Experimental techniques


2. **Materials and Methods**

This chapter presents a list of chemicals and the details of analytical, spectroscopic, electrochemical and biochemical techniques employed during the research work.

2.1. **Chemicals**

The metal salts of cobalt(II) chloride hexahydrate, cupric chloride anhydrate, Nickel(II) chloride hexahydrate, zinc chloride, 1,10-phenanthroline monohydrate (phen), 1,3-indandione, 1,4-naphthoquinone, adenine, L-histidine, L-methionine, ethidium bromide (EB), Tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) were of AR grade were purchased from E. Merck (India). Anhydrous sodium chloride, calcium hydride and the mineral acids such as hydrochloric acid, sulphuric acid, nitric acid were employed as a drying agent at various stages for purification which were of AR grade obtained from Aldrich Chemical Co. (U.S.A).

Methanol and ethanol were of spectroscopic grade from E. Merck (India). They were further purified by distilling over calcium hydride. The other solvents like dimethyl formamide, dimethyl-sulphoxide were purified according to the known procedures prior to use [1, 2]. Water used for the biochemical studies was triply distilled using a quartz distillation setup and then autoclaved before use.

Highly polymerized calf thymus DNA (CT-DNA), supercoiled pUC19 DNA (cesium chloride purified) and fish sperm DNA (FS-DNA) were obtained from Bangalore Genei (India). Agarose (low melt 65 °C, molecular biology grade for DNA), ethidium bromide, bromophenol blue, Tris- (hydroxymethyl) aminomethane, sodium chloride, ethylene diamine tetraacetic acid disodium salt
(EDTA-Na$_2$), sodium azide were of molecular biology grade, obtained from Himedia (India).

2.2. Physical Techniques Used for the Characterization of Complexes

All the synthesized compounds were characterized by elemental analysis, conductometric measurements, magnetic susceptibility, mass spectrometry, UV-Visible, Fourier transform infrared (FT-IR), electron spin resonance (EPR), proton nuclear magnetic resonance ($^1$H NMR) and carbon nuclear magnetic resonance ($^{13}$C NMR) spectroscopic techniques. These methods are briefly discussed below. Elemental analyses were done on a Euro Vector elemental analyzer. Acetanilide was used as a reference standard.

2.2.1. 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 ($\Lambda_m$) is made. Conventionally, solutions of $1 \times 10^{-3}$ M$^1$ strength were used for the conductance measurements. Molar conductance of all the complexes was measured in DMSO solution at room temperature on a deep vision 601 model digital conductometer.

2.2.2. Magnetic Susceptibility Measurements

Number of unpaired electrons possessed by the metal ion shows magnetic moment. This can be determined by the magnetic susceptibility measurements. Magnetic susceptibilities of the solid complexes were measured employing Lakeshore VSM 7410 magnetic balance at room temperature (28 ± 2 °C) using Hg[Co(SCN)$_4$] i.e., mercury(II) tetrathiocyanato cobaltate(II), as calibrant for standardizing the Gouy tube. Previously weighed and calibrated Gouy tube was
uniformly filled with finely powdered complexes up to the mark and recorded the weights with and without magnetic field. This process was repeated thrice and the mean of the three observations were taken as apparent change in weight.

2.2.3. **Infrared Spectroscopy**

Infrared spectral measurements are helpful to find out the atom/s through which the ligand is coordinated to the metal atom in a complex. Infrared spectra of the newly synthesized ligands and their respective M(II) complexes were recorded as KBr pellets on Perkin Elmer Spectrum RX I spectrophotometer. The formation of the ligands and complexes has been ascertained by scanning their infrared spectra in 4000-400 cm\(^{-1}\).

2.2.4. **Ultraviolet and Visible Spectroscopy**

The UV-visible spectral measurements of metal complexes provide useful information about the stereochemistry of metal ion in the complex, oxidation state and the nature of metal-ligand bond based on the positions and number of d-d transition peaks. Electronic spectra were recorded on Perkin Elmer Lambda 35 UV–visible spectrophotometer. A matched pair of quartz cuvettes was employed. The working range of the spectrophotometer is 800-200 nm. The spectra of the complexes were recorded using N,N’-dimethylformamide as solvent at 1 mM concentration. Data were reported in \(\lambda_{\text{max}}/\text{nm}\).

2.2.5. **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 \(^1\text{H}\) and \(^{13}\text{C}\). For a nucleus with \(I = 1/2\), there are two values for the nuclear spin angular momentum quantum number \(m_I = \pm 1/2\) 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 \( m_i \) corresponds to the lower energy state and negative value to higher energy state separated by \( \Delta E \). In NMR experiment, a strong homogeneous magnetic field is applied to cause the nuclei to précises. 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. The \(^1\)H and \(^{13}\)C NMR spectra were obtained on a Bruker AV-III FT NMR 500 MHz spectrometer operating at room temperature. Chemical shifts were reported on the \( \delta \) scale in parts per million (ppm).

2.2.6. **Mass Spectrometry**

Mass spectrometry is one of the most accurate microanalytical techniques which require only a few nanomoles of the sample to obtain characteristic information such as molecular mass. The molecule prefers to fragment from which the presence of recognizable group can be deduced from the peaks corresponding to the respective fragments. Mass spectrometry is complementary to FT-IR, NMR, UV-vis and EPR spectral techniques for structural determination of compounds. ESI mass spectrometry is relatively routine technique to analyze the inorganic and organometallic compounds. ESI mass spectra were recorded on JEOL GCMATE II spectrometer.

2.2.7. **Electron Paramagnetic Resonance (EPR) Spectroscopy**

Electron paramagnetic resonance spectra of the Cu(II) complexes were obtained on a JES-FA200 ESR spectrometer using tetracyanoethylene (TCNE) as
field marker. The spectra of complex solutions were recorded in appropriate solvents at liquid nitrogen temperature (LNT).

2.3. DNA Binding Experiments

All the experiments involving interaction of the complexes with CT-DNA were conducted in buffer containing Tris(hydroxymethyl)aminomethane (Tris Buffer) (0.01 M) adjusted to pH 7.2 with hydrochloric acid. The CT-DNA was dissolved in Tris HCl buffer and dialyzed against the same buffer overnight. Solutions of CT-DNA gave a UV absorbance at 260 and 280 nm in the ratio 1.8:1, indicating that the DNA is sufficiently free from protein [3]. The concentration of DNA was determined by absorption spectroscopy using the ε value of 6600 M⁻¹ cm⁻¹ at 260 nm [4]. The stock solution was stored at 4 °C.

2.3.1. Electronic Absorption Spectra

Absorption titration experiment was performed on Perkin Elmer Lambda 35 UV–visible spectrophotometer by keeping the concentration of the complex constant, while gradually increasing the concentration of DNA. When measuring the absorption spectra, an equal amount of DNA was added to both sample and the reference solutions to eliminate the absorbance of DNA. The solutions were allowed to incubate for 2 min before the absorption spectra was recorded. From the absorption data, the binding constant (Kₘ) was determined by plotting [DNA]/(εₐ₋ε₇) versus [DNA] according to Eq. 1 [5]:

\[
\frac{[DNA]}{(εₐ₋ε₇)} = \frac{[DNA]}{(εₐ₋ε₇)} + \frac{1}{Kₘ (εₐ₋ε₇)}
\]

....... (1)

Where, [DNA] is the concentration of DNA in base pairs, the apparent extinction coefficients εₐ, ε₇ and εₖ correspond to Aobs/[complex], the extinction coefficient for the free complex and the extinction coefficient for the complex in
fully bound form, respectively. The data were fitted to the above equation with a slope equal to \(1/(\varepsilon_b - \varepsilon_f)\) and intercept equal to \(1/[K_b(\varepsilon_b - \varepsilon_f)]\) and \(K_b\) was obtained from the ratio of the slope to the intercept.

### 2.3.2. Fluorescence Spectra

To carry out binding studies of metal complexes with DNA using fluorescence spectral titrations Hitachi F-2500 fluorescence spectrophotometer was used. The concentration of the complex was maintained constant \((10^{-6} \text{ M})\) while the concentration of the DNA was increased from \(0-10 \times 10^{-3} \text{ M}\). The excitation wavelength was fixed for each complex and a scan speed at 240 nm/min and a slit width of 10/10 nm before measurements. By monitoring the changes of fluorescence intensity with increasing concentration of DNA the binding constants \(K_b\) were calculated. For such calculations, Scatchard equation (Eq. 2) was employed [6]:

\[
\frac{r}{C_f} = K_b \left(1 - nr\right) \quad \text{........ (2)}
\]

where \(r = C_b/\text{[DNA]}\), \(C_f = C_t[(F - F_0)/(F_{\text{max}} - F_0)]\), \(n\) is the binding site size of base pairs, \(C_b\) and \(C_t\) are the concentration of free complex and the total complex, respectively. \(F\) is the observed fluorescence emission intensity at a given DNA concentration, \(F_0\) is the intensity in the absence of DNA, and \(F_{\text{max}}\) is the fluorescence intensity of the totally bound complex.

Further the intercalation of complexes to DNA was examined through competitive binding experiment using emission spectral studies. EB is a common fluorescent probe for DNA structure and has been employed in investigations of the mode of binding of metal complexes to DNA [7]. In the ethidium bromide (EB) fluorescence displacement experiment, 5 µL of the EB Tris–HCl solution
(1 mmol L\(^{-1}\)) was added to 1 mL of CT-DNA solution (at saturated binding levels) and stored in the dark for 2 h. Then the solutions of each complex were titrated with the DNA/EB mixture and diluted using Tris–HCl buffer to 5 mL to produce the solutions with the varied mole ratio of the metal complex to CT-DNA. The linear Stern-Volmer constant \(K_{sv}\) was calculated from the following Eq. 3 [8]:

\[
\frac{I_o}{I} = 1 + K_{sv} [Q] \quad \text{.........} \quad (3)
\]

where \(I_o\) and \(I\) are the fluorescence emission intensities in the absence and presence of quencher (complexes), respectively; \([Q]\) is the concentration of the quencher; \(K_{sv}\) is the Stern-Volmer constant, which is obtained by the ratio of the slope to the intercept from the plot of \(I_o/I\) vs \([Q]\).

### 2.3.3. Electrochemical Studies

To investigate the DNA binding property of the complexes through their electrochemical behaviour, cyclic voltammetry experiments were performed on a Princeton electrochemical analyzer at 25.0 ± 0.2 °C in a single compartment cell with a three-electrode configuration with glassy carbon as the working electrode, a platinum wire as auxiliary electrode and Ag/AgCl as the reference electrode. Solutions were deoxygenated by purging with \(N_2\) prior to measurements.

### 2.3.4. Circular Dichroic (CD) Study

The circular dichroic spectra of CT-DNA were recorded on a JASCO (J-810) Spectro polarimeter in absence and presence of ligand (L) and Cu(II) complex at room temperature with a quartz cell of 1 cm path length. Each sample solution was scanned in the range of 220–320 nm. The spectra were recorded by keeping the concentration of DNA (5 × 10\(^{-4}\) M) constant while varying the complex concentration from 0 to 3 × 10\(^{-5}\) M.
2.3.5. Viscosity Experiment

Viscosity measurements were made using an Ubbelodhe viscometer, immersed in a thermostatic water-bath that maintained at a constant temperature of 25.0 ± 0.1 °C. CT-DNA samples of approximately 0.5 mM were prepared by sonicating in order to minimize the complexities arising from CT-DNA flexibility [9]. The complexes (1–10 µM) were titrated into the CT-DNA solution (10 µM) present in the viscometer. The flow time of each sample was measured in triplicates, and an average one was calculated. Data were presented as $(\eta/\eta_0)^{1/3}$ vs. binding ratio, where $\eta$ and $\eta_0$ are the viscosity of CT-DNA in the presence and absence of complex, respectively. Viscosity values were calculated from the observed flow time of CT-DNA containing solutions corrected from the flow time of buffer alone ($t_0$), $\eta = t - t_0$ [10, 11].

2.3.6. Thermal Denaturation of DNA

Melting studies were carried out by monitoring the absorbance of the FS-DNA at different temperatures in the absence and presence of the complex. A solution of DNA ($1 \times 10^{-5}$ M) in 5 mM Tris buffer (pH~7.2) in the absence and presence of the complexes were continuously heated from 20-90 °C. The mixture of DNA–complex was incubated for 1 h to ensure complete interaction between DNA and the complex. During thermal experiments, the samples were kept in the holder for 2 min at a particular temperature and the absorbance values were measured at 260 nm. The melting temperature ($T_m$), at which 50% of double-stranded DNA becomes single-stranded was calculated as reported [12].

2.4. DNA Cleavage Studies (Gel Electrophoresis)

The samples were subjected to electrophoresis on 1% agarose gel prepared in TBE buffer (45 mM Tris, 45 mM boric acid and 1 mM EDTA, pH 7.3). Then 20 µl
from each of the incubated complex and DNA mixture was incubated for 30 minutes at 37 °C and it was loaded on the gel with tracking dye (0.25% bromophenol blue, 40% sucrose, 0.25% xylene cyanole, and 200 mM EDTA). Electrophoresis was performed at a constant voltage (100 V) for about 2 h in TBE buffer. At the end of electrophoresis, the electric current was turned off and the gel was stained by immersing it in water containing ethidium bromide (0.5 µg/ml) for 30–45 min at room temperature. Bands were visualized by UV light and photographed. To enhance the DNA cleaving ability by the complexes, hydrogen peroxide (100 µmol L⁻¹) was added into each sample [13, 14].

2.4.1. Buffers/Reagents for Agarose Gel Electrophoresis Experiments

2.4.1.1. Tris-borate ethylenediaminetetraacetate (TBE) Electrolyte Buffer (SOX Stock)

Tris base (54 g) and boric acid (27.5 g) was dissolved in water (100 ml). The pH was adjusted to 8.0 using EDTA disodium salt (20 ml, 0.5 mol) and the resulting buffer was stored at 4 °C.

2.4.1.2. Loading Buffer (6X)

This buffer was prepared by dissolving bromophenol blue (10 µg) in glycerol (3 ml) and xylene cyanol (2.5 ml). The volume was made up to 10 ml. The resulting buffer was stored at 4 °C.

2.4.1.3. Ethidium Bromide Stock Solution (10 mg/ml)

A 100 mg of ethidium bromide was dissolved in water (10 ml) by stirring in dark for several hours. The resulting solution was stored in a brown bottle at an ambient temperature. Working concentration of 0.5 µg/ml was used for staining the gels after electrophoresis.
2.4.1.4. Gel Configuration and Gel Casting

A horizontal slab gel electrophoresis chamber (6.0 × 7.5 inches) made of polystyrene snap lock box obtained from Systronics (India) was used to carry out the agarose gel electrophoresis. Platinum wire was used for each of the two electrodes. A platform composed of four lantern slides glued together in a stack is cemented in center of the box. A gel plate of 7.0 cm × 10.0 cm in size rests on this platform so that the gel is submerged just beneath the surface of the electrophoresis buffer. The details of gel casting are given below.

A 400 mg of low melt agarose (molecular biology grade) was added to TBE buffer (50 ml). The slurry was then heated on a boiling water bath until the agarose dissolved completely. The solution was cooled to 50 °C. Both ends of the gel mold were closed with a clean autoclaved tape and a small quantity of agarose solution was applied with a pipette along the edges of the gel mold so as to seal it completely. Remaining warm agarose solution was poured onto the gel mold and immediately the comb was clamped into position near one end of the gel. The teeth of the comb formed the sample wells. Care was taken to see that at least 0.5-1.0 mm of agarose was left between the bottom of the teeth and the base of the gel, so that the sample wells are completely sealed. After 35-40 min, the comb and the autoclaved tape were removed carefully and the gel was mounted in the electrophoresis tank. Working buffer (200 ml, TBE) was poured into the gel until the gel was covered to a depth of about 1 mm.

2.5. Software Details

MGL tools 1.5.4 with AutoGrid4 and AutoDock4 [15, 16] were used to set up and perform blind docking calculations between the synthesized compounds and DNA sequence. The crystal structure of CT-DNA sequence
d(CGCGAATTCGCG)\textsubscript{2} dodecamer (PDB ID: 1BNA) was acquired from the Protein Data Bank. Receptor (DNA) and ligand (complex) files were prepared using AutoDock Tools. Lamarckian genetic algorithms, as implemented in AutoDock, were used to perform docking calculations. For each of the docking cases, the lowest energy docked conformation, according to the AutoDock scoring function was selected as the binding mode. The output from AutoDock was rendered with PyMol [17].

2.6. DPPH Free Radical Scavenging Assay

The antioxidant activity was determined based on the ability of the complexes to scavenge the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH). DPPH free radical scavenging activity of test compounds were carried out by previously reported methods [18]. Different concentrations (10, 50 and 100 µg) of the test sample in dimethyl sulfoxide (DMSO) and ascorbic acid (AA) were taken and the volume was adjusted to 100 mL with methanol. 5 mL of 0.1 mM methanolic solution of DPPH was added to the test solutions and shaken vigorously. DMSO was used as negative and ascorbic acid was used as positive control, respectively. The solutions were allowed to stand in the dark for 30 min at 27 °C. After incubation, the absorbance of the samples was measured at 516 nm. Percent scavenging of DPPH free radical for each concentration of each compound was calculated using Eq. 4 [18].

\[
\text{% of free radical scavenging activity} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100 \quad \ldots \ldots (4)
\]

where \(A_{\text{control}}\) = absorbance of DPPH solution at 516 nm, \(A_{\text{sample}}\) = absorbance of DPPH solution containing metal complex at 516 nm.
2.7. **Antimicrobial Activity**

The *in vitro* evaluation of antimicrobial activity was carried out to provide antimicrobial efficiency of the synthesized ligand and the complexes. The antimicrobial effect was tested against the selected Gram-positive and Gram-negative bacteria and the selected fungal species. The minimum inhibitory concentration (MIC) was measured. Each result was compared with the action of a standard drug. The nutrient agar and dextrose agar in petri plates were served as the medium for the growth of bacteria and fungi, respectively. The DMSO solution of the complex was soaked in a filter paper disc of 5 mm diameter and 1 mm thickness. These discs were placed on the formerly seed plates and incubated at 37 °C. After 24 h., the inhibition zone around each disc was measured. Each test was carried out in triplicate in individual experiments and the average was reported.

2.8. **Cytotoxic Activity**

2.8.1. **3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) Assay**

3-[4,5-dimethylthiazol-2-yl]2,5-diphenyltetrazolium bromide (MTT) is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 h of incubation, 15 µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37 °C for 4 h. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The % cell inhibition was determined using Eq. 5.
% Cell Inhibition = 100 – Abs (sample) / Abs (control) × 100 ........(5)

Nonlinear regression graph was plotted between % cell inhibition and log (concentration) and IC$_{50}$ was determined using GraphPad Prism software.

2.8.2. **CellTiter-Blue® Cell Viability Assay**

The *in vitro* cytotoxicity of the ligand and the complexes on NIH/3T3 mouse fibroblast cells were examined. The cells were cultivated in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 1% antibiotics (penicillin, streptomycin) and 10% fetal calf serum. Incubation was carried out at 37 °C / 5% CO$_2$. The CTB analysis (CellTiter-Blue® Cell Viability Assay, Promega, USA) was performed according to the manufacturer’s protocol. In this assay, the viability of the cells can be indirectly measured through the reduction of resazurin to resofurin by metabolically active cells. For the assay, cells were incubated in a 96-Well plate and left for 24 h to adhere (8000 cells per well). Afterwards, the cells were incubated with the complexes in various concentrations. The complexes were first solubilized in DMSO and then diluted with DMEM. The cells were incubated with 100 µl of the respective dilution in a fourfold determination for additional 22-24 h. The medium was removed and the cells were incubated with a 10% CTB solution (100 µl, 90 µl DMEM plus 10 µl CTB). After 2 h, the plates were analyzed with a plate reader (excitation at 544 nm, emission at 590 nm). Analysis of the data was done with Microsoft Excel 2013 and Origin 9. Curve fitting was carried out for dose response using non-linear curve fitting with Origin 9.

2.9. **General Considerations**

Care was taken to avoid the entry of direct, ambient light into the samples in all the spectroscopic and electrochemical experiments. Care was also taken to avoid the direct human contact of DNA and ethidium bromide solution. The
solution containing ethidium bromide and other hazardous chemicals were decontaminated before disposal. Protective goggles, gloves and safety mask were used to minimize the exposure to obnoxious chemicals/biochemicals, ultraviolet light etc.

2.10. Conclusion

A brief account on purification of solvents and chemicals used during this work are described in this Chapter. Also given here is a description of the spectroscopic as well as other physical and biochemical methods employed in this study.

References


