Chapter IV

Synthesis of ferrocenyl hydrazone copper(I) complexes: A comparative study on the effect of heterocyclic hydrazides towards interaction with DNA/protein, free radical scavenging and cytotoxicity

Abstract

Two new copper(I) hydrazone complexes have been synthesised from bivalent copper precursor \([\text{CuCl}_2(\text{PPh}_3)_2]\) and ferrocene containing bidentate hydrazone ligands \(\text{HL}_1\) (1) or \(\text{HL}_2\) (2). Based on the elemental analyses and spectroscopic data, the complexes are best formulated as \([\text{CuL}_1(\text{PPh}_3)_2]\) (3) and \([\text{CuL}_2(\text{PPh}_3)_2]\) (4) of monovalent copper ion. Solid state structure of the ligand 2 and its corresponding complex 4 were also determined. The DNA/albumin interactions of all the synthesised compounds were investigated using absorption, emission and synchronous fluorescence studies. Further, antioxidant properties of all the compounds have also been checked against ABTS, \(\text{O}_2^–\) and OH radicals. Additionally, the \textit{in vitro} cytotoxic activity of 3 and 4 was assessed using tumour (HeLa, A431) and non-tumour (NIH 3T3) cell lines.

Bioinorganic chemistry is an emerging interdisciplinary field of science that utilizes transition metal complexes for various applications in biological, medical and environmental sciences. Copper ion, as center of the active sites of copper metalloproteins is involved in a number of important biological function, such as electron transfer, oxygen transport and substrate oxidation.\(^1\text{-}^3\) In recent years, considerable research efforts have been focused on the synthesis of copper complexes of hybrid ligands as provide new materials with useful properties such as magnetic exchange,\(^4\) electrical conductivity,\(^5\) photoluminescence,\(^6\) nonlinear optical property\(^7\) and antimicrobial activity.\(^8\) Active and well-defined Schiff base ligands containing azomethine group (–C=N–N=C–) are considered as ‘privileged ligands’ because of their capability to stabilize different metals in various oxidation states and their complexes are extensively studied due to synthetic flexibility, selectivity and sensitivity towards variety of metal ions.\(^9\text{-}^10\) The electronic, steric, and conformational effects imparted by the coordinated ligands such as various N, S, P and halide donors play an essential part in stabilizing the Cu(I) center and improving the chemical and physical properties of the as-synthesized cuprous complexes.
There are only a few groups of compounds that have captured the attention of chemists so intensively as ferrocene. Chemical studies of many classes of ferrocene containing organic compounds rely on sufficiently high accessibility of the starting ferrocenyl substituted unsaturated carbonyl compounds and served as the basis for the development of methods of synthesis of mono and polycyclic heterocycles (pyrazoles, pyrimidines, etc.), conjugated dienes, cross-conjugated trienes, etc.\textsuperscript{11,12} This led to an increasing attention on the introduction of ferrocene fragments in diverse organic molecules aimed at preparing iron-containing extended conjugated systems as representatives of an important category of materials. The presence of a ferrocenyl substituent at a multiple carbon-carbon bond in extended conjugated chains imparts valuable properties to these compounds that includes magnetic and electrochemical behavior, thermal stability, nonlinear optical (NLO) effects, electrical conductivity, and even superconductivity, etc.\textsuperscript{13-18}

It is well known that cancer is currently the second leading cause of death in industrialized countries, accounting for roughly a quarter of all deaths.\textsuperscript{19} Hence, the development of new anticancer therapies is one of the fundamental goals in medicinal chemistry. DNA is one of the main molecular targets in the design of anticancer compounds.\textsuperscript{20} Further, it has been demonstrated that free radicals can damage proteins, lipids, and DNA of bio-tissues, leading to increased rates of cancer and fortunately antioxidants can prevent this damage due to their free radical scavenging activity.\textsuperscript{21,22} Hence, it is very important to develop compounds with both strong DNA binding and antioxidant properties for effective cancer therapy. Copper complexes have shown remarkable performance in antioxidant,\textsuperscript{23} DNA binding and anticancer studies.\textsuperscript{24,25}

As known well, serum albumins are the class of proteins involved in the transport of metal ions and metal complexes along with drugs through the blood stream.\textsuperscript{26} Many drugs, including anticoagulants, tranquilizers, anti-inflammatory and general anaesthetics are transported in the blood via combination with albumin.\textsuperscript{27,28} The nature and magnitude of drug-albumin interactions significantly influence the pharmacokinetics of drugs and the binding parameters are useful in studying protein-drug binding as they greatly influence absorption, distribution, metabolism and excretion properties of typical drugs. Despite the wealth of information available on this topic, only very few reports are
available on the related copper(I) complexes. Hence, in view of the aforementioned facts, the present article embodies the spectroscopic and single crystal characterization of the newly synthesized copper(I) complexes containing ferrocenyl hydrazone ligands. In addition, biological experiments on both DNA and albumin binding, antioxidant and cytotoxicity activities of all the synthesised compounds were also carried out.

Experimental section

Materials

Reagent grade chemicals CuCl$_2$·2H$_2$O, triphenylphosphine, ferrocene-2-carbaldehyde, furoic acid hydrazide and thiophene-2-carboxylic acid hydrazide were purchased from Sigma-Aldrich company and used as received. Calf thymus DNA (CT DNA) and bovine serum albumin (BSA) were purchased from Himedia Company. The human cervical cancer cell line (HeLa), human skin cancer cell line (A431) and NIH 3T3 mouse embryonic fibroblasts was obtained from National Centre for Cell Science (NCCS), Pune, India. All the other chemicals and reagents used for DNA binding, protein binding, antioxidant and cytotoxicity assays were of high quality.

Physical measurements

Microanalyses (% C, H & N) were performed on a Vario EL III CHNS analyzer. IR spectra of the samples were recorded using KBr pellets on a Nicolet Avatar instrument in the frequency range of 400-4000 cm$^{-1}$. $^1$H NMR spectra of ligands and their copper complexes were recorded on a Bruker AMX 500 spectrometer operating at 500 MHz using CDCl$_3$ as solvent and tetramethylsilane as an internal standard. The electronic absorption and emission spectra were recorded in DMSO-buffer (5:95) solution on a Jasco V-630 spectrophotometer and Jasco FP 6600 spectrofluorometer respectively at room temperature.

The X-ray diffraction data of ligand 2, complexes 3a and 4 were collected at 100, 100 and 223 K respectively on a Rigaku AFC-12 Saturn 724+ CCD diffractometer equipped with a graphite-monochromated Mo K$_\alpha$ radiation source ($\lambda = 0.71075$ Å, 0.7107 Å and $\lambda = 0.71073$ Å, respectively) and a Rigaku XStream low-temperature device cooled to 100 K. For each compound, a crystal of suitable quality was removed.
from the vial, covered with mineral oil and mounted on a nylon thread loop. Corrections for Lorentz and polarization effects were applied. The structure was solved by direct methods and refined by full-matrix least-squares cycles on $F^2$ using the Siemens SHELXTL PLUS 5.0 (PC) software package and PLATON. All non-hydrogen atoms were refined anisotropically and the hydrogen atoms were placed in fixed, calculated positions using a riding model.

**Synthesis of starting precursor complex**

The precursor complex $[\text{CuCl}_2(\text{PPh}_3)_2]$ was prepared according to the literature method.

**Preparation of dichlorobis(triphenylphosphine)copper(II), $[\text{CuCl}_2(\text{PPh}_3)_2]$**

To a warm solution of CuCl$_2$.6H$_2$O (0.8524 g; 0.005 mol) a hot solution of triphenylphosphine (2.623 g; 0.01 mol) in minimum amount of ethanol was added slowly with constant stirring to give a white precipitate. The reaction mixture was boiled for 5 minutes and kept at room temperature for 24 hours. The precipitate was filtered, washed with dry acetone and dried in a vacuum desiccator. The complex was further washed with petroleum ether to remove traces of free triphenylphosphine.

Yield: 78%. Colour: White; mp: 222 °C.

**Synthesis of hydrazone ligands**

The reactions involved in the synthesis of hydrazone ligands were given in scheme 4.1.

![Scheme 4.1 Synthesis of hydrazone ligands 1 and 2.](image)
Synthesis of ferrocenyl hydrazone ligand HL$^1$ (1)

Ferrocene containing hydrazone ligand 1 was prepared by refluxing an equimolar mixture of ferrocene-2-carbaldehyde (0.214 g; 1 mM) with furoic acid hydrazide (0.126 g; 1 mM) (1) in 50 mL of absolute ethanol for 8 h as given in scheme 4.1. The reaction mixture was then cooled to room temperature and the solid obtained was filtered, washed several times with distilled water and recrystallized from EtOH to afford the ligand 1 in pure form with good yield. Slow evaporation of a MeOH / CHCl$_3$ solution of ligand 1 failed to yield suitable crystals suitable for X-ray diffraction studies.

Yield: 78%. Colour: Orange; mp: 174 °C. Anal. Found (%) for C$_{16}$H$_{14}$N$_2$O$_2$Fe$^1$ (Mol wt = 322.138): C, 59.93; H, 4.23; N, 8.76. Calculated (%): C, 59.66; H, 4.38; N, 8.70. Selected IR bands ($\nu_{\text{max}}$ in cm$^{-1}$): 3216 (NH); 1650 (C=O); 1567 (C=N); 1025 (N–N). UV-visible (DMSO-buffer): $\lambda_{\text{max}}$ (nm): 263 & 306 (ILCT); 361 (LMCT); 449 (MLCT). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ (ppm): 9.21 (s, 1H, NH); 8.22 (s, 1H, HC=N); 7.51 - 6.58 (2d & 1t, 3H, Ar–H); 4.73-4.23 (3s, 9H, cp–H).

Synthesis of ferrocenyl hydrazone ligand HL$^2$ (2)

Ferrocene containing hydrazone ligand 2 was prepared by refluxing an equimolar mixture of ferrocene-2-carbaldehyde (0.214 g; 1 mM) with thiophene-2-carboxylic acid hydrazide (0.142 g; 1 mM) (2) in 50 mL of absolute ethanol for 8 h as given in scheme 4.1. The reaction mixture was then cooled to room temperature and the solid obtained was filtered, washed several times with distilled water and recrystallized from EtOH to afford the ligand 2 in pure form with good yield. Slow evaporation of a MeOH / CHCl$_3$ solution of ligand 2 afforded red crystals suitable for X-ray diffraction studies.

Yield: 75%, Colour: Orange; mp: 179 °C. Anal. Found (%) for C$_{16}$H$_{14}$N$_2$O$_1$S$_1$Fe$^1$ (Mol wt = 338.205): C, 56.93; H, 4.29; N, 8.35. Calculated (%): C, 56.82; H, 4.17; N, 8.28. Selected IR bands ($\nu_{\text{max}}$ in cm$^{-1}$): 3215 (NH); 1636 (C=O); 1572(C=N); 1034 (N–N). UV-visible (DMSO-buffer): $\lambda_{\text{max}}$ (nm): 263 & 306 (ILCT); 361 (LMCT); 449 (MLCT). $^1$H NMR (500 MHz, CDCl$_3$): $\delta$ (ppm): 9.60 (s, 1H, NH); 8.25 (s, 1H, HC=N); 7.51-6.58 (2d & 1t, 3H, Ar–H); 4.73-4.23 (3s, 9H, cp–H).
Synthesis of copper hydrazone complexes

The reactions involved in the synthesis of copper ferrocenyl complexes are given in scheme 4.2.

![Scheme 4.2 Synthesis of copper complexes.](image)

**Synthesis [Cu(L₁)(PPh₃)₂] (3)**

Complexes [Cu(L₁)(PPh₃)₂] (3) and [CuCl(PPh₃)₃] (3a) were prepared in a single reaction by refluxing equimolar quantities of [CuCl₂(PPh₃)₂] (0.658 g; 1 mM) and the hydrazone ligand (HL₁) (1) (0.322 g; 1 mM) in 40 mL of methanol (scheme 4.2). After few minutes of the mixing up of the above reactants, 2 or 3 drops of methanolic KOH was added and continuously refluxed for 5h. It is then cooled to room temperature, and the resulting product was filtered off, washed with methanol and dried under vacuum. Purity of the above product checked by TLC showed the presence of two distinct spots, i.e., complexes 3 and 3a that were subsequently separated by column chromatography using a mixture of petroleum ether and ethyl acetate as an eluent. Isolation of crystals suitable for single crystal XRD studies went unsuccessful in the case of complex 3 whereas complex 3a afforded white crystals on crystallization and were identified to be [CuCl(PPh₃)₃]. The data corresponding to complex 3 were as follows.

Yield: 45%. Colour: Red; mp: 283 °C. Anal. Found (%) for C₅₀H₄₆N₂O₂Fe₃P₂Cu₁ (Mol wt = 909.252): C, 68.61; H, 4.63; N, 3.16. Calculated (%): C, 68.69; H, 4.77; N, 3.08. Selected IR bands (ν max in cm⁻¹): 1612 & 1513 (C=N–N=C); 1348 (enolic C–O); 1009 (N–N). UV-visible (DMSO-buffer): λ max (nm): 263 & 298 (ILCT); 371 (LMCT). ¹H NMR (500 MHz, CDCl₃): δ (ppm): 8.58 (s, 1H, HC=N); 7.63-7.22 (1m, 33H, Ar–H); 4.74-3.92 (3s, 9H, cp–H).
Synthesis of [Cu(L²)(PPh₃)₂] (4)

Complexes [Cu(L²)(PPh₃)₂] (4) and [CuCl(PPh₃)₃] (4a) were prepared in a single reaction by refluxing equimolar quantities of [CuCl₂(PPh₃)₂] (0.658 g; 1 mM) and the hydrazone ligand (HL²) (2) (0.338 g; 1 mM) in 40 mL of methanol (scheme 4.2). After few minutes of the mixing up of the above reactants, 2 or 3 drops of methanolic KOH was added and continuously refluxed for 5h. It is then cooled to room temperature, and the resulting product was filtered off, washed with methanol and dried under vacuum. Purity of the above product checked by TLC showed the presence of two distinct spots, i.e., complexes 4 and 4a that were subsequently separated by column chromatography using a mixture of petroleum ether and ethyl acetate as an eluent. Slow evaporation of both 4 in MeOH / CHCl₃ mixture afforded single crystals suitable for X-ray diffraction studies and the compound 4a was identified to be [CuCl(PPh₃)₃] as obtained in previous reaction. The data corresponding to complex 4 were as follows,

Yield: 43%. Colour: Red; mp: 289 °C Anal. Found (%) for C₅₂H₄₃N₂O₁₁S₁Fe₁P₂Cu₁ (Mol wt = 925.319): C, 67.88; H, 4.52; N, 3.13. Calculated (%): C, 67.50; H, 4.68; N, 3.03. Selected IR bands (νmax in cm⁻¹): 1603 & 1535 (C=N–N=C); 1336 (enolic C–O); 1001 (N–N). UV-visible (DMSO-buffer): λmax (nm): 265 & 347 (ILCT). ¹H NMR (500 MHz, CDCl₃): δ (ppm): 8.67 (s, 1H, HC=N); 7.47–7.24 (m, 33H, Ar–H); 4.76–3.85 (3s, 9H, cp–H).

DNA binding studies

Electronic absorption experiments

Electronic absorption titration experiments were performed by maintaining the concentration of the test compounds (ligands / complexes) as constant (25 μM) but with variable nucleotide concentration from 0 to 25 μM. While measuring the absorption spectra, equal amounts of DNA were added to both complex and reference solutions to eliminate the absorbance of DNA itself. The data were then fit into the following equation and the intrinsic binding constant K₆ was calculated in each case.³³

\[
\frac{[\text{DNA}]}{[\varepsilon_a-\varepsilon_i]} = \frac{[\text{DNA}]}{[\varepsilon_b-\varepsilon_i]} + \frac{1}{K_b[\varepsilon_b-\varepsilon_i]}
\]
where, [DNA] is the concentration of DNA in base pairs, $\varepsilon_a$ is the extinction coefficient of the complex at a given DNA concentration, $\varepsilon_f$ is the extinction coefficient of the complex in free solution and $\varepsilon_b$ is the extinction coefficient of the complex when fully bound to DNA. A plot of $[\text{DNA}]/(\varepsilon_b - \varepsilon_f)$ versus $[\text{DNA}]$ gave a slope and an intercept equal to $1/(\varepsilon_a - \varepsilon_f)$ and $(1/K_b)(\varepsilon_b - \varepsilon_f)$, respectively. The intrinsic binding constant $K_b$ is the ratio of the slope to the intercept.

**Competitive binding measurements**

The apparent binding constant ($K_{app}$) of ligands 1 and 2 and their copper complexes 3 and 4 was determined by a fluorescence spectral technique using ethidiumbromide (EB) bound CT DNA solution in Tris-HCl buffer solution (pH, 7.2). The changes in fluorescence intensities at 602 nm (545 nm excitation) of EB bound to DNA were measured with respect to different concentration of the test compounds. EB was non-emissive in Tris-HCl buffer solution (pH, 7.2) due to fluorescence quenching of free EB by the solvent molecules. In the presence of DNA, EB showed enhanced emission intensity due to its intercalative binding to DNA. A competitive binding of the metal complexes to CT DNA resulted in the displacement of the bound EB thereby decreasing its emission intensity. The quenching constant ($K_q$) was calculated using the classical Stern-Volmer equation.

$$\frac{I_0}{I} = K_q [Q] + 1$$

where, $I_0$ is the emission intensity in the absence of quencher, $I$ is the emission intensity in the presence of quencher, $K_q$ is the quenching constant obtained from the slope of $I_0/I$ vs $[Q]$ and $[Q]$ is the quencher concentration. The apparent binding constant ($K_{app}$) has been calculated from the equation,

$$K_{EB} [\text{EB}] = K_{app} [\text{complex}]$$

where, the complex concentration was obtained from the value at a 50% reduction of the fluorescence intensity of EB, $K_{EB} = 1.0 \times 10^7 \text{ M}^{-1}$ and $[\text{EB}] = 5 \mu\text{M}$.

**Protein binding studies**

Binding of free hydrazone ligands and their copper complexes with bovine serum albumin (BSA) was studied using fluorescence spectra recorded with an excitation at 280
nm and corresponding emission at 345 nm assignable to that of free bovine serum albumin (BSA). The excitation and emission slit widths and scan rates were constantly maintained for all the experiments. Samples were carefully degassed using pure nitrogen gas for 15 minutes by using quartz cells (4×1×1 cm) with high vacuum Teflon stopcocks. Stock solution of BSA was prepared in 50 mM phosphate buffer (pH, 7.2) and stored in dark at 4 °C for further use. Concentrated stock solutions of test compounds were prepared by dissolving them in DMSO and diluted suitably with phosphate buffer to get required concentrations. 2.5 mL of BSA solution (1 μM) was titrated by successive additions of a 5 μL stock solution of test compounds (10^−4 M) using a micropipette. Synchronous fluorescence spectra was also recorded using the same concentration of BSA and complexes as mentioned above with two different Δλ (difference between the excitation and emission wavelengths of BSA) values such as 15 and 60 nm.

**Antioxidant studies**

Total antioxidant activity assay using ABTS cationic, superoxide and hydroxyl radicals of the synthesised compounds were studied according to the following procedures.  

**ABTS cationic radical scavenging activity**

ABTS (2,2′-Azino-3-ethylbenzthiazoline-6-sulfonic acid diammonium salt) was dissolved in water to a 5 mM concentration and its cationic radical was produced by reacting with 5 mM potassium persulfate. The resulting mixture was kept in dark at room temperature for 12-16 h before use. Prior to assay, the solution was diluted in ethanol (about 1:79 v/v) and equilibrated to 30 °C to give an absorbance of 0.70±0.02 at 734 nm. After the addition of 2 mL of diluted ABTS cationic radical solution to different concentration (10-50 μM) of the compounds, the absorbance was taken at 30 °C exactly 30 min after the initial mixing and the reaction mixture without test sample was used as control.

**Superoxide radical scavenging activity**

The superoxide (O_2^-) radical scavenging assay was done based on the capacity of the compounds to inhibit formazan formation by scavenging the superoxide radicals...
generated in riboflavin-light-NBT system. Each 3 mL reaction mixture contained 50 mM sodium phosphate buffer (pH, 7.6), 20 µg riboflavin, 12 mM EDTA and 0.1 mg NBT. Reaction was started by illuminating the reaction mixture with different concentration of the test compounds (10-50 µM) for 90 seconds. Immediately after illumination, the absorbance was measured at 590 nm. The entire reaction assembly was enclosed in a box lined with aluminum foil. The above reaction mixture without test sample was used as control.

**Hydroxyl radical scavenging activity**

The hydroxyl (OH) radical scavenging activities of test compounds have been investigated using the Nash method. *In vitro* hydroxyl radicals were generated by Fe³⁺ / ascorbic acid system. The detection of hydroxyl radicals was carried out by measuring the amount of formaldehyde formed from the oxidation reaction with DMSO. The formaldehyde produced was detected spectrophotometrically at 412 nm. A mixture of 1.0 mL of iron-EDTA solution (ferrous ammonium sulphate (0.331 mM) and EDTA (0.698 mM), 0.5 mL of EDTA solution (0.048 mM) and 1.0 mL of DMSO (10.83 mM) DMSO (v/v) in 0.1 M phosphate buffer, pH 7.4) were sequentially added to the test tubes containing the test compounds with different concentrations in the range of 10-50 µM. The reaction was initiated by adding 0.5 mL of ascorbic acid (1.25 mM) and incubated at 80-90 °C for 15 min in a water bath. After incubation, the reaction was terminated by the addition of 1.0 mL of ice cold TCA (107 mM). Subsequently, 3.0 mL of Nash reagent was added to each tube and left at room temperature for 15 min. The reaction mixture without sample was used as control. The intensity of the colour formed was measured spectrophotometrically at 412 nm against reagent blank.

In the case of above three assays, all the tests were run in triplicate. The percentage of activity was calculated using the formula,

\[
\% \text{ of activity} = \left(\frac{A_0 - A_C}{A_0}\right) \times 100
\]

where, \(A_0\) and \(A_C\) are the absorbance in the absence and presence of the tested compounds respectively. The IC\(_{50}\) can be calculated using the percentage of activity results.
Cytotoxicity

The *in vitro* cytotoxicity assay (IC$_{50}$) was performed on the human cervical cancer cell line HeLa, human skin cancer cell line A431 and the non-tumours NIH 3T3 mouse embryonic cell line. The HeLa and A431 tumor cell lines used in this work were grown in Eagles Minimum Essential Medium containing 10% fetal bovine serum (FBS) and the NIH 3T3 fibroblasts were grown in Dulbeccos Modified Eagles Medium (DMEM) containing 10% FBS. For the screening experiments, the cells were seeded into 96 well plates in 100 mL of the respective medium containing 10% FBS, at a plating density of 10,000 cells/well. The cells were incubated at 37 °C in 5% CO$_2$ and 95% air at a relative humidity of 100% for 24 h prior to the addition of the test compounds 3 and 4. The compounds were solubilized in dimethylsulfoxide and diluted in the respective serum free medium. After 24 h, 100 mL of the medium containing the test compounds with various concentrations (e.g. 15-500 μM) was added and incubated at 37 °C in an atmosphere of 5% CO$_2$ and 95% air with 100% relative humidity for 48 h. All measurements were made in triplicate and the medium containing no test compounds served as the control. After 48 h, 15 mL 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 formazan crystals that had formed were solubilized in 100 mL of DMSO and the absorbance at 570 nm was measured using a micro plate reader. The % cell inhibition was determined using the following formula,

\[
\text{% cell Inhibition} = 100 - \frac{\text{Abs}_{(\text{sample})}}{\text{Abs}_{(\text{control})}} \times 100.
\]

The IC$_{50}$ values were calculated from the graph plotted between % cell inhibition and concentration.$^{38}$

Results and discussion

The reactions of [CuCl$_2$(PPh$_3$)$_2$] in equimolar amounts with hydrazone ligand either HL$^1$ (1) and HL$^2$ (2) yielded complexes of the type [Cu(L$^1_0$(PPh$_3$)$_2$] (3), [Cu(L$^2_0$(PPh$_3$)$_2$] (4) and [CuCl(PPh$_3$)$_3$] 3a / 4a (scheme 4.2). The analytical data of the complexes are in good agreement with the proposed molecular formulae with 1:1 metal to ligand stoichiometry (presented under the experimental part). All the compounds are quite stable in air and light and soluble in most of the common organic solvents and are well
characterised using several physico-chemical techniques.

**Infrared spectra**

The IR spectra of the metal hydrazone complexes were compared with those of respective free hydrazone ligands in the region 4000-400 cm\(^{-1}\). The spectra of the ligands 1 and 2 displayed characteristic absorption bands at 3216/3215, 1650/1636, 1567/1572 and 1025/1034 cm\(^{-1}\) due to \(v_{(N-H)}\), \(v_{(C=O)}\), \(v_{(C=N)}\) and \(v_{(N-N)}\) vibrations, respectively. The bands due to \(v_{(N-H)}\) and \(v_{(C=O)}\) vibrations of the free ligands were absent for complexes 3 and 4, thus indicating that enolisation and deprotonation had taken place prior to coordination. This fact was further confirmed by the appearance of two new bands in the spectra of complexes around 1612-1513 cm\(^{-1}\) and 1316-1348 cm\(^{-1}\) that corresponds to \(v_{(C=N-N=C)}\) and \(v_{(C-O)}\) stretching vibrations, respectively. The bands attributed to \(v_{(C=N)}\) stretching was shifted to higher frequencies while a positive shift of about \(\sim 15-33\) cm\(^{-1}\) was observed for \(v_{(N-N)}\) stretching vibration in comparison with that of their respective free ligands, thus implying that the nitrogen atom of the azomethine group is coordinated to the metal in these complexes. All these facts suggested that the hydrazone ligands behaves as a monobasic bidentate (NO) chelating ligand in both of the complexes 3 and 4.

**Electronic spectra**

Electronic spectrum of ligand 1 showed four well resolved bands at 263, 306, 361 and 449 nm. Similarly, the spectrum of ligand 2 exhibited absorption bands at 306, 360 and 456 nm. The bands appeared in the range of 263-306 nm are assigned to intra ligand charge transfer (ILCT) transitions. A broad shoulder found at 360 and 361 nm corresponds to ligand to metal (iron) charge transfer (LMCT) transitions and the band at 449 and 456 nm was assigned to charge transfer transition from iron to either a non-bonding or an antibonding orbital of the cyclopentadienyl ring. Corresponding electronic spectrum of complexes 3 and 4 exhibited two or three bands in the range of 263-371 nm. Complex 3 exhibited three different absorptions positioned at 263, 298 and 371 nm whereas complex 4 showed only a couple of bands at 265 and 347 nm. In general, the bands appeared in 263-347 nm range in the case of both the complexes are...
due to intraligand $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ charge transfer (ILCT) transitions\textsuperscript{40} and the band at 371 nm for the complex 3 was due to LMCT transition.

$^1$H NMR spectra

$^1$H NMR spectra of the free hydrazone ligands and their complexes were assigned on the basis of observed chemical shift. The spectra of the ligands 1 and 2 displayed a weak singlet respectively at 9.21 and 9.60 ppm due to NH proton. But, the NMR spectra of complexes 3 and 4 (Figs. 4.1 and 4.2) did not register any signal corresponds to NH and revealing that the ligands adopted enol form, followed by deprotonation prior to coordination with the metal ion. In addition, both the ligands showed a sharp singlet for azomethine (HC=N) proton at 8.22 and 8.25 ppm, three singlets in the range of 4.73-4.23 ppm for cyclopentadienyl moieties, respectively. However, in the case of spectra of copper(I) hydrazone complexes, the signal corresponds to the azomethine protons gets shifted to downfield due to the participation of azomethine nitrogen in coordination with metal ion and observed at 8.58 and 8.67 ppm, respectively. Similarly, the
cyclopentadienyl signals were also underwent downfield shift upon coordination of the ligands with that of the respective metal ions. The signals corresponding to the protons of aromatic moieties of the ligands and their complexes were observed as multiplets in the range of 6.5-8 ppm.

![Fig. 4.2 1H NMR spectrum of complex 4.](image)

**X-ray crystallography**

From the elemental analyses, IR, electronic and 1H NMR spectroscopic studies it is understood that the complexes 3 and 4 are structurally similar to each other. Hence, the exact structure of both the complexes has been studied by using single crystal X-ray diffraction method.

**Crystallographic study of HL²**

Needle shaped dark orange crystals of hydrazone ligand 2 crystallized in the monoclinic space group $P2_1/n$. The crystal structure revealed the presence of two molecules of the compound 2 each containing a solvent molecule CHCl₃ in its asymmetric unit with similar molecular arrangements (Fig. 4.3).

The crystallographic data, along with a selection of bond lengths and bond angles are given in Tables 4.1 and 4.2, respectively. The Fe–C contact lengths ranged from 2.031(2) Å [Fe1–C7] to 2.052(2) Å [Fe1–C10] in the molecule 1, and from 2.024(4) Å [Fe2–C28] to 2.046 (3) Å [Fe2–C31] in the molecule 2. The distance between the iron atom and cyclopentadienyl carbon atoms are almost similar in both the asymmetric
molecules. The average value of the cyclopentadienyl C–C bonds is 1.424(3) Å in the molecule 1 and 1.417(4) Å in the molecule 2. The dihedral angle between the least square planes calculated through the atoms of the cyclopentadienyl and the neighbouring heterocyclic rings is significantly larger in the molecule 2 (17.32°) than in 1 (12.71°). This leads to the conformational difference between two molecules in the asymmetric unit which is further supported by the values of the torsion angles [S1–C4–C5–N1] 177.4(1) and its counterpart in the molecule 2 [S2–C20–C21–N3] 179.5(1). The crystal packing diagram of the ligand 2 is shown in Fig. 4.4. There exists hydrogen bonding between a pair of asymmetric molecules through O2 atom of one unit to the N1–H atom of the other unit. Further, the contact is extended to the neighboring set of asymmetric molecules via N2 and N3–H atoms that led to the formation of a novel structure with a
syndiotactic type of molecular configuration leading to 1-D chains through parallel array of ligand moieties (Fig. 4.5).
Table 4.1 Crystal and structure refinement data.

<table>
<thead>
<tr>
<th>Description</th>
<th>Ligand 2</th>
<th>Complex 4</th>
</tr>
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<tbody>
<tr>
<td>Formula</td>
<td>C_{16}H_{14}FeN_{2}OS·CHCl_{3}</td>
<td>C_{52}H_{43}CuFeN_{2}OP_{2}S</td>
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<tr>
<td>Formula weight</td>
<td>457.57</td>
<td>925.27</td>
</tr>
<tr>
<td>Temperature (K)</td>
<td>100(2)</td>
<td>223(2)</td>
</tr>
<tr>
<td>( \lambda ) (Å)</td>
<td>0.71075</td>
<td>0.71073</td>
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<td>Crystal system</td>
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</tr>
<tr>
<td>Space group</td>
<td>( P2(1)/n )</td>
<td>( P2_1/c )</td>
</tr>
<tr>
<td>Cell dimensions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a (Å)</td>
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<td>13.766(2)</td>
</tr>
<tr>
<td>b (Å)</td>
<td>14.4886(6)</td>
<td>17.839(3)</td>
</tr>
<tr>
<td>c (Å)</td>
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<td>20.779(3)</td>
</tr>
<tr>
<td>( \alpha ) (°)</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>( \beta ) (°)</td>
<td>105.2480(10)</td>
<td>119.372(3)</td>
</tr>
<tr>
<td>( \gamma ) (°)</td>
<td>90</td>
<td>90</td>
</tr>
<tr>
<td>Z</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>hkl limits</td>
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<td>-17&lt;=h&lt;=12</td>
</tr>
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<td>-22&lt;=k&lt;=23</td>
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<td></td>
<td>-26&lt;=l&lt;=27</td>
<td>-19&lt;=l&lt;=26</td>
</tr>
<tr>
<td>( D_{calc} ) (Mg/m^3)</td>
<td>1.600</td>
<td>1.382</td>
</tr>
<tr>
<td>F(000)</td>
<td>1856</td>
<td>1912</td>
</tr>
<tr>
<td>Crystal size (mm^3)</td>
<td>0.32x0.31x0.29</td>
<td>0.25x0.21x0.19</td>
</tr>
<tr>
<td>Independent reflections</td>
<td>8677 [R(int) = 0.0250]</td>
<td>10180 [R(int) = 0.0800]</td>
</tr>
<tr>
<td>Data / restraints / parameters</td>
<td>8677 / 37 / 473</td>
<td>10180 / 0 / 541</td>
</tr>
<tr>
<td>Goodness-of-fit on F^2</td>
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<td>1.035</td>
</tr>
<tr>
<td>Final R indices [I&gt;2sigma(I)]</td>
<td>R1 = 0.0284, wR2 = 0.0729</td>
<td>R1 = 0.0453, wR2 = 0.1017</td>
</tr>
<tr>
<td></td>
<td>R1 = 0.0315, wR2 = 0.0748</td>
<td>R1 = 0.0670, wR2 = 0.1142</td>
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</table>

Table 4.2 Selected bond lengths (Å) and bond angles (°).

<table>
<thead>
<tr>
<th>Bond lengths (Å)</th>
<th>Bond angles (°)</th>
<th>Bond lengths (Å)</th>
<th>Bond angles (°)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1–C4</td>
<td>1.726(2)</td>
<td>O1–C5–N1</td>
<td>123.5(2)</td>
</tr>
<tr>
<td>C4–C5</td>
<td>1.478(2)</td>
<td>N1–N2–C6</td>
<td>114.1(1)</td>
</tr>
<tr>
<td>C5–O1</td>
<td>1.234(2)</td>
<td>O2–C21–N3</td>
<td>122.8(1)</td>
</tr>
<tr>
<td>C5–N1</td>
<td>1.352(2)</td>
<td>N3–N4–C22</td>
<td>115.0(1)</td>
</tr>
<tr>
<td>N1–HN</td>
<td>0.880(1)</td>
<td>S1–C4–C5–N1</td>
<td>177.4(1)</td>
</tr>
<tr>
<td>N1–N2</td>
<td>1.388(2)</td>
<td>C4–C5–N1–N2</td>
<td>-178.8(1)</td>
</tr>
<tr>
<td>N2–C6</td>
<td>1.285(2)</td>
<td>N1–N2–C6–C7</td>
<td>-177.8(1)</td>
</tr>
<tr>
<td>C6–C7</td>
<td>1.450(2)</td>
<td>S2–C20–C21–N3–N4</td>
<td>-174.3(1)</td>
</tr>
<tr>
<td>S2–C20</td>
<td>1.723(2)</td>
<td>C20–C21–N3–N4–N4</td>
<td>178.9(1)</td>
</tr>
<tr>
<td>C20–C21</td>
<td>1.472(2)</td>
<td>N3–N4–C22–C23</td>
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</tr>
<tr>
<td>C21–O2</td>
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<td>N3–N4–C22–C23</td>
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<td>N3–N4–C22–C23</td>
<td>-176.9(1)</td>
</tr>
<tr>
<td>N3–HN</td>
<td>0.880(1)</td>
<td>N3–N4–C22–C23</td>
<td>-176.9(1)</td>
</tr>
<tr>
<td>N4–C22</td>
<td>1.284(2)</td>
<td>N3–N4–C22–C23</td>
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</tr>
<tr>
<td>C22–C23</td>
<td>1.458(2)</td>
<td>N3–N4–C22–C23</td>
<td>-176.9(1)</td>
</tr>
</tbody>
</table>
Crystallographic study of \([\text{Cu}(\text{L}^2)(\text{PPh}_3)]\) (4)

Reactions done with 1:1 metal to ligand stoichiometric ratios between the starting precursor complex of copper(II) ion and the ferrocene containing hydrazone ligand 2 yielded complex 4 that contains a single unit of coordinated hydrazone ligand in which the central metal ion is reduced from its original cupric to cuprous state along with the formation of another copper(I) complex \([\text{CuCl}(\text{PPh}_3)_3]\) (4a) as a minor product in the reaction. The observed reduction of \(\text{Cu}^{2+}\) to \(\text{Cu}^{1+}\) ion is accounted partly due to the presence of hydrazone ligand which generally acts as a good reducing agent and also due to the attainment of more stable \(t^6_e^4\) \((d^{10})\) configuration corresponding to copper(I) with tetrahedral geometry rather than less favourable \(t^6_e^3\) \((d^9)\) configuration of copper(II) with tetrahedral arrangements. An ORTEP diagram of complex 4 is depicted in Fig. 4.6.

![Fig. 4.6 Molecular structure of complex 4, with displacement ellipsoids drawn at the 25% probability level and the solvent molecule is omitted for clarity.](image-url)
Diamond shaped red crystals obtained from chloroform contained two molecules of CHCl₃ in lattice sites that are not interacting with the complex. The crystallographic data, along with a selection of bond lengths and bond angles collected are given in Tables 4.1 and 4.2, respectively. X-ray results of complex 4 revealed that the crystals are comprised of monoclinic unit cells in the space group $P2_1/c$ with $Z = 4$. The unit cell dimensions are $a = 12.9853(6)\,\text{Å}$, $b = 14.4886(6)\,\text{Å}$, $c = 20.9329(9)\,\text{Å}$; $a = \gamma = 90^\circ$, $\beta = 105.2480(10)^\circ$, $V = 3799.6(3)\,\text{Å}^3$. The central copper(I) ion adopts a distorted tetrahedral coordination sphere comprising an imine nitrogen (N2), an enolate oxygen (O1) of the hydrazone ligand and two phosphorous atoms of the triphenylphosphine. Furthermore, the bond angles of $[\text{P}1–\text{Cu}–\text{P}2]$ 114.9(3)$^\circ$, $[\text{P}1–\text{Cu}–\text{O}1]$ 117.33(5)$^\circ$, $[\text{P}1–\text{Cu}–\text{N}2]$ 125.30(6)$^\circ$, $[\text{N}2–\text{Cu}–\text{O}1]$ 78.84(8)$^\circ$, $[\text{O}1–\text{Cu}–\text{P}2]$ 94.25(5)$^\circ$ and $[\text{P}2–\text{Cu}–\text{N}2]$ 115.29(6)$^\circ$ are deviated from the theoretical value of 109.28$^\circ$. The bond lengths of $[\text{Cu}–\text{O}1]$ 2.101(2) Å, $[\text{Cu}–\text{N}2]$ 2.046(2) Å, $[\text{Cu}–\text{P}1]$ 2.224(8) Å and $[\text{Cu}–\text{P}2]$ 2.296(8) Å are almost equal to the tetrahedral bond lengths and are comparable with that of the other tetrahedral

![Unit cell packing diagram of the complex 4.](image-url)
copper(I) complex.\textsuperscript{41} The unit cell packing of complex 4 is shown in Fig. 4.7.

Structure of the minor product 4a obtained along with complex 4 was solved using XRD and found to be [CuCl(PPh\textsubscript{3})\textsubscript{3}]. The crystal structure, unit cell parameters, bond lengths of 4a was found to be in good agreement with the Cu(I) monovalent species reported earlier.\textsuperscript{42} Fig. 4.8 represents the crystal structure of the complex 4a.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{complex_4a.png}
\caption{Molecular structure of the complex 4a showing the atom-numbering scheme with thermal ellipsoids at 25\% probability level.}
\end{figure}

\section*{Biological properties}

\subsection*{DNA binding experiments}

\subsection*{Electronic absorption measurements}

Electronic absorption spectroscopy is universally employed to determine the binding characteristics of compounds with DNA, as binding to the macromolecule leads to changes in the electronic spectra of the same. Compounds that bound to DNA through intercalation is characterized by the change in absorbance (hypochromism) and bathochromic shift in wavelength, due to a strong stacking interaction between the aromatic chromophore of the test compounds and DNA base pairs. The extent of hypochromism is commonly consistent with the strength of intercalative interaction. The absorption spectra of ligands 1 and 2 and copper hydrazone complexes 3 and 4 in the absence and presence of CT DNA are shown in Fig. 4.9, respectively.
Electronic spectrum of ligand 1 showed four well resolved bands at 263, 306, 361 and 449 nm. Similarly, the spectrum of ligand 2 exhibited absorption bands at 306, 360 and 456 nm. Complex 3 exhibited three different absorptions positioned at 263, 298 and 371 nm whereas complex 4 showed only a couple of bands at 265 and 347 nm. Hence, these observations were subjected to ILCT, LMCT and MLCT that were discussed in previous section. With increasing CT DNA concentration, the absorption bands corresponding to hydrazone ligands 1 and 2 showed hypochromism of about 6.53, 9.05, 7.03, 7.09, 11.73, 2.24 and 7.98% respectively at 263, 306, 361, 449, 306, 360 and 456 nm. 

Fig. 4.9 Electronic absorption spectra of ligands 1 and 2 and its complexes 3 and 4 (25 μM) in the absence and presence of increasing amounts of CT DNA (2.5, 5.0, 7.5, 10.0, 12.5, 15.0, 17.5 and 20.0, 22.5 and 25 μM). Arrows show the changes in absorbance with respect to an increase in the DNA concentration.
nm without any wavelength shift. However, in the case of complex 3, we observed the same phenomenon of hypochromism (45.39, 40.53 and 33.68%) with a bathochromic shift of about 3, 5 and 2 nm corresponding to the absorptions at 263, 298 and 371 nm, respectively. Similarly, complex 4 showed hypochromism of about 56.47% at 265 nm with hypsochromic shift (3 nm) whereas at 347 nm, there observed hypochromism of about 53.69% together with a bathochromic shift of 7 nm. These results suggested that all the tested compounds were bound with DNA helix through intercalation. Once the compound intercalate into the base pairs of DNA, the π* orbital of the intercalators may couple with π orbital of the base pairs, thus decreasing the π→π* transition probabilities and hence a hypochromism is observed in the above cases. These results are similar to those reported earlier for various classical intercalators suggesting that the metal hydrazone complexes used in this study showed better intercalative binding to DNA when compared to that of the free hydrazone ligands. In order to determine quantitatively the binding strength of the complexes with CT DNA, intrinsic binding constants were obtained by monitoring the changes in both the wavelength as well as their corresponding intensity of absorption of the high energy bands of compounds 1-4, respectively upon increasing concentration of added DNA. The following equation was applied to calculate the binding constant: 

\[ \frac{[\text{DNA}]}{[\varepsilon_a - \varepsilon_f]} = \frac{[\text{DNA}]}{[\varepsilon_b - \varepsilon_f]} + \frac{1}{K_b[\varepsilon_b - \varepsilon_f]} \]

and the value of intrinsic binding constant \( K_b \) was found to be 3.655×10^4 M⁻¹, 3.878×10^4 M⁻¹, 7.565×10^4 M⁻¹ and 2.225×10^5 M⁻¹ corresponding to the ligands 1, 2 and its complexes 3 and 4, respectively. The magnitude of the binding constant value clearly showed that complex 4 bound more strongly with CT DNA than the complex 3 through an intercalative mode.

**Competitive binding measurements**

The ability of a ligand / metal complexes to affect the fluorescence intensity of EB-DNA adduct is a reliable tool for the measurement of its affinity towards DNA. Intense fluorescent light is emitted from EB in presence of DNA owing to its strong intercalation between adjacent DNA base pairs. Any compound that binds with DNA by the displacement of EB bound to DNA causes emission quenching. The emission spectra of EB-DNA system in the absence and presence of the test compounds 1-4 are shown in
Fig. 4.10. Upon increasing the concentration of ligands 1 and 2 added to EB bound DNA solution, 22.49 and 26.90% decrease in the fluorescence intensity of EB–bound DNA without any shift in wavelength was observed, respectively. However, upon the addition of complexes 3 and 4 to EB-bound DNA solution showed the phenomenon of hypochromism with a magnitude of 41.62 and 51.32% accompanied with bathochromic shift of 1 nm wavelength in each case. These factors strongly indicate that the EB molecules that were originally bound with DNA got displaced from their binding sites by the added compounds 1-4 under investigation. In general, the binding affinities of metal chelates towards DNA are better than that of the ligands due to chelate effect.

Fig. 4.10 Emission spectra of DNA-EB (5 µM), in the presence of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140 and 150 µM of compounds 1-4. Arrow indicates the change in the emission intensity as a function of compound concentrations. Inset: Stern-Volmer plot of the fluorescence titration data corresponding to the test compounds.
According to the Stern-Volmer equation, relative fluorescence is directly proportional to the concentration of the quencher. The ratio of the slope to the intercept obtained by plotting \( \frac{I_0}{I} \) versus \([Q]\) yielded the value of quenching constant \((K_q)\) corresponding to the test compounds 1-4 as \(1.8203 \times 10^3 \text{ M}^{-1}\), \(2.2529 \times 10^3 \text{ M}^{-1}\), \(4.4703 \times 10^3 \text{ M}^{-1}\) and \(6.9395 \times 10^3 \text{ M}^{-1}\), respectively. The linearity observed in the quenching plots illustrated that quenching is in good agreement with the Stern-Volmer equation (Fig. 4.10 with insets). Further, the \(K_{\text{app}}\) values obtained for the compounds 1-4 using the equation \(K_{\text{EB}} [\text{EB}] = K_{\text{app}} [\text{test compound}]\) (where the compound concentration has the value at a 50% reduction of the fluorescence intensity of EB, \(K_{\text{EB}} = 1.0 \times 10^7 \text{ M}^{-1}\) and \([\text{EB}] = 5 \mu\text{M}\) were in the magnitude of \(9.4032 \times 10^4 \text{ M}^{-1}\), \(1.2060 \times 10^5 \text{ M}^{-1}\), \(2.3059 \times 10^5 \text{ M}^{-1}\) and \(3.421 \times 10^5 \text{ M}^{-1}\), respectively.

The overall order of binding affinity is increased in the order of \(1 < 2 < 3 < 4\). Also, it was clear that among the two ligands \(\text{HL}_1^1\) (1) and \(\text{HL}_2^2\) (2), the one (ligand 2) with heterocyclic moiety, i.e., thiophene ring instead of furan ring in acid hydrazide part of the other (ligand 1) in their molecular skeleton showed higher efficiency to leach out more number of EB molecules those were originally bound to DNA. Similar observations have also been noted among the copper complexes containing respective hydrazone ligand.

With the simultaneous possibility of the two atoms i.e., oxygen and sulphur, present in the ligands donating their electron pair (as delocalized electrons) for the formation of coordinate bonds with that of the central metal ion, chances are that the sulfur atom would donate its pair of electrons more easily than the oxygen atom. This can be explained on the basis that compared to the O atom, the less electronegative S atom possesses more tendencies to donate electrons and hence the central copper atom becomes more electron rich center that increases the interacting ability with the nucleic acids. These results did indicate that in addition to the chelation, the type of hetero atom present in the molecular skeleton of the complexes have also influenced their affinity towards DNA binding.
Protein binding studies

Fluorescence quenching measurements

It is important to consider the interactions of drugs with plasma proteins particularly with serum albumin, which is the most abundant protein in plasma. Binding to these proteins may lead to loss or enhancement of the biological properties of the original drug or provide paths for drug transportation. Bovine serum albumin (BSA) is the most extensively studied serum albumin due to its structural homology with human serum albumin (HSA). Generally, the fluorescence of a protein is caused by three intrinsic characteristics of the protein, namely tryptophan, tyrosine and phenylalanine residues. Fluorescence quenching refers to any process that decreases the fluorescence intensity of a fluorophore due to a variety of molecular interactions including excited-state reactions, molecular rearrangements, energy transfer, ground state complex formation and collision quenching. Qualitative analysis of chemical compounds bound to BSA can be undertaken by examining the respective fluorescence spectra. Addition of the test compounds 1-4 to BSA resulted in the quenching of fluorescence emission intensity of about 42.44, 43.90, 45.70 and 51.58% from the initial fluorescence intensity together with a hypsochromic shift of 6 to 7 nm due to the formation of a complex between the test compounds and BSA. Fig. 4.11 showed the effect of increasing the concentration of test compounds on the fluorescence emission intensity of BSA.

The fluorescence quenching is described by Stern-Volmer relation: $I_0/I = 1 + K_{SV} [Q]$; where $I_0$ and $I$ are the fluorescence intensities of the fluorophore in the absence and presence of quencher respectively, $K_{SV}$ is the Stern-Volmer quenching constant and $[Q]$ is the quencher concentration. $K_{SV}$ value obtained from the plot of $I_0/I$ vs $[Q]$ was found to be $7.432 \times 10^5 \ M^{-1}$, $7.932 \times 10^5 \ M^{-1}$, $8.421 \times 10^5 \ M^{-1}$ and $1.056 \times 10^6 \ M^{-1}$ corresponding to the free ligands 1 and 2 and respective complexes 3 and 4. The calculated value of $K_{SV}$ for the test compounds indicates their good BSA binding propensity with copper complexes 3 and 4.
UV-visible absorption measurements

UV-visible absorption measurement is a simple method applicable to explore the structural changes and to know the complex formation in the donor-acceptor systems i.e., absorption characteristic of BSA-test compounds. The UV-visible spectral measurement can also distinguish the type of quenching exist i.e., static or dynamic quenching. Dynamic quenching only affects the excited states of the fluorophores and hence there are no changes in the absorption spectra. However, ground-state complex formation will frequently result in perturbation of the absorption spectrum of the fluorophore. From the absorption spectra of pure BSA and BSA+test compounds, it can be seen that upon the
addition of compounds 1-4 to a fixed concentration of BSA, a gradual increase in the intensity of BSA absorption was observed while keeping the location of the peak unchanged (as shown in Fig. 4.12) demonstrating that an interaction between compounds and BSA occurred through static quenching.44

**Fig. 4.12** Absorption spectra of BSA (1×10⁻⁵ M) and BSA-compounds 1-4 (BSA= 1×10⁻⁵ M and compounds 1-4 = 1×10⁻⁶ M).

### Binding analysis

For the static quenching interaction, if it is assumed that there are similar and independent binding sites in the biomolecule, the binding constant (Kₖ) and the number of binding sites (n) can be determined according to the method described by using the following equation:45 \[ \log \left( \frac{F_0 - F}{F} \right) = \log [K] + n \log [Q] \], where, in the present case, K is
the binding constant for free hydrazones or metal hydrazone chelates with BSA and n is the number of binding sites per albumin molecule that can be determined from the slope and intercept of the double logarithm regression curve of log \([(F_0-F)/F]\) vs log [compound] (Fig. 4.13) based on the above equation. The binding constants (K) obtained from the above said plot corresponding to the free ligands 1, and 2 and its copper complexes 3 and 4 were calculated as 2.2612×10^5 M\(^{-1}\), 6.080×10^3 M\(^{-1}\), 2.3957×10^5 M\(^{-1}\) and 5.7488×10^5 M\(^{-1}\), respectively.

![Fig. 4.13 Plot of log \([(F_0-F)/F]\) vs log [Q].](image)

The values of n, 0.8937, 0.8999, 0.9482 and 0.9819 found are approximately equal to 1, which indicates that there is a single mode of binding of the test compounds with BSA. From the protein interaction studies, it can be concluded that the thiophene containing complex 4 showed higher binding affinity than that of the furan containing complex 3 due to better electron donating ability of the former ligand as observed in the DNA binding experiments discussed in the previous section.

**Characteristics of synchronous fluorescence spectra**

Synchronous fluorescence spectroscopy is a very useful method to study the microenvironment of amino acid residues by measuring the emission wavelength shift and the technique has several advantages such as sensitivity, spectral simplification, spectral bandwidth reduction and avoiding different perturbing effects.\(^{46}\) Vekshin\(^{47}\)
suggested a useful method to study the environment of amino acid residues by measuring
the possible shift in wavelength emission maximum and also the shift in position of
emission maximum corresponding to the changes of the polarity around the chromophore
molecule. As is known, synchronous fluorescence spectra show tyrosine residues of BSA
only at the wavelength interval $\Delta \lambda$ of 15 nm and tryptophan residues of BSA only at $\Delta \lambda$
of 60 nm. Figs. 4.14 and 4.15 show the synchronous fluorescence spectra of both tyrosine
and tryptophan residues in BSA with various amounts of test compounds.

With increasing concentration of ligands 1 and 2, the intensity of tyrosine is
decreased very slightly without affecting the emission wavelength was observed

![Synchronous spectra of BSA (1×10^{-6} M) as a function of concentration of the ligands 1 and 2 (0, 2, 4, 6, 8
and 10×10^{-3} M) with wavelength difference of $\Delta \lambda = 15$ nm (A) and $\Delta \lambda = 60$ nm (B). Arrow indicates the changes in
emission intensity w.r.t various concentration of compounds.](image-url)
At the same time, the tryptophan fluorescence emission is decreased significantly with a hypsochromic shift of 1 nm. These experimental results indicate that the ligands 1 and 2 do not affect microenvironment of tyrosine residues during the binding process but affects the tryptophan microenvironment and hence, the polarity around the same residue is decreased with increase in hydrophobicity. However, under the identical experimental conditions, the copper(I) hydrazone complex 3 showed a very slight increase in intensity at tyrosine residues, whereas complex 4 displayed a significant increase in intensity of the same (Fig. 4.15). Moreover, the above complexes exhibited remarkable decrease in intensity at the tryptophan residues with a hypsochromic shift of 2

![Fig. 4.15 Synchronous spectra of BSA (1x10^-6 M) as a function of concentration of the complexes 3 and 4 (0, 2, 4, 6, 8 and 10x10^-7 M) with wavelength difference of Δλ = 15 nm (A) and Δλ = 60 nm (B). Arrow indicates the changes in emission intensity w.r.t various concentration of complexes.](image-url)
nm. From the above results, it can be deduced that the complex 3 affects only tyrosine microenvironment but complex 4 affects both the tyrosine and tryptophan residues by increasing the hydrophobicity around them. Similar behaviour regarding the interaction between the BSA and metal hydrazone complexes was reported earlier.\textsuperscript{48}

**Antioxidant studies**

The antioxidant activity of free hydrazone ligands 1 and 2 and their corresponding metal complexes 3 and 4 were evaluated in a series of *in vitro* assays involving ABTS cationic, superoxide and hydroxyl radicals in a dose dependent manner. IC\textsubscript{50} values of the ligands 1 and 2 in relevance to ABTS cationic, O\textsubscript{2}\textsuperscript{-} and OH radical assays were 32.17, 42.38, >50, 31.85, 40.56 and >50 μM, respectively, whereas, the corresponding complexes 2 and 3 showed their IC\textsubscript{50} values at 23.12, 28.17, 32.65, 20.08, 25.82 and 31.78 μM, respectively. The results of these experiments are shown in Fig. 4.16.

![Graphs showing antioxidant activity of ligands and complexes](image)

**Fig. 4.16** Trends in the inhibition of ABTS, superoxide and hydroxyl radicals by ligands 1 and 2 and corresponding metal complexes 3 and 4 at various concentrations.

In general, metal complexes exhibited greater antioxidant activity than the free ligand due to the chelate effect. Also, it was clear that among the two ligands HL\textsuperscript{1} (1) and HL\textsuperscript{2} (2), the one (ligand 2) with thiophene ring instead of furan ring in acid hydrazide part of the other (ligand 1) in their molecular skeleton showed higher scavenging efficiency. Similar observations have also been noted among the respective copper complexes containing furan or thiophene moiety in the coordinated ligand. The antioxidant activity of the ligands and its copper complexes against the free radicals i.e., ABTS cationic, O\textsubscript{2}\textsuperscript{-} and OH was found to decrease in the order of 4 > 3 > 2 > 1. Further, the results obtained against the different radicals confirmed that all the compounds are more effective to
arrest the formation of the ABTS than the other radicals studied and complexes 2 and 3 possess excellent antioxidant activities that are comparable to those of standard antioxidant butylated hydroxyl anisole (BHA).

In vitro cytotoxicity

Copper hydrazone complexes 3 and 4 were evaluated for their cytotoxicity versus a pair of human tumour cell lines (HeLa and A431) and normal cells NIH 3T3 by means of a colorimetric assay (MTT assay) that measures mitochondrial dehydrogenase activity as an indication of cell viability. The effects of the above said complexes to arrest the proliferation of tumor cells without causing any damage to normal cells were evaluated after an exposure period of 48 h. The results were analyzed by means of cell viability curves and expressed with IC\textsubscript{50} values in the studied concentration range from 15-500 μM. The activity corresponding to inhibition of cancer cell growth at maximum level, are shown in Fig. 4.17.

Upon increasing the concentration of complexes, the results of MTT assays revealed that the complexes 3 and 4 have very moderate cytotoxic potencies, with IC\textsubscript{50} values generally in the low micromolar concentrations (Fig. 4.18). Cisplatin was used as a standard to assess the cytotoxicity of both the complexes 3 and 4 (not shown in graph). Among the two different cell lines used in this study, the proliferation of A431 was arrested to a greater extent than HeLa cells by the complexes 3 and 4. As a general observation, the complex possessing thiophene moiety in the hydrazide part showed more effectiveness than the complex containing furan moiety against both the tested cell lines. A simple structure activity relationship (SAR) analysis suggest that, in both the complexes, as the hetero atom of the hydrazide moiety gets changed there is a change in the cytotoxic potency. So, it can be said that the hetero atom in hydrazides is a part of the pharmacophore and the cytotoxicity is mainly governed by the hydrazide ligands that are highly cytotoxic themselves, while complexation to metal ions rather serves to modulate their mode of action and activity. Though the above set of complexes are active against the tumor cell lines under in vitro cytotoxicity experiments, none of them could reach the effectiveness shown by the standard drug cisplatin (IC\textsubscript{50} = 14.6 and 12.6 μM, respectively).\textsuperscript{48} Upon treatment of the complexes 3 and 4 against normal NIH 3T3 cells,
it did not cause any significant damage to healthy cells (Fig. 4.17 and 4.18). The results of the cell viability tests imply that the complexes 3 and 4 have the ability to arrest the proliferation of tumor cells without causing perceptible damage to the normal cells.

**Fig. 4.17** % Cell inhibition of NIH 3T3, HeLa and A431 cell lines as a function of concentration of complexes 3 and 4.

**Fig. 4.18** Comparison of IC\textsubscript{50} values between complexes 3 and 4 on the inhibition of NIH 3T3, HeLa and A431 cell lines.
Conclusion

A set of new copper(I) hydrazone complexes have been synthesised from Cu(II) starting precursor and ferrocene containing hydrazone ligands. The synthesised compounds were characterised by elemental analyses, spectroscopic and single crystal XRD methods. The bidentate hydrazone ligand and two units of triphenylphosphines bound to copper(I) ions present a distorted tetrahedral geometry around it in the synthesized complexes. Ligands with different electronic properties have been included in order to study their influence on the biological activity of the respective complexes that includes DNA/albunin binding, antioxidant and cytotoxicity studies. UV spectroscopy and emission studies have demonstrated the binding of the complexes as well as ligands with DNA. The binding strength of the compounds with CT DNA calculated with UV-visible spectroscopic titrations have shown that \( \text{[CuL}^2(\text{PPh}_3)_2]\) (4) exhibits the highest \( K_b \) value among the compounds examined. Competitive binding studies with EB have showed the potential of the compounds to displace EB from the EB-DNA complex and confirmed the intercalation as the most possible binding mode to DNA. The examined compounds showed good binding affinity to BSA proteins giving relatively high binding constants in the order of \( 10^5 \text{ M}^{-1} \). Synchronous fluorescent measurements revealed that all the test compounds affect the tryptophan microenvironment leaving the tyrosine unaffected by compounds 1, 2 and 3. Free ligands and their corresponding complexes tested for in vitro antioxidant studies presented significantly high scavenging activity against ABTS cationic radicals than the superoxide and hydroxyl radicals, with complex 4 being the most active one. In vitro cytotoxicity of complexes 3 and 4 against HeLa and A431 tumour cells and NIH 3T3 non-tumorous cells showed that the copper hydrazone complex 4 has more potency towards the inhibition of tumour cell growth without significant damage to the normal cells. From the biological activity experiments, we concluded that the complex 4 containing less electronegative S atom in the thiophene moiety of the hydrazone became more electron rich center that increases the interacting ability with biomolecules than complex 3 with more electronegative O atom in furan moiety of the hydrazone. Hence, in addition to chelation, the type of hetero atom present in the molecular skeleton of the complexes has also influenced their affinity towards bimolecular interactions.
Reference


47. N.L. Vekshin, Biophysics, 1996, 1176-1182.