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DNA cleavage, structural elucidation and anti-microbial studies of three novel mixed ligand Schiff base complexes of copper(II)

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Abstract: Three new copper complexes of mixed ligands derived from Schiff bases (condensation of *p*-aminoacetanilide and substituted benzaldehydes) with 1,10-phenanthroline have been synthesized and characterized by elemental analysis, IR, UV–Vis, magnetic moments, conductivity and electrochemical measurements. The spectral techniques suggest that all the copper complexes exhibit octahedral geometry. The low electrical conductance of the complexes supports their neutral nature. The monomeric nature of the complexes was assessed from their magnetic susceptibility values. The *in vitro* biological screening effects of the investigated compounds were tested against the bacteria *Escherichia coli, Staphylococcus aureus,* and *Salmonella typhi* and the fungi *Rhizopus stolonifer* and *Candida albicans* by the serial dilution method. A comparative study of the MIC values of the Schiff bases and their copper complexes indicates that the metal complexes exhibited higher antibacterial activity than the free ligands. The DNA cleavage ability of the complexes was monitored by the gel electrophoresis technique. It was found that electron withdrawing group substituted copper complex had higher DNA cleavage activity than the other copper complexes.

Keywords: *p*-aminoacetanilide, benzaldehyde, *p*-methoxybenzaldehyde, 2-chlorobenzaldehyde, CT DNA, copper(II) complexes.

INTRODUCTION

Transition metals are essential for the normal functioning of living organisms. Therefore, it is not surprising that transition metal compounds are of great interest as potential drugs. Many complexes, including the platinum group, have been synthesized and tested in a number of biological systems after the discovery of the inorganic anti-cancer agent, cisplatin. Thus, interest in the complexes of copper has arisen for the following reasons: copper complexes are known to have a broad spectrum of biological action[1] and copper is considered as an essential trace element but its concentrations as free metal ion inside cells should be lower than 10^{-15} M (calculated)/10^{-12} M (observed), since concentrations higher than 10^{-9} M in the cytoplasm can be poisonous.[2] The free copper concentration in the cyto-

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plasm is regulated by different mechanisms, including pumps, exchangers and by protein expression, which use copper in their active sites.[3] The great majority of the copper proteins are involved mainly in oxidation/reduction reaction as well as in dioxygen transport.[4] The development of mimic system for copper metalloenzymes has provided similar activities to that of natural metalloenzymes.[5–7] Copper has proved to be a valuable metal center in the development of artificial nuclease systems, as reported in the literature.[8–11] Thus, synthetic copper compounds have been investigated on their ability to promote nucleic acid cleavage, as well as receiving special attention for their action on DNA.[12] The potential scope of the utility of these compounds is enormous and ranges from the creation of synthetic restriction enzymes for use by molecular biologists to the development of chemotherapeutic agents which may be effective against a variety of diseases, sensitive chemical probes for DNA structure in solution and tools for the molecular biologist to dissect genetic systems.[13] Especially, a number of metal complexes of a variety of ligands have been studied in view of their possibility to lead to advanced functional materials, tuning the redox potentials, affinity towards DNA, and specificity for DNA base sequence recognition.[14–16] Investigations of the interaction of DNA with small molecules are basic work in the design of new types of pharmaceutical molecules. Some types of metal complexes interacted with DNA could induce the breakage of DNA strands as shown by the gel electrophoresis technique. Thus, in cancer genes, after a cleavage of a DNA strand, the DNA double strands breaks. The replication ability of cancer gene is thereby destroyed.

It is well known from the literature that compounds containing an amide moiety have a strong ability to form metal complexes and exhibit a wide range of biological activities. Hence, in this work, the synthesis of three Schiff base copper complexes containing an amide moiety and 1,10-phenanthroline was attempted and the biological activities, such as anti-microbial and DNA cleavage, of the resulting complexes were studied.

EXPERIMENTAL

Synthesis of the Schiff bases

The Schiff base was prepared by the dropwise addition of an ethanolic solution (50 ml) of *p*-aminoacetanilide into a solution of benzaldehyde (L^1)/*p*-methoxybenzaldehyde (L^2)/2-chlorobenzaldehyde (L^3) (1:1 molar ratio of 10 mM) in ethanol. After completion of the addition, the solution was refluxed on a water bath for 1 h and allowed to cool by standing at room temperature. The formed solid product was removed by filtration and recrystallized from ethanol (Yield: 75 % for L^1; 70 % for L^2; 78 % for L^3).

Synthesis of the copper complexes

A solution of Schiff base and 1,10-phenanthroline in ethanol was added to a solution of $CuCl_2 \cdot 2H_2O$ (1:1:1 molar ratio of 10 mM) in ethanol and the mixture was stirred for 1 h. The solid product so-formed was separated by filtration and washed thoroughly with ethanol and dried *in vacuo* (Yield: 52 % for $[CuL^1(Phen)Cl_2]$; 55 % for $[CuL^2(Phen)Cl_2]$; 60 % for $[CuL^3(Phen)Cl_2]$).

Nuclease activity

The DNA cleavage experiment was conducted using CT DNA by gel electrophoresis with the corresponding copper complex in the presence of H_2O_2 as an oxidant. The reaction mixture was incubated at 35 °C for 1.5 h before the electrophoresis experiment as follows: CT DNA 10 μM, 5 μM each complex, 1 μM H_2O_2 in 50 mM tris–HCl buffer (pH 7.0) and 34 μM of double distilled water. The samples were electrophoresed for 2 h at 50 V on 1 % agarose gel using tris–acetic acid–EDTA buffer, pH 8.3. After electrophoresis, the gel was stained using 1 μg cm^3 EB and photographed under UV light.

Anti-microbial activity

The *in vitro* biological screening effects of the investigated compounds were tested against *Staphylococus aureus, Escherichia coli, Salmonella typhi* by the well diffusion method using agar nutrient as the medium and amphotericin as the control.

The *in vitro* anti-fungal assay was performed by the disc diffusion method. The complexes and ligand were tested against the fungi *Rhizopus stolonifer* and *Candida albicans*, cultured on potato dextrose agar as medium. In a typical procedure, a well was made on the agar medium inoculated with the fungi. The well was filled with the test solution using a micropipette and the plate was incubated at 30 °C for 72 h. During this period, the test solution diffused and the growth of the inoculated fungi was affected. The inhibition zone developed on the plate was measured. The MIC of the complexes was determined by the serial dilution technique.

Apparatus and reagents

All reagents were Merck products and were used as supplied. For the voltammetric experiments, the tetrabutylammonium perchlorate (TBAP) used as the supporting electrolyte, was purchased from Sigma. Anhydrous grade methanol and DMSO were obtained from Fisher Scientific Company. The metal contents of the complexes were estimated gravimetrically as their oxides,[17] by fusion with AnalaR ammonium oxalate. Microanalytical data and FAB mass spectra of the compounds were recorded at the Regional Sophisticated Instrumentation Center, Central Drug Research Institute (RSIC, CDRI), Lucknow. Microanalyses were performed using a Carlo Erba 1108 CHN Elemental Analyser. The FAB mass spectra of the complexes were recorded on a JEOL SX 102/DA-6000 mass spectrometer/data system using argon/xenon (6 kV, 10 mA) as the FAB gas. The accelerating voltage was 10 kV and the spectra were recorded at room temperature using *m*-nitrobenzyl alcohol (NBA) as the matrix. The IR spectra of the samples were recorded on a Perkin-Elmer 783 spectrophotometer in 4000–200 cm^{-1} range using the KBr pellet technique. The UV–Vis spectra were recorded on a Shimadzu UV-1601 spectrophotometer using DMSO as the solvent in the wave range of 200–800 nm. The X-band ESR spectra of the complexes were recorded at 77 K at IIT, Mumbai using TCNE (tetracyanoethylene) as the g-marker. Magnetic susceptibility measurements of the complexes were carried out using a Guoy balance with copper sulfate as the calibrant. Electrochemical studies were carried out using a EG&G Princeton Applied Research Potentiostat/Galvanostat Model 273A, controlled by M270 software. The measurements were carried out under oxygen-free condition using a three electrode cell, in which glassy carbon was the working electrode, a saturated Ag/AgCl was the reference electrode and a platinum wire was used as the auxiliary electrode. All solutions (10^{-3} M) were purged with N_2 for 30 min prior to each set of experiments. The molar conductivity was measured with a Systronic conductivity bridge, using freshly prepared solutions of the complexes in DMSO solution (10^{-3} M). Solutions of CT-DNA in 50 mM NaCl/50 mM tris–HCl (pH 7.0) gave a ratio of UV absorbance at 260 and 280 nm, A260/A280 of *ca.* 1.8–1.9, indicating that the DNA was sufficiently free of protein contamination. The DNA concentration was determined by the UV absorbance at 260 nm after 1:100 dilutions. Stock solutions were kept at 4 °C and doubly distilled H_2O was used to prepare the buffer.

RESULTS AND DISCUSSION

All the complexes were air stable, insoluble in water and soluble in DMSO. They were identified by their physical and analytical data. The analytical data (Table I) are in good agreement with the general formula $[CuL(Phen)Cl_2]$. The monomeric nature of the complexes was evidenced from their magnetic susceptibility values. The study of the magnetic and electronic spectral data was quite informative in characterizing the geometry of the complexes. These complexes are non-electrolytic due to their low conductivity values.[18]

TABLE I. Physical characterization, analytical, molar conductance and magnetic susceptibility data of the complexes

Compound	Color	Composition found (calcd), %				$\Lambda_m \times 10^3$ S cm^2 mol^{-1}	μ_{eff} / μ_B
		M	C	H	N		
L^1	Yellow	–	75.69 (75.63)	5.81 (5.88)	11.80 (11.76)	1.3	–
$[CuL^1(Phen)Cl_2]$	Dark green	11.49 (11.50)	58.60 (58.64)	3.95 (3.98)	10.11 (10.13)	1.6	1.85
L^2	White	–	71.10 (71.91)	5.65 (5.62)	10.42 (10.49)	1.2	–
$[CuL^2(Phen)Cl_2]$	Brown	10.80 (10.85)	57.61 (57.68)	4.10 (4.12)	9.63 (9.61)	1.8	1.75
L^3	Glittering white	–	67.10 (67.16)	4.80 (4.85)	8.99 (8.95)	1.4	–
$[CuL^3(Phen)Cl_2]$	Pale green	10.86 (10.85)	55.15 (55.33)	3.52 (3.59)	9.51 (9.56)	1.7	1.79

The FAB mass spectra of the Schiff bases and their copper complexes were used to compare their stoichiometric composition. The Schiff base L^2 shows a molecular ion peak M^+ at $m/z = 268$. The molecular ion peak, observed at $m/z = 581$ for the copper complex confirms the molecular formula of the copper complex as $[CuL^2(Phen)Cl_2]$. This was also supported by the FAB mass spectra of the other complexes.

The elemental analysis values are in close agreement with the values calculated from molecular formula assigned to these complexes, which is further supported by the FAB-mass studies of the complexes.

The electronic spectra of the Schiff base ligands and their copper complexes were recorded in DMSO solution at 300 K. The spectra of copper complexes show a d-d transition in the region 700−750 nm, assigned to a $^2B_{1g} \rightarrow {}^2A_{2g}$ transition. The position of this band in the spectra suggests a distorted octahedral geometry for the complexes.[19] A moderately intensive band observed in the range 330–360 nm is due to a ligand-to-metal charge transfer transition[20] and the strong band observed in the range 250–280 nm is due to an intra-ligand charge transfer transition for these copper complexes.[21]

The IR spectra of the free Schiff base ligands were compared with the spectra of the complexes. The IR spectra of the Schiff bases show a prominent absorption peak at *ca*. 1700 cm^{-1} corresponding to ν(C=O) of acetanilide, which is shifted to a lower frequency 1680 cm^{-1} in the spectra of the copper complexes, indicating coordination to the metal ion. The ligands show their characteristics ν(C=N) bands in the region 1610–1590 cm^{-1}, which are also shifted to lower frequencies in the spectra of all the complexes (1580–1550 cm^{-1}). The IR spectra of the copper complexes show some new bands in the region 490–510 cm^{-1} and 450––460 cm^{-1}, which are probably due to Cu–O and Cu–N bonds respectively.[22] The weak band at 350 cm^{-1} is probably due to the formation of Cu–Cl bonds.[23]

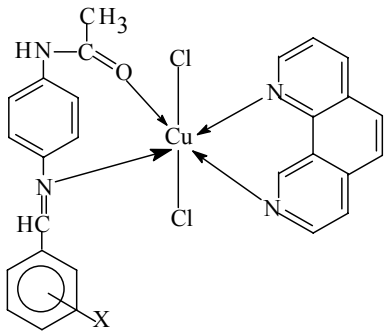
The ESR spectrum of metal complexes provides information about hyperfine and superhyperfine structures which are of importance in the study of the environment of the metal ion in the complex, *i.e.*, the geometry and nature of the ligating sites of the Schiff base and the metal. The X-band ESR spectrum of the polycrystalline copper complex recorded at 77 K shows a well-resolved hyperfine splitting and exhibits two different *g*-values, indicating the magnetic anisotropy in the complex. The magnetic susceptibility value reveals that the copper complex has a magnetic moment, 1.85 μ_B, corresponding to that of one unpaired electron, indicating that the complex is mononuclear. This fact was also evident from the absence of a half field signal, observed in the spectrum at 1600 G due to ms = ±2 transitions, ruling out any Cu–Cu interaction.[24]

The *g*-tensor value of the $[CuL^3(Phen)Cl_2]$ complex can be used to derive the ground state. In octahedral complexes, the unpaired electron lies in the $d_{x^2-y^2}$ orbital. In the present copper complex, the observed *g*-tensor values are $g_{\|}(2.256) > g_{\perp}(2.052) > g_e(2.002)$, which suggest that this complex has a distorted octahedral geometry. The ESR parameters of the complex also coincide well with related systems for which it was suggested that the complexes have a distorted octahedral geometry and that the systems are axially symmetric.[25] In the axial spectra, the *g* values are related to the exchange interaction coupling constant (*G*) by the expression:

$$G = g_{\|} - \frac{2}{g_{\perp} - 2}$$

According to Hathaway,[26] if the *G* value is larger than four, the exchange interaction is negligible because the local tetragonal axes are aligned parallel or are slightly misaligned. If its value is less than four, the exchange interaction is considerable and the local tetragonal axes are misaligned. For the present copper complex, it is 4.9, which suggests that the local tetragonal axes are aligned parallel or are slightly misaligned and are consistent with a $d_{x^2-y^2}$ ground state.

Based on the above spectral data, the structures of these complexes are assigned as shown in Fig. 1.



X = -H, -OCH_3, -Cl

Fig. 1. Proposed structure of the synthesized copper(II) complexes.

Cyclic voltammetry is the most versatile electrochemical technique for the study of electroactive species. The cyclic voltammogram of the [$CuL^3(Phen)Cl_2$] (0.01 M) complex in DMSO at 300 K in the potential range 0.8 to –0.4 V at a scan rate 100 mV s^{-1} is shown in Fig. 2, which shows one quasi-reversible reduction peak at 0.31 V (E_{pc}), corresponding to the formation of Cu(III)/Cu(II) and an oxidation peak at 0.49 V (E_{pa}), corresponding to the formation of the Cu(II)/Cu(III) couple. The peak potential difference (ΔE_p = 130 mV) shows one quasi-reversible couple. The number of electrons of the complex transferred was established by the current heights (I_{pc}/I_{pa} = 0.91) which shows that the copper complex has one electron transfer.

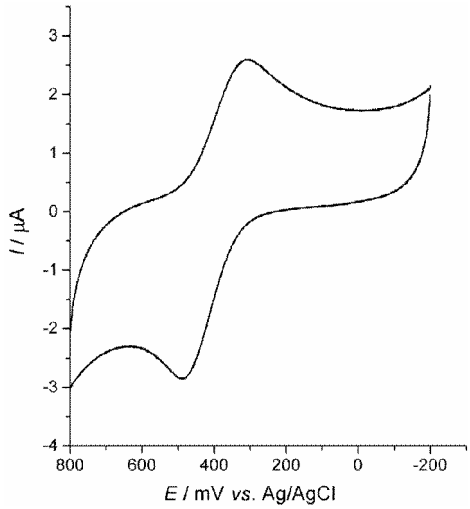


Fig. 2. Cyclic voltammogram of [$CuL^3(Phen)Cl_2$] in DMSO at room temperature.

The *in vitro* anti-microbial activity of the investigated compounds was tested against the microorganisms *E. coli, S. typhi, S. aureus, C. albicans* and *R. stolnifer* by the serial dilution method. The minimum inhibitory concentration (MIC) values of the compounds against the growth of micro-organisms are summarized

in Table II. A comparative study of the ligands and their copper complexes (MIC values) indicates that all the copper complexes exhibited higher anti-microbial activity than the free ligands. Such increased activity of the complexes can be explained on the basis of the Overtone concept[27] and the Tweedy chelation theory.[28] According to the Overtone concept of cell permeability, the lipid membrane surrounding the cell favors the passage of only lipid-soluble materials, due to which liposolubility is an important factor controlling the anti-microbial activity. On chelation, the polarity of the metal ion will be reduced to a greater extent due to the overlap of the ligand orbital and partial sharing of the positive charge of the metal ion with donor groups. Furthermore, the mode of action of the compound may involve formation of a hydrogen bond through the azomethine group with the active centre of cell constituents, resulting in interference with normal cell processes.

TABLE II. Anti-microbial data of the investigated compounds (MIC×10^3 M)

Compound	*E. coli*	*S. typhi*	*S. aureus*	*R. stolonifer*	*C. albicans*
L^1	4.2	5.9	5.1	4.6	3.8
$[CuL^1(Phen)Cl_2]$	3.2	0.8	1.6	2.2	2.6
L^2	4.5	3.9	5.9	4.0	6.2
$[CuL^2(Phen)Cl_2]$	2.6	1.5	1.0	2.2	1.8
L^3	4.0	2.9	4.2	3.4	5.2
$[CuL^3(Phen)Cl_2]$	1.4	1.8	2.1	0.6	2.8
Amphotericin	3.2	2.0	1.8	1.5	2.5

The nuclease activity of the present copper complexes was investigated using CT DNA by agarose gel electrophoresis in the presence of an oxidant (H_2O_2), as shown in Fig. 3.

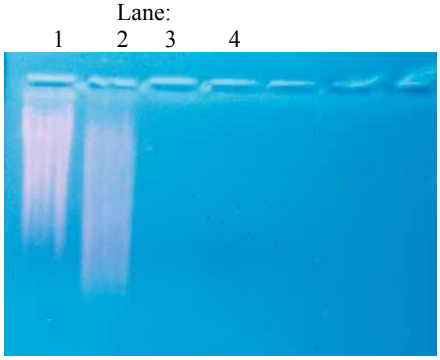


Fig. 3. Agarose gel (1 %) showing the changes in the agarose gel electrophoretic pattern of CT DNA induced by H_2O_2. Lane 1: DNA alone, Lane 2: DNA + + $[CuL^1(Phen)Cl_2]$, Lane 3: DNA + $[CuL^2(Phen)Cl_2]$ and Lane 4: DNA + $[CuL^3(Phen)Cl_2]$.

As can be seen from the results (Fig. 3), the electron withdrawing substituted complex had a higher DNA cleavage activity than the other copper complexes (*i.e.*, $[CuL^3(Phen)Cl_2] > [CuL^2(Phen)Cl_2] > [CuL^1(Phen)Cl_2]$). Due to the presence of an electron withdrawing group in the ligand, the positive charge of the copper ion increases, and this enhances the ability of the copper ion to interact

with DNA. The samples in lanes 3 and 4 were completely degraded. This shows that a slight increase in the concentration over the optimal value (*i.e.*, the value at which a 100 % cleavage efficiency was observed) led to extensive degradation, resulting in the disappearance of the bands on the agarose gel. The other copper complex (lane 2) degrades DNA efficiently. To understand the active oxygen species in the reactions, inhibition experiments were performed with standard scavengers for reactive oxygen intermediates using degradation experiments with the Rhodamine B dye test (Fig. 4).

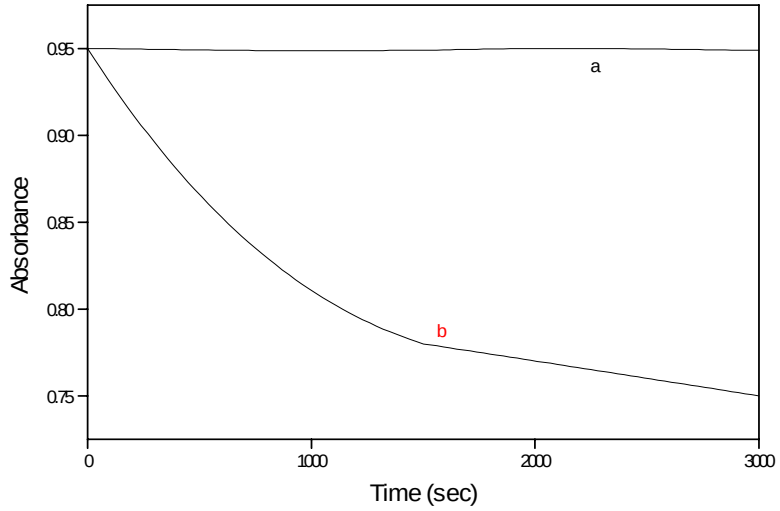


Fig. 4. Rhodamine B degradation followed by the decrease of the absorbance at 552 nm at pH 8.1 in 10 mM phosphate buffer: (a) in the presence of 0.1 mM $[CuL^1(Phen)Cl_2]$; (b) in the presence of 0.1 mM $[CuL^1(Phen)]^{2+}$, 10 mM H_2O_2, 1 mM ascorbic acid.

The degradation of the dye provides a direct measure of the concentrations of hydroxyl radicals in the reaction mixture. From the observation, it is suggested that reactive oxygen species can be produced by the $[CuL^1(Phen)Cl_2]$ complex under redox conditions.[29]

CONCLUSIONS

A new series of mixed ligand complexes of copper was synthesized and their octahedral geometry was inferred from their spectral data. A comparative study of the MIC values of the ligands and their complexes indicates that the copper complexes exhibit higher anti-microbial activity than the free ligands. The Rhodamine B dye test clearly indicates that the increase of the cleavage reactivity is related to the presence of reactive radicals. Electrochemical experiments neatly demonstrate that the present ligand system is ideally suited for stabilizing the higher oxidation states of the copper ion.

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И З В О Д

РАЗЛАГАЊЕ ДНК, ОДРЕЂИВАЊЕ СТРУКТУРЕ И АНТИМИКРОБНА ИСПИТИВАЊА ТРИ НОВА МЕШОВИТА КОМПЛЕКСА БАКРА(II) СА ЛИГАНДИМА ЗАСНОВАНИМ НА ШИФОВИМ БАЗАМА

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Синтетисана су три нова комплекса бакра са мешовитим лигандима од Шифових база (кондензација *p*-аминоацетанилида и супституисаних бензалдехида) са 1,10-фенантролином и окарактерисана елементалном анализом, IR, UV–Vis, одређивањем магнетних момената и проводљивости, као и електрохемијским мерењима. Спектралне технике указују на то да су сви комплекси бакра октаедарске геометрије. Ниска електрична проводљивост комплекса указује на њихову неутралну природу. Вредности магнетне сусцептибилности указују на мономерну природу комплекса. Биолошка активност добијених једињења испитивана је *in vitro* на бактеријама *Escherichia coli, Staphylococcus aureus* и *Salmonella typhi* и гљивама *Rhizopus stolonifer* и *Candida albicans* методом серијског разблажења. Упоредна анализа MIC вредности Шифових база и њихових комплекса бакра указују на то да су метални комплекси антибактеријски активнији од слободних лиганада. Способност комплекса за везивање ДНК испитивана је техником гел-електрофорезе. Утврђено је да супституисани комплекси бакра мање електронске густине јаче везују ДНК од осталих комплекса бакра.

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