluted to 50% by volume in acetonitrile. The optimum pH range is between 3 and 4. In the same manner, other alkali metal solutions can also be prepared by titrating TFA with 10 mM/L of alkali metal hydroxide solution.

2.7 (b) Sample preparation for ESI MS studies

Before recording the ESI MS spectra of G quadruplex, to standardize the ESI MS, a standard oligonucleotide (supplied by Applied Biosystems USA) with
known mass (7691 Da) is injected into the machine along with 1:1 acetonitrile water and 5 mM ammonium acetate. The sequence of the standard oligonucleotide used is d(5'-AGGCATGCAAGCTTGAGTATCTAT-3'). The spectra are recorded in the negative ion mode. After recording the ESI MS spectra of the standard oligonucleotide (Figure 9) and calculating the mass of the oligonucleotide, accuracy and calibration of the ESI MS instrument are verified. G quadruplex samples are injected into the ESI MS instrument after finding the monoisotopic mass of the standard oligonucleotide (7691 Da). Figure 10 shows the ESI MS spectra of a 22 mer oligonucleotide namely d(T8G16). Each time about 2 µl of oligonucleotide d(T8G16) is injected into the nano ESI MS instrument along with a mixture containing 1:1 acetonitrile water and 5 mM ammonium acetate. From Figure 10 it is clear that the oligonucleotide is pure and the experimental molecular mass matches with the theoretically calculated monoisotopic mass (7027 Da) using the software called Oligo II Mass Calculator. Ammonium ion in the ESI MS buffer solution does not interfere with G quadruplex structure as it require a 16 hours of incubation at 4°C. The results obtained from the ESI-MS of the standard oligonucleotide and d(T8G16) are shown in the Table 2 and Table 3 respectively. (Table 2 & Table 3 are shown in Chapter III (section IV)). For calculating the exact monoisotopic mass of oligonucleotides, average masses of each base in the oligonucleotide are considered as dA - 313 209 Da, dG - 329 208 Da, dC - 289 184 Da, and dT 304 209 Da.
Figure 9  ESI MS spectra of standard oligonucleotide
Figure 10  ESI MS spectra of the oligonucleotide d(T₆G₁₆)