CHAPTER II

Experimental
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EXPERIMENTAL METHODS

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1. Characterization techniques

1.1 Infrared spectroscopy

The infrared spectroscopy is a useful technique to characterize a compound. It results from transition between vibrational and rotational energy levels. IR region of the
electromagnetic spectrum covers a wide range of wavelength from 200 to 4000 cm$^{-1}$. It has been found that in IR absorption, some of the vibrational frequencies are associated with specific groups of atoms and remain same irrespective of the molecules in which the group is present. These are called characteristic frequencies [173] and their constancy results from the constancy of bond force constants from molecule to molecule. The important observation that the IR spectrum of a complex molecule consists of characteristic group frequencies makes IR spectroscopy, unique and powerful tool in structural analysis.

1.2 Ultraviolet and visible spectroscopy

When a molecule absorbs radiation, its energy is increased. This increased energy is equal to the energy of the incident photon expressed by the relation

$$E = hv$$

$$E = \frac{hc}{\lambda}$$

where h is Planck’s constant, v is the frequency, λ is the wavelength of the radiation and c is the velocity of light. Most of the compounds absorb light in the spectral region between 200 and 800 nm resulting in the excitation of electrons of the molecules from ground state to higher electronic states. In transition metals, all the five 'd' orbitals viz. $d_{xy}$, $d_{yz}$, $d_{xz}$, $d_{z}^2$ and $d_{x^2-y^2}$ are degenerate. However, in coordination compounds due to the presence of ligands, this degeneracy is destroyed and d orbitals split into two groups $t_{2g}$
(d_{xy}, d_{yz} and d_{xz}) and e_g (d_z^2 and d_{x^2-y^2}) in an octahedral complex and t and e in a tetrahedral complex. The set of t_{2g} orbitals goes below the original level of degenerate orbitals in octahedral complexes and the case is reversed in tetrahedral complexes (Figure 30a-b).

**Figure 30a.** Splitting of the d energy levels in an octahedral complex.

**Figure 30b.** Ligand field splitting of a tetrahedral complex, ‘g’ subscript is omitted in T_d symmetry.
At energy higher than the ligand field absorption bands, we commonly observe one or more very intense bands that go off scale unless log ε is plotted. These are the charge transfer bands, corresponding to electron transfer processes that might be either ligand → metal (L → M) or metal → ligand (M → L). In case of octahedral complexes M → L transitions occur for metal ion complexes that have filled, or nearly filled, t2g orbitals with ligands that have low lying empty orbitals. The L → M charge-transfer (C-T) spectra have been studied more thoroughly. The electron density is transferred from the ligand to the metal. This is favoured when the central metal has a high oxidation state, and is ‘short of electrons’. The ligand π orbitals are lower in energy than the metal t2g orbitals. No net oxidation-reduction usually occurs, because of the short lifetime of the excited state [174].

1.3 Nuclear magnetic resonance spectroscopy

The nuclei of certain isotopes possess a mechanical spin or angular momentum. The NMR spectroscopy is concerned with nuclei having nuclear spin quantum number I = 1/2, example of which include ^1H, ^12C, ^31P, ^119Sn and ^19F.

For a nucleus with I = 1/2, there are two values for the nuclear spin angular momentum quantum number mI = ±1/2 which are degenerate in the absence of a magnetic field. However, in the presence of the magnetic field, this degeneracy is destroyed such that the
positive value of $m_1$ corresponds to the lower energy state and negative value to higher energy state separated by $\Delta E$.

In an NMR experiment, one applies strong homogeneous magnetic field causing the nuclei to press. Radiation of energy comparable to $\Delta E$ is then imposed with radio frequency transmitter equal to precision or Larmor frequency and the two are said to be in resonance. The energy can be transferred from the source to the sample. The NMR signal is obtained when a nucleus is excited from low energy to high energy state.

1.4 Electron paramagnetic resonance spectroscopy

EPR spectroscopy [175] is the branch of absorption spectroscopy in which radiation having frequency in the microwave region is absorbed by molecules possessing electrons with unpaired spins. The unpaired electrons in radicals or in complexes of transition metal centers with only partially filled $d$ orbitals features a spin, the orientation of which in a magnetic field can give rise to two energetically different states. For an electron of spin $S = 1/2$, the spin angular momentum quantum number will have values of $m_s = \pm 1/2$. In absence of magnetic field, the two values of $m_s$ i.e. $+1/2$ and $-1/2$ will give rise to a doubly degenerate spin energy state. If a magnetic field is applied, this degeneracy is lifted and leads to the non-degenerate energy levels. The low energy level will have the spin magnetic moment aligned with the field and correspond to the quantum number $m_s = -1/2$. On the other hand, the high energy state will have the spin magnetic moment
opposed to the field and correspond to the quantum number \( m_s = +1/2 \). The energy \( E \) of
the transition is given by:

\[
E = hv = g\beta H_0
\]

Where \( h \) is Plank's constant, \( v \) the frequency of radiation, \( \beta \) the Bohr magneton, \( H_0 \) the
field strength and \( g \) the spectroscopic splitting factor.

The magnitude of \( g \) is characteristic for the paramagnetic system and depends upon the
orientation of the molecule with respect to the magnetic field. From the observed value of
\( g \) it is possible to determine oxidation and spin states or even details in the coordination
sphere of the metal centers. If the paramagnetic radical or ion has \( g \) value independent of
the orientation of the crystal it is said to be isotropic and if the value of \( g \) depends on the
orientation of the crystal and is said to be anisotropic.

1.5 Mass spectroscopy

At electron beam energy of about 9 to 15 electron volts, depending on the molecule
involved, a molecular ion is formed by interaction with the beam electrons. Recognition
of the parent ion (actually a radical ion) is of great importance because it gives the
molecular weight of the sample [176]. At this point, the molecular weight is an exact
numerical molecular weight not the approximation obtained by the all other molecular
weight procedures. Mass spectra is usually obtained at an electron beam energy of 70
electron volts and under these conditions numerous fragment ions (including the parent
ions) versus their relative concentrations constitutes the mass spectrum of the samples. The fragmentations occur on the basis of mass/charge (m/z). The largest peak in the spectrum is called base peak and assigned a value of 100%. The other peaks are reported as percentage of the base peak.

1.6 Conductance measurements

The conductivity measurement is one of the simplest and easily available techniques used to study the nature of the complexes. It gives direct information regarding whether a given compound is ionic or covalent. For this purpose, the measurement of molar conductance \( \Lambda_m \) which is related to the conductance value in the following manner is made.

\[
\Lambda_m = \frac{\text{cell constant} \times \text{conductance}}{\text{concentration of solute expressed in mol cm}^{-3}}
\]

Conventionally, solutions of \( 1\times10^{-3} \) M strength are used for the conductance measurements. Molar conductance values of different types of electrolytes in a few solvents are given as, 1:1 electrolyte has a value of 80-115 ohm\(^{-1}\) cm\(^2\) mol\(^{-1}\) in MeOH, 65-90 ohm\(^{-1}\) cm\(^2\) mol\(^{-1}\) in DMF, 78-80, 50-70 ohm\(^{-1}\) cm\(^2\) mol\(^{-1}\) DMSO and 35-45 ohm\(^{-1}\) cm\(^2\) mol\(^{-1}\) in EtOH [177,178]. Similarly a solution of 2:1 electrolyte has a value of 160-
220 ohm\(^{-1}\) cm\(^{2}\) mol\(^{-1}\) in MeOH, 130-170 ohm\(^{-1}\) cm\(^{2}\) mol\(^{-1}\) in DMF and 70-90 ohm\(^{-1}\) cm\(^{2}\) mol\(^{-1}\) in EtOH.

2. DNA binding studies

Calf thymus DNA was procured from Sigma Chemical Company and Tris-base was obtained from E. Merck. All the experiments involving interaction of the complexes with CT DNA were conducted in buffer containing Tris (0.01 M) adjusted to pH 7.2 with hydrochloric acid. The CT DNA was dissolved in Tris HCl buffer and was dialyzed against the same buffer overnight. Solutions of CT DNA gave ratios of UV absorbance at 260 and 280 nm above 1.8, indicating that the DNA was sufficiently free of protein [179]. DNA concentration per nucleotide was determined by absorption spectroscopy using the molar absorption coefficient 6600 dm\(^{3}\) mol\(^{-1}\) cm\(^{-1}\) at 260 nm [180]. The stock solution was stored at 4\(^{0}\)C.

2.1 Cyclic voltammetry

Cyclic voltammetry involves the measurement of current-voltage curves under diffusion controlled, mass transfer conditions at a stationary electrode, utilizing symmetrical triangular scan rates ranging from a few millivolts per second to hundred volts per second. The triangle returns at the same speed and permits the display of a complete polarogram with cathodic (reduction) and anodic (oxidation) waveforms one above the other. Two seconds or less is required to record a complete polarogram [181].
Consider the reaction

\[ O + ne \rightarrow R \]  \hspace{2cm} (i)

Assuming semi-infinite linear diffusion and a solution containing initially only species O.

With the electron held at a potential \( E_i \) where no electrode reaction occurs. The potential is swept linearly at \( v \) v/sec so that the potential at any time is

\[ E(t) = E_i - vt \]

or \( E_{\text{peak}} = E^0 - 0.0285 \)

The rate of electron transfer is so rapid at the electrode surface that species O and R immediately adjust to the ratio according to the Nernst equation, which is as follows,

\[ C_o (0,t) = C_o^* - [nF A (\pi D_0)^{1/2}]^{-1} \int I(\pi)(t-\tau)^{-1/2} \, d\tau \]  \hspace{2cm} (ii)

\[ I = nF AC_o^* (\pi D_0 \sigma)^{1/2} x (\sigma t) \]  \hspace{2cm} (iii)

Redox (electron-transfer) reactions of metal complexes can be investigated by cyclic voltammetry. An electrode is immersed in a solution of the complex and voltage is swept while current flow is monitored. No current flows until oxidation or reduction occurs. After the voltage is swept over a set range in one direction, the direction is reversed and swept back to the original potential. The cycle may be repeated as often as desired. Figure 31 shows the cyclic voltammograms (CV) for a reversible one-electron redox reaction such as,

\[ \text{CpFe(CO)LMe} \rightarrow \text{CpFe(CO)LMMe}^+ + e^- \]
Sweeping the potential in an increasing direction oxidize the complex as the anodic current $I_a$ flows; reversible reduction of CpFe(CO)LMe* generates cathodic current $I_c$ on the reverse sweep. The magnitude of the current is proportional to the concentration of the species being oxidized or reduced.

The measured parameters of interest on these cyclic voltammograms are $I_{pa}/I_{pc}$ the ratio of peak currents, $E_{pa} - E_{pc}$ the separation of peak potentials and the formal electrode potential $E^\circ$. For a Nernstian wave with stable product, the ratio $I_{pa}/I_{pc} = 1$ regardless of scan rate, $E^\circ$ and diffusion coefficient, when $I_{pa}$ is measured from the decaying current as a base line. The difference between $E_{pa}$ and $E_{pc}$ ($\Delta E_p$) is a useful diagnostic test of a Nernstian reaction. Although $\Delta E_p$ is slightly a function of $E^\circ$, it is always close to $2.3RT/nF$.

**Figure 31.** (a) Cyclic potential sweep (b) Resulting cyclic voltammogram

The technique yields information about reaction reversibilities and also offers a rapid means of analysis for suitable systems. The method is particularly valuable to study interaction of metal ions to DNA as it provides a useful compliment to other methods of
investigation, such as UV/Vis spectroscopy. Cyclic voltammetric studies were accomplished on a CH Instrument Electrochemical analyzer using a three-electrode configuration comprised of a Pt wire as the auxiliary electrode, a platinum micro-cylinder as the working electrode and Ag/AgCl as the reference electrode. Electrochemical measurements were made under nitrogen atmosphere. All electrochemical data were collected at 298 K and are uncorrected for junction potentials.

2.2 Absorption spectral studies

In a closed constant volume system, the rate of a chemical reaction is defined as the rate of change of the concentration of any of the reactants and products with time. The concentration can be expressed in any units of quantity per unit volume e.g. moles per liter, moles per cubic centimeter. The rate will be defined as positive quantity regardless of the component whose concentration change is measured.

The rate of a chemical reaction is not measured directly instead the concentration of one of the reactants or products is determined as a function of time. A common procedure for determining the reaction order is to compare the experimental results with integrated rate equations for reactions of different orders. For a first order rate equation, integrating by separate variables using integration limits such that at $t = 0$, $c = c_0$ and at $t = t$, $c = c$. [182].

$$-\frac{dc}{dt} = kc$$
or \[ \ln \left( \frac{c_0}{c} \right) = kt \]

The intrinsic binding constant \( K_b \) of the complex to CT DNA was determined from equation (1), through a plot of \([\text{DNA}] / (e_a - e_f) \) vs \([\text{DNA}] \), where \([\text{DNA}] \) represents the concentration of DNA, and \( e_a, e_f \), and \( e_b \) the apparent extinction coefficient \((A_{obs} / [M])\), the extinction coefficient for free metal complex \((M)\), and the extinction coefficient for the free metal complex \((M)\) in the fully bound form, respectively. In plots of \([\text{DNA}] / e_a - e_f \) vs. \([\text{DNA}] \), \( K_b \) is given by the ratio of slope to intercept [183,184]. These absorption spectral studies were performed on USB 2000 Ocean Optics and Shimadzu UV-1700 PharmaSpec UV/Vis spectrophotometers.

\[
[\text{DNA}] / |e_a - e_f| = [\text{DNA}] / |e_a - e_f| + 1 / K_b |e_a - e_f| \tag{1}
\]

Absorption spectral titration experiments were performed by maintaining a constant concentration of the complex and varying the nucleic acid/nucleotide concentration. This was achieved by diluting an appropriate amount of the metal complex solutions and CT DNA/5′GMP stock solutions while maintaining the total volume constant. This results in a series of solutions with varying concentrations of CT DNA/5′GMP but a constant concentration of the complex. The absorbance \((A)\) was recorded after successive additions of CT DNA/5′GMP. While measuring the absorption spectra an equal amount of CT DNA/5′GMP was added to both the compound solution and the reference solution to eliminate the absorbance of the CT DNA/5′GMP itself.
2.3 Fluorescence spectral studies

When molecules that have absorbed light are in a higher electronic state, they must lose their excess energy to return back to the ground state. If the excited molecule returns to the ground state by emitting light, it exhibits fluorescence and spectrum thus obtained is called emission spectrum. This phenomenon is generally seen at moderate temperature in liquid solution. The emission spectrum is obtained by setting the excitation monochromator at the maximum excitation wavelength and scanning with emission monochromator. Often an excitation spectrum is first made in order to confirm the identity of the substance and to select the optimum excitation wavelength. Further experiments were carried out to gain support for the mode of binding of complexes with CT DNA. Non-fluorescent or weakly fluorescent compounds can often be reacted with strong fluorophores enabling them to be determined quantitatively. On this basis molecular fluorophore EthBr was used which emits fluorescence in presence of CT DNA due to its strong intercalation. Quenching of the fluorescence of EthBr bound to DNA were measured with increasing amount of metal complexes as a second molecule and Stern-Volmer quenching constant K was obtained from the following equation 2 [185].

\[ \frac{I_o}{I} = 1 + Kr \]  \hspace{1cm} (2)

where \( r \) is the ratio of total concentration of complex to that of DNA and \( I_o \) and \( I \) are the fluorescence intensities of EthBr in the absence and presence of complex. Binding
constant K of the metal complexes was also determined from equations 3 and 4 (Scatchard equations) by emission titration [186,187].

\[ C_F = C_T \left( \frac{I}{I_o} - P \right) (1-P) \]  
\[ \frac{r}{C_F} = K (n-r) \]

Where \( C_F \) is the free probe concentration, \( C_T \) is the total concentration of the probe added, \( I \) and \( I_o \) are fluorescence intensities in presence and absence of CT DNA, respectively and \( P \) is the ratio of the observed fluorescence quantum yield of the bound probe to that of the free probe. The value \( P \) was obtained as the intercept by extrapolating from a plot of \( I/I_o \) vs \( 1/ [DNA] \), \( r \) denotes ratio of \( C_B (=C_T-C_F) \) to the DNA concentration i.e., the bound probe concentration to the DNA concentration, \( K \) is the binding constant and \( C_F \), is the free metal complex concentration and "n" is the binding site number. Emission intensity measurements were carried out using Hitachi F-2500 spectrofluorometer at room temperature.

**2.4 Gel electrophoresis**

Gel electrophoresis is a technique widely used for separation and analysis of charged biomolecules like nucleic acids [188,189]. Any charged biomolecule migrates when placed in an electric field. The ratio of migration of a molecule depends on its net charge, size, shape and the applied current. This can be represented as follows

\[ V = E \cdot q / f \]
Where \( V \) = velocity of migration of the molecule, \( E \) = electric field in volts/cm, \( q \) = net charge on the molecule, \( f \) = frictional coefficient which is function of mass and shape of molecule. The movement of a charged molecule in an electric field is often expressed in terms of electrophoretic mobility (\( \mu \)), which is defined as the velocity per unit of electric field.

\[
\mu = \frac{V}{E} = E \cdot \frac{q}{f} \cdot E
\]

\[
\mu = \frac{q}{f}
\]

For molecules with similar conformation, "\( f \)" varies with size but not with shape. Thus electrophoretic mobility (\( \mu \)) of a molecule is directly proportional to the charge density (charge/mass ratio). Molecules with different charge/mass ratio migrate under the electric field at different rates and hence get separated. This is the basic principal for all the electrophoretic techniques. Depending upon the nature of support medium, electrophoresis is of different types such as paper, starch, polyacrylamide and agarose gel electrophoresis. We have opted agarose gel electrophoresis, because agarose gels are more porous as compared to polyacrylamide gels and are, therefore, used to fractionate large macromolecules such as DNA that cannot be readily penetrate into and move through other types of supporting materials. Agarose is a linear polymer of D-galactose and 3,6-anhydro-L-galactose. When an electric field is applied across agarose gel, DNA molecules that are negatively charged at neutral pH, migrates towards oppositely charged electrode at rates determined by their molecular size and conformation. DNA molecules of the same size but with different conformation travel at different rates. The order of
migration velocity in the increasing order of various forms of DNA is: supercoiled DNA > linear double strand DNA > open circular DNA.

2.5 Viscometry studies

The hydrodynamic changes are the consequence of the change in length of the molecule, the diminished bending between layers and the diminished length-specific mass. Viscosity measurements were carried out using Ostwald's viscometer at 29 ± 0.01 °C. Flow time was measured with a digital stopwatch. Each sample was measured three times and an average flow time was calculated. Data were presented as ($\eta/\eta_o$) versus binding ratio ([M]/[DNA]), [190,191] where $\eta$ is a viscosity of DNA in the presence of complex and $\eta_o$ is the viscosity of DNA alone. Viscosity values were calculated from the observed flow time of DNA containing solution ($t > 100s$) corrected for the flow time of buffer alone ($t_o$), $\eta = t - t_o$. 