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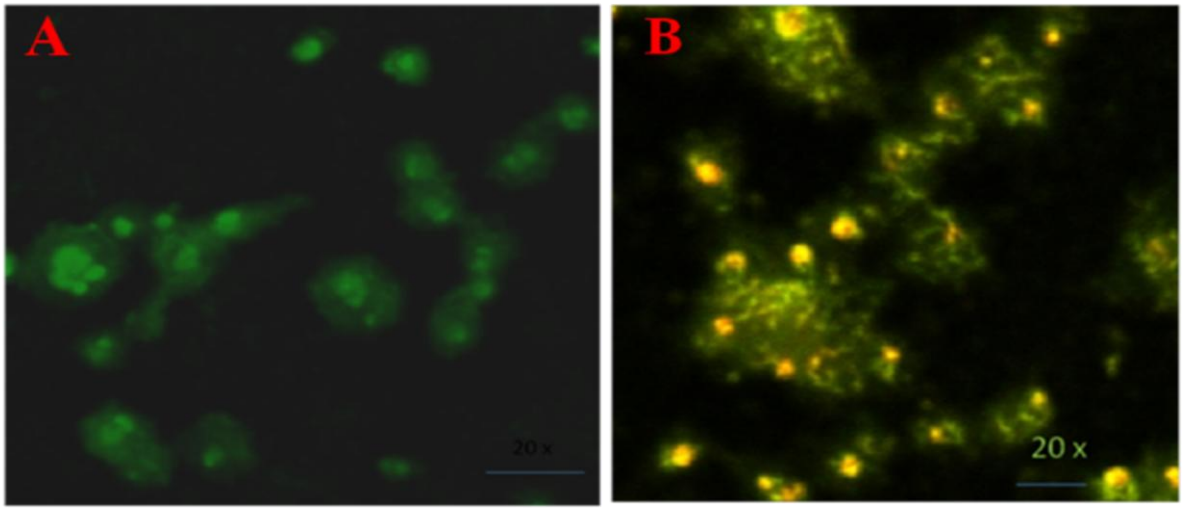
GRAPHICAL ABSTRACT



Untreated control cells

Surfactant copper(II) complex treated Control cells

Nucleic acids Binding study of Surfactant Copper(II) Complex Containing dipyrido[3,2-a:2'-3'-c]phenazine ligand as Intercalator: In Vitro Antitumor Activity of Complex in Human Liver Carcinoma (HepG2) Cancer Cells

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Abstract: A new surfactant copper(II) complex, $[Cu(dppz)_2DA](ClO_4)_2$, where dppz = dipyrido[3,2-a:2'-3'-c]phenazine and DA-dodecylamine, has been synthesized and characterized by physico-chemical and spectroscopic methods. The critical micelle concentration (CMC) value of this surfactant copper(II) complex in aqueous solution was found out from conductance measurements. Specific conductivity data at different temperature served for the evaluation of the temperature-dependent CMC and the thermodynamics of micellization (ΔG^0m, ΔH^0m and ΔS^0m). The binding interaction of this complex with nucleic acids (calf thymus DNA and yeast t-RNA) was investigated using electronic absorption, fluorescence spectroscopy, viscometry, cyclic voltammetry (CV) and thermal denaturation studies. In presence of the nucleic acids UV-Vis spectrum of our complex showed red shift of the absorption band at 268 nm along with significant hypochromicity indicating intercalation of our complex with nucleic acids. The intrinsic binding constant values are $K_b = 1.1 \times 10^6\ M^{-1}$ for DNA and $1.6 \times 10^6\ M^{-1}$ for RNA. The viscosity measurements confirm that the complex–nucleic acid interaction is through intercalation. A competitive binding study with ethidium bromide (EB) show that the complex exhibits the ability to displace the nucleic acid-bound EB indicating that the complex binds to nucleic acids in strong competition with EB for the intercalative binding site. CV results also confirm this mode of binding. Some significant thermodynamic parameters of the binding of the titled complex to DNA have also been determined. The antimicrobial and antifungal screening tests of this complex have shown good results. The copper(II) complex shows more pronounced activity against human liver carcinoma(HepG2) cancer cell line.

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1. Introduction

The past two decades have seen extensive studies in the DNA and DNA site-specific cleavage.[1] several reports of biological studies have proven that DNA is the major intracellular target of some anticancer drugs. Small molecules interact with DNA may create DNA damage in cancer cells, stopping the division of cancer cells and finally in cell death.[2] Because of this there are efforts which stem from the search for an understanding of drug–nucleic acid interaction on a molecular level and based on them to develop novel chemotherapeutics and diagnostic agents.[3] There are several types of sites in the DNA molecule where binding of small molecules can occur among base pairs[4], groove and helix.[5] Among the small molecules studied transition metal complexes are very important to detect the possible new generation drugs. A number of useful applications of transition metal complexes refer that the complex binds to DNA via an intercalative mode with the ligand intercalates into the DNA base pairs. Because of the uncommon binding properties and general photoactivity, these coordination compounds are probably as DNA secondary structure probe[5], photocleavers and antitumor drugs.[6] In contrast to DNA, very little attention has been paid to the binding properties of transition metal complexes with RNA. These metal complexes have been used as catalysts of RNA hydrolysis cleavage,[7] shape-selective probes of RNA tertiary structure, agents of RNA oxidation cleavage and approval of mismatches in RNA.[8] But only a few reports have investigated the interaction between transition metal complexes and RNA.[9]

The ligand (dppz = dipyrido[3,2-a:2'-3'-c]phenazine) (dppz) is a familiar heterocyclic aromatic compound, which has been principally used in synthetic inorganic chemistry to layout supramolecular motifs.[10] This ligand can be synthetically modified to produce different derivatives which are also reported detailed in the literature.[11] This class of ligands containing multiple pyridine rings, is commonly known as 'polypyridines' and has been used in coordination chemistry on large scale. These transition metal-dppz compounds have been under extensive investigations due to their differential electrochemical, optical and photophysical properties.[12] Transition metal-dppz interact with different biological substrates either by intercalation or by coordinative binding.[13] The multidirectional applications include use as antitumour agents, protein probing agents, and radiotherapeutics.[14]

Copper is found in all living organisms and is a resolvable trace element in redox chemistry, growth and development.[15] It is a biologically related element and many enzymes that depend on copper for their activity have been identified. Because of its biological relevance, a large number of copper(II) complexes have been synthesized and searched for their biological activities.[16] Among the copper complexes, attention has been mainly focused on the copper(II) complexes with modified-phenanthroline ligands due to their high nucleolytic efficiency, anti-tumor, and antimicrobial activities.[17] The number of publications based on phenanthroline and modified phenanthroline containing coordination compounds has been growing extensively in the last decade. Among this detailed study of articles, only a very limited number of articles deal with biological aspects of these groups of ligands or compounds. Some derivatives of modified phenanthroline and their respective coordination compounds with copper have been synthesized but DNA interaction studies, or cell viability assays are not done.

In our laboratory, we have been focusing on designing, development and interaction of surfactant metal complexes with nucleic acids.[18,19] Surfactant metal complexes with chelating ligands are of attraction for metallobiomolecules in the search for appropriate systems for binding and activating simple molecules, catalysis and magnetic interactions.[20] In these surfactants, the metal complex containing the central metal ion with its primary coordination sphere acts as the head group and the hydrophobic part of one or more ligands acts as the tail. Like any other surfactant these surfactant metal complexes also form micelles. The critical micelle concentration (CMC) is the concentration of the surfactant above which a surfactant aggregates into micelles.Thus, the CMC represents a phase separation between single molecules of surfactant and surfactant aggregates in dynamic equilibrium.[21] Understanding micelle behavior in biological systems is important because the state of aggregation or micelle phase of naturally occurring molecules, drug molecules or added surfactant may influence the biological effects.[22] We have established recently that the complexes of $[Co(diimine)(DA)_2](ClO_4)_3$ [diimine = bipyridine (bpy), 1,10-phenanthroline (phen), etc.] interact with DNA through their long chains. In the present investigation, the complex $[Cu(dppz)_2(DA)]^{2+}$ containing

ligand with extended aromaticity and long aliphatic chain has been prepared and its interaction with calf thymus (CT) DNA and yeast tRNA was studied by UV-Visible absorption, emission spectroscopy, viscosity, thermal denaturation and cyclic voltammetric methods. The anti-microbial and antibacterial characteristics of this surfactant copper(II) complex was also tested against Gram +ve and Gram -ve bacteria and fungus. Besides the anticancer activity of this complex has been tested against human liver carcinoma cancer cell (HepG2).

2. Experimental

2.1. Materials and methods

All the reagents used in the preparation of ligand and its metal complex were of reagent grade (Sigma). The solvents used in the synthesis of ligand and metal complex were distilled before use. Calf thymus DNA (CT-DNA), dodecylamine (DA) and ethidium bromide (EB) were purchased from Sigma Chemicals Co. (USA). All the experiments involving interaction of the surfactant complex with nucleic acids were carried out in buffer containing 5mMTris and 50mM NaCl and adjusted to pH 7.2 with hydrochloric acid. A solution of CT-DNA gave a ratio of UV absorbance at 260 and 280nm of about 1.8–1.9, indicating that the CT-DNA was sufficiently free of protein. [24] Ligands were prepared by the Schiff-base condensation of 1,10-phenanthroline-5,6-dione with the appropriate diamino compound in ethanol under reflux.[25] The dione was prepared by oxidation of 1,10-phenanthroline following the method of Gillard *et al.*[26]

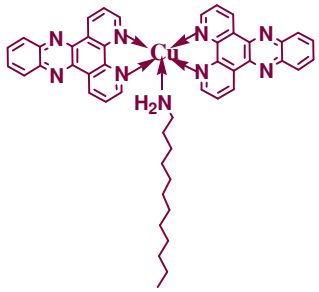
The elemental analyses (C, H, and N) of the sample were determined at SAIF, Cochin University, Cochin, Kerala. The electronic spectra were recorded on a Shimadzu UV-3101PC spectrophotometer using cuvettes of 1 cm path length, and emission spectra were recorded on a JASCO FP 770 spectrofluorimeter. Conductivity measurements were carried out in aqueous solutions of the complex with an Elico conductivity bridge type CM 82 and a dip-type cell with a cell constant of 1.0. FT-IR spectra were recorded on FT-IR JASCO 460 PLUS spectrophotometer with samples prepared as KBr pellets. EPR spectra were recorded on JEOL-FA200 EPR spectrometer at room temperature and at LNT in methanol solution.

2.2. Preparation of $[Cu(dppz)_2(Cl)]Cl$

The precursor copper(II) complex, $[Cu(dppz)_2(Cl)]Cl$ was prepared in a similar method to that described in the literature.[27] The complex was prepared by reacting $CuCl_2 \cdot 2H_2O$ (0.17 g; 1.0 mmol) with dppz (0.74 g, 2.5 mmol) in methanol (50 cm^3). The solution was stirred at 45°C for 2 h followed by cooling to an ambient temperature. The solid product thus obtained was isolated, washed with ethanol and diethyl ether and finally dried in vacuum dessicator.

2.3. Synthesis of surfactant copper(II) complex

To a solution of $[Cu(dppz)_2(Cl)]Cl$ in 15 cm^3 of water slightly more than the calculated amount of dodecyl amine in 3 cm^3 of ethanol was added dropwise over a period of 30 min. The green solution gradually became red and the mixture was set aside at 40^0 C for 2 days until no further change in colour was observed. Afterwards a saturated solution of sodium perchlorate in very dilute perchloric acid was added. Slowly the complex $[Cu(dppz)_2(DA)](ClO_4)_2$ was separated as a pasty solid mass and was filtered off, washed with small amounts of alcohol and acetone, and then dried over air. The semidried solid was further dried in a drying pistol over fused calcium chloride and stored in vacuum desiccator. The structure of $[Cu(dppz)_2(DA)](ClO_4)_2$ is shown in Scheme 1.



Surfactant copper(II) complex

Scheme 1: Structure of surfactant copper(II) complex

2.4. CMC determination

The measurements of conductivities of the appropriate concentration range of surfactant copper(II) complex was studied by continuous dilution of a concentrated solution into water. The conductivity of these solutions was measured at 303, 308, 313, 318 and 323 K. The conductivity was recorded when its fluctuation was less than 1% within 2 min. After each addition, the solution was mixed carefully without the formation of foam. The break point in the plot of specific conductance versus surfactant concentration was taken as CMC.

2.5. Nucleic acid binding experiments

The DNA/RNA binding experiments were performed at 25.0 ± 0.2^0C. The nucleic acid concentration per nucleotide was determined by UV-Visible spectroscopy using the known molar extinction coefficient value of 6600 M^{-1} cm^{-1} and 9250 M^{-1} cm^{-1} for DNA and RNA at 260 nm respectively.[28] Absorption titration was performed with fixed concentration of the copper(II) surfactant complex to which increasing the concentration of the DNA/RNA stock solution was added to both complex solution and reference solution to eliminate the absorbance of nucleic acid itself.

Thermal denaturation studies were carried out on a JASCO V530 spectrophotometer using cuvettes of 1 cm path length with JULABO F32H peltier systems (±0.1^0 C) in buffer. With the use of the thermal melting program, the temperature of the cell containing the cuvette was ramped from 40 to 110^0 C. The absorbance at 260 nm was monitored by every 1^0 C for solutions of CT-DNA (80 µM) in the absence and presence of the title complex at different concentrations. The melting temperature T_m, which is defined as the temperature where half of the total base pairs is unbounded, was determined from the midpoint of the melting curves. ΔT_m values were calculated by subtracting T_m of the DNA alone from that of DNA–complex adduct.

Cyclic voltammetry measurements were made on Princeton EG&G-PARC model potentiostate. The supporting electrolyte was 50 mM NaCl /10 mM Tris HCl buffer (pH = 7.2). A standard three-electrode system was used containing a glassy carbon as working electrode, platinum-wire auxillary electrode and a saturated calomel reference electrode (SCE). Solutions were deoxygenated by purging with N_2 prior to measurements.

Ethidium bromide emits intense fluorescence in the presence of DNA/RNA due to its strong intercalation between the adjacent DNA/RNA base pairs. It has been previously reported that this fluorescence can be quenched by the addition of a second molecule.[29] The extent of fluorescence quenching of EB bound to nucleic acids can be used to determine the extent of binding between the second molecule and nucleic acids. This competitive binding experiments were used to find out the extend of

binding of the surfactant copper(II) complex of the present study with nucleic acids. The fluorescence spectra of EB were measured using an excitation wavelength of 520nm and the emission range was set between 550 and 750 nm.

Viscosity experiments were carried out on a Ubbelodhe viscometer, immersed in a thermostatic water bath maintained at 30 ± 0.1^{0}C. Flow time was recorded three times for each sample, and an average flow time was calculated. Data were presented as $(\eta/\eta_0)^{1/3}$ versus the ratio of the concentration of the complex to nucleic acid, where η is the viscosity of nucleic acid in the presence of complex, and η_0 is the viscosity of nucleic acid alone. Viscosity values were calculated from the observed flow time of nucleic acid containing solutions (t > 100 s) corrected for the flow time of the buffer alone (t_0), $\eta = (t - t_0) / t_0$.[30]

2.6. Cytotoxicity assay

The cytotoxicity of the surfactant copper(II) complex was measured in the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay as described earlier.[31] The complex was first dissolved quantitatively in dimethyl sulfoxide (DMSO, Sigma, USA) to make the stock solution. Briefly, cells were seeded at a density of 5 x 10^4 HepG2 liver cancer cells /well into 96-well plates. After 24 h, the cells were treated with surfactant copper(II) complex at various concentrations (10, 30, 60, 90 µg/ml) and incubated for 24 and 48 hours as indicated. At the end of the incubation, 10µl of 3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide (MTT) (5 mg/ml) per well was added and incubated in dark at 37°C for 4 hours. The formazan crystals formed after 4 hours were solubilized in 100µl of DMSO after aspirating the medium. The absorbance was monitored at 570 nm (measurement) and 630 nm using a 96 well plate reader (Bio-Rad, Hercules, CA, USA). The IC_{50} value was defined as the concentration of compound that produced a 50% reduction of cell viability.

2.6.1. Evaluation of apoptosis (Acridine orange and ethidium bromide staining)

Acridine orange and ethidium bromide staining was performed as described by Spector *et al.*[32] Twenty-five microliters of cell suspension of each sample (both attached, released by trypsinization, and floating), containing 5 × 10^5 cells, was treated with AO and EB solution (one part of 100 mg/mL AO and one part of 100 mg/mL EO in PBS) and examined under a fluorescent microscope (Carl Zeiss, Germany) using an UV filter (450-490 nm). Three hundred cells per sample were counted in tetraplicates for each dose point. Cells were scored as viable, apoptotic or necrotic as judged by the staining, nuclear morphology and membrane integrity, and percentages of apoptotic and necrotic cells were then calculated. Morphological changes were also observed and photographed.

2.6.2. Dye preparation and drug preparation

The amount of 200 µL of dye mixture (100 µL/mg AO and 100 µL/mg EB in distilled water) was mixed with 2 mL cell suspension (30,000 cells/mL) in 6-well plate. The suspension was immediately examined and viewed under Olympus inverted fluorescence microscope (Ti-Eclipse) at 200× and 400 x magnification. We observed untreated cells as controls and cells treated with testing material IC_{50} concentrations for 24 h of exposure.

2.6.3. Drug treatments

HepG2 were seeded in a 24-well plate (50,000 cells per well). After 24 h of cells incubation, the medium was replaced with 100 µL medium containing IC_{50} dose of testing material. Untreated cells served as the control. After 24 h, aspirate the media and treat with prepared dye and observe under the fluorescent microscope.

2.6.4. Hoechst 33342 staining

This procedure is very sensitive to cell concentration and pH of the media. Cells should be approximately 1- 2 x 10^6 ml, in buffered media, pH 7.2. It is also helpful to include 2% fetal calf serum to maintain the cells.

Drug was added and incubated for 24 and 48 hours. Homogenously aspirated and spent media was removed and 1 ml of saline was added and centrifuged at 1500 rpm for 10 mins. The cells were stained with 0.5mL of Hoechst 33342 solution (3.5 μg/mL in PBS) and incubated for 30min at 37^0C incubator. After 30 min the Hoechst 33342 solution was discarded and the cells observed at 490-520nm of fluorescent microscope. Time is a critical factor due to the transport of the dye. Typically, 30 minutes is a minimum, but it is important to remember that the signal may begin to degrade after ~120 minutes. It is recommended that the staining kinetics be empirically defined. Analyze apoptosis under fluorescent microscope after incubation. Washing is not recommended.

3. Results and discussion

3.1. Characterization

The surfactant copper(II) complex synthesized in the present study was characterized by UV-Visible, IR, and EPR spectral techniques. The purity of the complex was checked by microanalyses (C, H and N) and the results were found to be in good agreement with the calculated values (Found: C, 56.59%; H, 4.57%; N, 12.27% $C_{48}H_{47}Cl_2CuN_9O_8$ calcd: C, 56.95%; H, 4.68%; N, 12.45%). The electronic absorption spectra are often very helpful in the evaluation of results furnished by other methods of structural investigation. The electronic spectral measurements were used to assign the stereochemistries of the metal ions in the complexes based on the positions and number of d-d transition peaks. The electronic absorption spectum of the complex was recorded at room temperature. The spectrum shows multiple transitions in the ultraviolet region due to intraligand transitions at 362 nm and 382 nm due to metal to ligand charge transfer transitions (MLCT). This type of MLCT band around 350 nm has been reported for copper complexes of phenanthroline.[33] Besides a broad band is observed at the lower frequency of about 648 nm in the spectrum, corresponding to the d-d transitions of copper(II) indicating square pyramidal geometry around copper(II).[34,35] Similar observations were observed by Pradeep *et al.*[35] IR region our surfactant copper(II) complex shows bands around 1586cm^{-1}, 1351cm^{-1} and 3392cm^{-1} which can be attributed to the ring stretching frequencies [γ(C=C), γ(C=N) and γ(N-H)] of the dipyrido[3,2-a:2'-3'-c]phenazine ligand whose values at the free state are 1594cm^{-1}, 1383cm^{-1} and 3483 cm^{-1} respectively. These shifts can be explained by the fact that each of the two nitrogen atoms of dipyrido[3,2-a:2'-3'-c]phenazine ligands donates a pair of electrons to the central copper metal forming a coordinate covalent bond. The other bands observed for this complex around 2920cm^{-1} and 2850 cm^{-1} can be assigned to C-H asymmetric and symmetric stretching vibrations of aliphatic CH_2 of dodecylamine. Perchlorate bands appear around 1100 and 620 belong to an ionic species means that the counter-ion is not involved in the copper-ligand coordination.[36,37] The solid state EPR spectra of our surfactant copper(II) complex were recorded in X-band frequencies at room temperature (SI Fig. 1) as well as in frozen solution (77K). The spectral features at both temperatures are quite similar. The complex exhibits well defined single isotropic features near g = 2.09 in the solid state at RT as well as at LNT (SI Fig. 2), revealing that such isotropic lines are usually the results of intermolecular spin exchange, which broaden the lines suggesting that dx^2-dy^2 is the ground state with the d^9 (Cu^{2+}) configuration and square pyramidal geometry.

3.2. Critical micelle concentration (CMC)

The CMC value of our surfactant copper(II) complex, was determined by conductivity method as reported earlier [38] at five different temperatures (303, 308, 313, 318 and 323K). Determination of the value of CMC from the conductivity measurements was carried out through a change in the slope when the specific conductivity versus surfactant concentration for surfactant solutions was plotted. These conductivity measurements (SI Fig. 3 of supplementary information) were repeated three

times and the accuracy of CMC values (SI Table 1 of supplementary information) was found to be within ± 2%. Similar to our previous reports,[18,38] the CMC value for the copper(II) surfactant complex of the present study is also very low compared to that of the simple organic surfactant, dodecylammonium chloride (CMC = 1.59 x 10^{-2} mol dm^{-3}) indicating that our surfactant metal complex has more capacity to associate into micellar aggregates. Besides the CMC value of our complex containing modified phenanthroline ligand is lower than that of the corresponding phenanthroline coordinated complex (9.75 x 10^{-5}). [38] This is expected as dppz ligand is more hydrophobic than phenanthroline ligand which facilitates higher micellization.

3.3. Thermodynamics of micelle formation

Various thermodynamic quantities like the free energy (ΔS^0_m,) the enthalpy (ΔH^0_m) and the entropy (ΔS^0_m) of micellization were obtained by using the following relationships and temperature dependence of CMC fitted equations:

$$\Delta G^0_m = RT\ln CMC \Delta H^0_m = -RTd\ln CMC/dT \Delta S^0_m = (\Delta H^0_m - \Delta G^0_m)/T$$

The standard free energy of micelle formation per mole of monomer, ΔG^0_m, is given by

$$\Delta G^0_m = RT(2-\alpha_{ave})\ln CMC, \quad (1)$$

where R, T and α_{ave} are gas constant, absolute temperature and average degree of micellar ionization, respectively.
The enthalpy and entropy of micelle formation can be obtained by applying the Gibbs–Helmholtz equation to Eq. (1)

$$\Delta H^0_m = -RT^2(2-\alpha_{ave})d\ln CMC/dT. \quad (2)$$

$$\Delta S^0_m = (\Delta H^0_m - \Delta G^0_m)/T \quad (3)$$

The thermodynamic parameters thus obtained for the surfactant copper(II) complex are shown in SI Table 1(supplementary information available). As seen from the table the Gibbs free energy of micellization is found to be negative which indicates that the micellization was spontaneous. A linear correlation between enthalpy and entropy of micellization was observed for this surfactant copper(II) complex as shown in SI Fig. 4 (Supplementary information). As the temperature is increased, the enthalpy contribution to the free energy increased whereas the entropic contribution decreased. Further ΔH^0_m of micellization is negative and ΔS^0_m of micellization is positive. Nusselder and Engberts [39] have suggested that negative ΔH^0_m values will indicate London-dispersion forces as a major force in the micelle formation. Positive values of ΔS^0_m indicate that the micellization of the surfactant complex in aqueous solution is governed mainly by hydrophobic interactions between the surfactant cations, resulting in the breakdown of the structured water surrounding the hydrophobic groups.

3.4. Binding studies with nucleic acids

3.4.1. Absorption studies

The binding of intercalated complexes to the DNA helix can be characterized through absorption spectral titration, by following the changes in absorbance and wavelength. Due to the intercalative mode involving a strong stacking interaction between the aromatic chromophore and the DNA base pairs hypochromism along with red shift can be observed. The extent of the hypochromism is commonly consistent with the strength of the intercalative interaction.[40,41] Thus, in order to provide evidence for the intercalative binding of surfactant copper(II) complex to DNA, the binding process was monitored by absorption spectroscopy by following the changes in absorption band intensity and its position. The absorption spectra of the complex in the

absence and presence of nucleic acids is shown in Figures 1 and 2. As seen from these figures it is observed that with the increase in the concentration of CT-DNA or yeast tRNA, the absorption spectrum of the surfactant copper(II) complex showed strong hypochromism (H% = 100% (A_{free} – A_{bound}) / A_{free}) in absorbance bands indicating a strong stacking interaction (intercalation) between the aromatic chromophore and the base pairs of the nucleic acid. With DNA binding, the hypochromism reaches as high as 29.16% with slight red shift, and with yeast tRNA binding the hypochromism reached as high as 38.77% and a slight red shift. Hypochromism was suggested to be due to a strong interaction between the extended aromaticity of the ligand and that of the DNA bases.[42,43]This affinity is basically consistent with data found for phenanthroline-based complexes of copper.[19] Our results may suggest that the ligand, dppz, structurally provides one aromatic moiety extending from the metal center to overlap with the DNA base pairs by intercalation. The long aliphatic chain present in the surfactant copper(II) complex enhances this intercalation with the base pairs of DNA through strong hydrophobic effect. The observed spectroscopic changes are thus, consistent with intercalation of complex into the DNA base stacks. From the absorption titration data, the binding constant (K_b) was determined using the following equation(Eq. 4), [28]

$$[NA]/ (\varepsilon_a-\varepsilon_f) = [NA]/(\varepsilon_b-\varepsilon_f)+1/K_b(\varepsilon_b-\varepsilon_f) \quad (4)$$

where, [NA] is the concentration of nucleic acid expressed in base pairs ; ε_a, ε_f and ε_b are the apparent, free and fully bound copper(II) complex extinction coefficients. In plots of [NA] / (ε_a-ε_f) versus [NA], K_b is given by the ratio of the slope to intercept. The K_b values thus obtained for surfactant copper(II) complex with CT-DNA and yeast tRNA are given in Table 1. The large hypochromism and K_b values observed in the electronic absorption titration experiments of the surfactant copper(II) complex with yeast tRNA binding compared to CT-DNA indicate a strong stacking interaction between the aromatic chromophore (dppz) and the base pairs of RNA. Besides this binding constant of the surfactant complex of the present study is higher than that of the surfactant complexes containing bipyridine, phenanthroline and dpq ligands reported by us earlier.[18,19] Because compared to these ligands dppz ligand can provide more aromaticity extending from the metal center to overlap with the DNA base pairs by intercalation. Also the K_b of our surfactant copper(II) complex is very much higher than that for the ordinary copper(II)/cobalt(III) complexes, like $[Cu(dppz)_2(Cl)]Cl$,[27] $[Co(bpy)_3]^{3+}$ (K_b, 9.3×10^3 M^{-1}) [44] $[Co(bpy)_2(imp)]^{3+}$ (K_b, 1.1×10^4 M^{-1}).[45] This indicates that the long aliphatic chain amine ligand present in the surfactant copper(II) complex play a definite role in the intercalative binding between the surfactant copper(II) complex and nucleic acids through their hydrophobic effect.

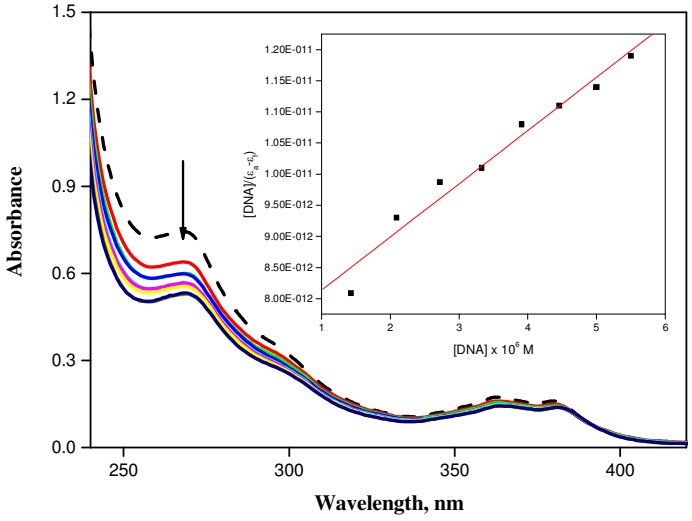


Figure 1. Absorption spectra of surfactant complex in the absence (dotted lines) and in the presence of increasing amounts of CT DNA (solid lines), [Complex] = 3.0 × 10^{-5} M, [DNA] = 0–5.5 × 10^{-6} M. Arrow shows the absorbance changes upon increasing DNA concentrations. Inset: plot of [DNA]/ ($\varepsilon_a - \varepsilon_f$) versus [DNA].

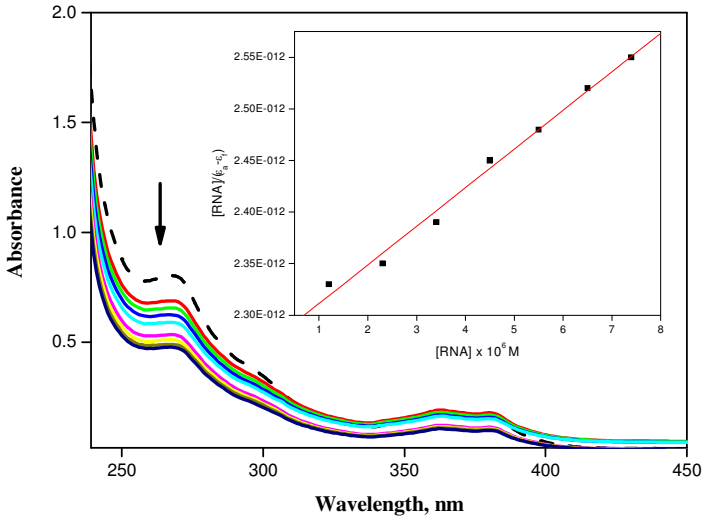


Figure 2: Absorption spectra of complex in the absence (dotted lines) and in the presence of increasing amounts of RNA (solid lines), [Complex] = 3.0 × 10^{-5} M, [RNA] = 0–7.4 × 10^{-6} M. Arrow shows the absorbance changes upon increasing RNA concentrations. Inset: plot of [RNA]/ ($\varepsilon_a - \varepsilon_f$) versus [RNA].

Table 1. The binding constant (K_b) and K_{SV} of $[Cu(dppz)_2DA](ClO_4)_2$ with Nucleic acid using tris buffer.

Complex	K_b (M^{-1})		% Hypochromism		K_{sv} M^{-1}	
	DNA	RNA	DNA	RNA	DNA	RNA
$[Cu(dppz)_2DA](ClO_4)_2$	1.1 X 10^6± 0.17	1.6 X 10^6± 0.26	29.16	38.77	7.88 x 10^3	1.13 x 10^4

3.4.2. Thermal denaturation studies

The DNA melting experiments are useful in establishing the extent of intercalation, because the intercalation of the complex into DNA base pairs causes stabilization of base stacking and therefore raises the melting temperature of the double-

stranded DNA.[46] It is well-accepted that: when the temperature in the solution increases, the double-stranded DNA gradually dissociates to single strands, generating a hyperchromic effect in the absorption spectra of the DNA bases (λ_{max} = 260 nm). So the transition temperature of double strands to single strands can be determined by monitoring the absorbance of the DNA bases at 260 nm as a function of temperature.[47] Literature reports,[48] show that the intercalation of the complexes into DNA generally result in a considerable increase of Tm. The melting curves of CT-DNA in the absence and presence of $[Cu(dppz)_2DA](ClO_4)_2$ are presented in SI Fig. 4 (Supplementary information). A melting temperature (T^0_m) of CT-DNA in buffer was determined as 74.0 ± 0.2^0 C under our experimental conditions. The DNA intrinsic binding constant of the title complex at T_m was calculated by using McGhee's equation (Eq. (5)),[49,2] where T^0_m is the melting temperature of CT-DNA alone, T_m is the melting temperature in the presence of the Cu(II) complex, ΔH_m is the enthalpy of DNA (per base pair), R is the gas constant, K is the DNA-binding constant at T_m, L is the free complex concentration (approximate by the total complex concentration) at T_m, and n is the size of the binding site. For the CT-DNA used in these studies, under identical solution conditions, a melting enthalpy of 6.9 kcal mol^{-1} was determined by differential scanning calorimetry.[2] On the basis of the neighbor-exclusion principle, the value of n for the title complex was assumed to be 2.0 bp.[50]

$$1/T^0_m - 1/T_m = (R/\Delta H_m)\,[\ln(1+KL)]^{1/n} \quad (5)$$

By substitution of the required parameters into Eq. (5), K was determined to be 6.68 x 10^5 M^{-1} for the title complex at 85^0C.

3.4.3. Thermodynamic parameters

Only few thermodynamic parameters such as free energy, enthalpy and entropy changes upon binding of metal complexes to DNA have been measured, although there have been many reports on the interaction of metal complexes with DNA. In fact, the thermodynamic parameter of DNA-complex formation is essential for a thorough understanding of driving forces of the binding of metal complexes to DNA.[51] The change of standard enthalpy was determined according to the van't Hoff's equation (Eq. (6)). The changes of standard free energy and standard entropy of the binding of the title complex to DNA were determined according to Eqs. (7) and (8), where K_1 and K_2 are the DNA-binding constants of the complex at the temperatures T_1 and T_2, respectively. ΔG^0_T, ΔH^0, and ΔS^0 are the changes of standard free energy, standard enthalpy, and standard entropy of the binding of the title complex to CT-DNA, respectively.

$$\ln(K_1/K_2) = \Delta H^0/R\,(T_1\text{-}T_2/T_1T_2) \quad (6)$$

$$\Delta G^0_T = -RT \ln K \quad (7)$$

$$\Delta G^0_T = \Delta H^0 - T\Delta S^0 \quad (8)$$

The value of ΔH^0 is derived to be -82.8 k J mol^{-1} by substituting K_1 = 1.17 x 10^6 M^{-1} (T_1 = 298K) and K_2 = 6.68 x 10^5 M^{-1} (T_2 = 358K) into Eq. (6). By substituting K_1 = 1.17 x 10^6 M^{-1}(T_1 = 298K) and ΔH^0 = -82.8 k J mol^{-1} into Eqs. (7) and (8), $\Delta G^0_{298\,K}$ = -34.6 k J mol^{-1} and ΔS^0 = -16.2 J mol^{-1} K^{-1} at 25^0 C were derived.

3.4.4. Binding mode between surfactant copper(II) complex and CT DNA

It is clearly observed from the experimental results that the complex formation for all the cases was spontaneous with negative ΔG^0 values. The negative binding free energy suggests that the energy of the complex–DNA adduct is lower than the sum of the energies of the free complex and DNA, and the binding of the title complex to CT-DNA is favorable at room

temperature. The negative enthalpy means that the binding at 25^0 C is exothermic and thus it is driven by enthalpy. The negative entropy change implies that the degree of freedom of the title complex and DNA conformation is reduced upon complex–DNA binding. According to the thermodynamic data, interpreted as follows, the model of interaction between a drug and biomolecule can be [52]: (1) ΔH<0 and ΔS<0, hydrophobic forces; (2) ΔH>0 and ΔS>0, van der Waals interactions and hydrogen bonds; (3) ΔH>0 and ΔS<0, electrostatic interactions.[53] In order to elucidate the interaction of our complex with DNA, the thermodynamic parameters were calculated. When we apply this analysis to the binding of the complex with CT-DNA, we find that ΔH < 0 and ΔS < 0. Therefore, intercalations via hydrophobic interactions are probably the main forces in the binding of the investigated complex to CT-DNA. These results provide an additional support that the complex $[Cu(dppz)_2DA](ClO_4)_2$ undoubtedly interacts with CT-DNA in an intercalation mode.

3.4.5. Competitive binding studies

No luminescence is observed for the surfactant copper(II) complex in any solvent or even in the presence of nucleic acid. So the competitive binding experiments using surfactant complex as quencher may afford further information for study the binding of complex to nucleic acids. Ethidium bromide (EB) is known to emit intense fluorescence in the presence of nucleic acids due to its strong intercalation between the base pairs of nucleic acids. It has been reported that the enhanced fluorescence can be quenched, at least partially by the addition of a second molecule.[54] The extent of quenching of fluorescence of nucleic acids-bound EB can be used to determine the extent of binding between the second molecule and nucleic acids. The emission spectra of nucleic acids-bound EB in the absence and the presence of complex are shown in Figures 3 and 4. The binding was analyzed through Stern-Volmer equation, $I_0/I = 1 + K_{sv}[Q]$, where I_0 and I are the fluorescence intensities in the absence and presence of the complex, respectively, K_{sv} is the linear Stern-Volmer constant and Q is the concentration of surfactant copper(II) complex.[55] A plot of I_o/I vs. [Q] was drawn and K_{sv} was obtained from slope (Table 1). As seen from this table the K_{sv} values of our surfactant copper(II) complex are 7.88 x 10^3 with DNA and 1.13 x 10^4 with RNA suggesting that our complex binds with RNA more strongly than with DNA as observed through absorption spectral method.

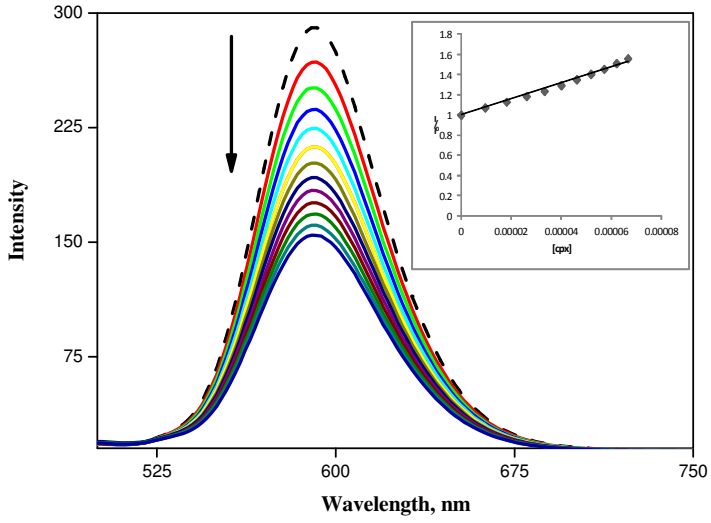


Figure 3. Emission spectra of EB bound to CT DNA in the absence (- - -) and in the presence (—) of complex [EB] = 2×10-5 M, [DNA] = 1×10-4 M, [Complex] = 0-7.04 × 10-5 Arrow shows intensity changes upon increasing the concentration of the complex.

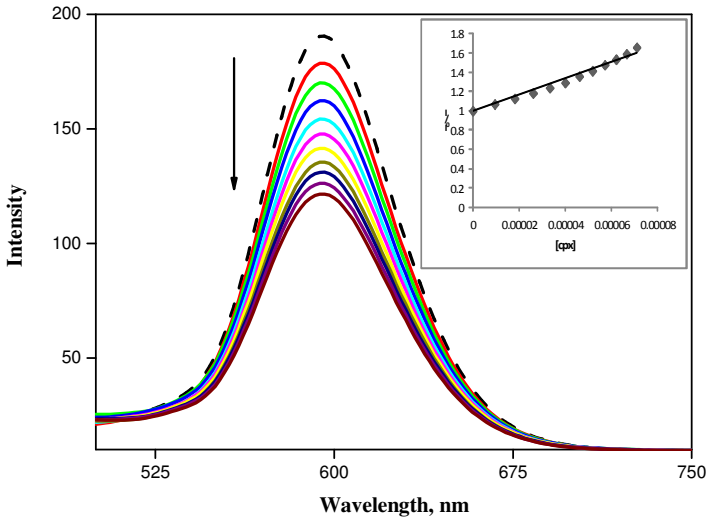


Figure 4. Emission spectra of EB bound to RNA in the absence (- - -) and in the presence (—) of complex [EB] = 2×10-5 M, [RNA] = 1×10-4 M, [Complex] = 0-7.43 × 10-5 Arrow shows intensity changes upon increasing the concentration of the complex.

3.4.6. Electrochemical studies

The change in the electrochemical properties on binding of surfactant copper(II) complex with nucleic acids were studied by cyclic voltammetry in tris buffer (containing 5mM Tris-HCl / 50 mm NaCl at pH 7.0.) solution. Based on the shift of the formal potentials in the cyclic voltammograms the relative binding modes of the metal complexes with DNA can be determined.[46] The cyclic voltammogram (CV) of our complex exhibits one redox couple in the potential range, +1V to –1.5V. The typical cyclic voltammograms (CV) of surfactant copper(II) complex in the absence and presence of nucleic acids are shown in SI Figures 5 and 6 (Supplementary informations) and the peak potentials are provided in the Tables 2 and 3. When DNA is added to a solution of complex both the anodic and cathodic peak current heights of the complex increased in the same manner of increasing additions of RNA. Also during nucleic acids addition the anodic peak potential (E_{pa}), cathodic peak potential (E_{pc}), and $E_{1/2}$ (calculated as the average of E_{pc} and E_{pa}) all showed positive shifts. These positive shifts are considered as evidences for intercalation of the complex into the nucleic acids, because this kind of interaction is due to hydrophobic interaction. Therefore, the positive shift in the CV peak potentials of complex is indicative of intercalative binding mode of the complex with nucleic acids.[47]

Table 2. Electrochemical parameters for the interaction of RNA with $[Cu(dppz)_2DA](ClO_4)_2$

Surfactant copper(II) complex	E_{pc}	E_{pa}	ΔE_p	$E_{1/2}$	I_{pa}/I_{pc}
$[Cu(dppz)_2DA](ClO_4)_2$	-0.78	0.13	4.88	-0.31	0.22
$[Cu(dppz)_2DA](ClO_4)_2$ + CT DNA	-0.64	-0.04	0.91	-0.32	0.41

Table 3. Electrochemical parameters for the interaction of DNA with $[Cu(dppz)_2DA](ClO_4)_2$

Surfactant copper(II) complex	E_{pc}	E_{pa}	ΔE_p	$E_{1/2}$	I_{pa}/I_{pc}
$[Cu(dppz)_2DA](ClO_4)_2$	-0.69	0.09	0.78	-0.49	0.20
$[Cu(dppz)_2DA](ClO_4)_2$ + RNA	-0.57	-0.06	0.63	-0.25	0.28

3.4.7. Viscosity studies

Viscosity measurements were carried out for further clarifying the CT DNA binding nature of the complex. According to the classical intercalation concept put forward by Lerman [48], the presence of the intercalation bond between a drug and the base pairs of DNA forces these base pairs away from each other and therefore, unwinding the double helix and lengthening a given amount of DNA which in turn, increases the viscosity of the DNA solution. In contrast, groove binding or electrostatic interactions typically cause less pronounced (positive or negative) or no change in the DNA solution viscosity.[45,2] In the absence of crystallographic structural data, these hydrodynamic methods are the suitable methods to support an intercalative binding model. The effects of surfactant copper(II) complex on the viscosity of nucleic acid solution is given in SI Fig. 7 (Supplementary information). The results showed that the presence of the surfactant copper(II) complex increased the relative viscosity of nucleic acids solutions, indicating that the complex interacted with nucleic acid by an intercalative mode, which is in good agreement

with the above findings obtained by electronic absorption, thermal denaturation, fluorescence binding and cyclic voltammetry methods.

3.5. Cytotoxity Studies

3.5.1. MTT assay

The cytotoxicity of the effects of the surfactant copper(II) complex on cultured HepG2 liver cancer cells by exposing cells for 24 and 48 h to the medium containing the complex at 90µg/mL concentration. In vitro antitumor activity of this complex was determined according to the percentage of nonviable cells (%NVC) which was calculated by the following equation:

NVC% = [number of NVC/total number of cells] x 100

The increasing concentration of surfactant copper(II) complex was accompanied by progressive decrease in the VC%. This is due to the fact that by increasing the concentration of cationic surfactant complex the adsorption of ions on cell membranes increases, leading to increase in penetration and antitumor activity.

The inhibition of cell viability percent showed that the surfactant copper(II) complex is the most active one at a concentration of 90 µg/ml, the VC % reaching up to 7.3 (± 0.11)%. This means that the drug at this concentration causes the death of most of the tumor cells. For 24 h treatment period, higher concentrations of the complex were required to kill the cells whereas for 48 h treatment the cell killing occurred at lower concentrations. The results of the cytotoxic activity on human tumor cell lines was determined according to the dose values of drug exposure required to reduce survival in the cell lines to 50% (IC_{50}). The IC_{50} value of the complex was slightly higher for the 24 h treatment groups, i.e., in the range of 7.3 (± 0.11)-90µg/mL, whereas for the 48 h treatment groups the IC_{50} value fell in the range of 5.9 (± 0.19)-90µg/mL. It should be noted that the action of the complex as antitumor agents is found to be dependent on the type of tumor cell line tested but, as shown from the results, surfactant copper(II) complex show good cytotoxic activity against tumor cell lines and, at very low concentrations, reduces the survival to 50%. This is due to the fact that copper(II) complex has a capacity to reduce the energy status in tumors as well as to enhance tumor hypoxia, which also influences their antitumor activities. It is known that phenanthroline-containing metal complexes have a wide range of biological activities such as antitumor, antifungal, apoptosis [56] and interaction with DNA inhibiting replication, transcription and other nuclear functions and arresting cancer cell proliferation so as to arrest tumor growth. In general, the high selectivity of action by copper(II) complex upon tumors is due to their specific reactivity.[57] From these results, surfactant copper(II) complex seems to offer promise due to the high electron affinity of the metal (which increases its ability to bind DNA) and the ready reducibility of the compounds.[58]

3.5.2. Apoptosis studies (AO and EB staining)

AO/EB staining adopting fluorescence microscopy also revealed apoptosis from the perspective of fluorescence. After HepG2 liver cancer cells were exposed to the concentrations of surfactant copper(II) complex for 24 h. In this study, we used acridine orange/ethidium bromide (AO/EB) double staining assay.[59] Acridine orange is taken up by both viable and nonviable cells and emits green fluorescence if interrelated into double stranded nucleic acid (DNA) or red fluorescence if bound to single stranded nucleic acid (RNA). Ethidium bromide is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA. We distinguished four types of cells according to the fluorescence emission and the morphological aspect of chromatin condensation in the stained nuclei: (1) viable cells showing light green fluorescing nuclei with highly organized structure; (2) early apoptotic cells having bright green fluorescing nuclei with chromatin condensation and nuclear fragments; (3)

late apoptotic cells having orange to red fluorescing nuclei with condensed or fragmented chromatin; and (4) necrotic cells having red fluorescing without chromatin fragmentation. Viable cells have uniform bright green nuclei with organized structure. Apoptotic cells have orange to red nuclei with condensed or fragmented chromatin. Necrotic cells have a uniformly orange to red nuclei with condensed structure (Fig. 5). Our results indicate that surfactant copper(II) complex induced apoptosis at the concentrations evaluated, in agreement with the cytotoxic results. The results suggest that the complex treatment caused more cells to take to death in HepG2 liver cancer cells.

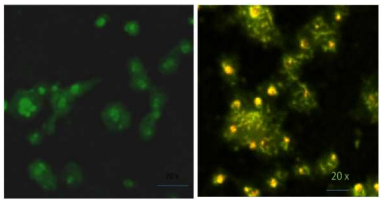
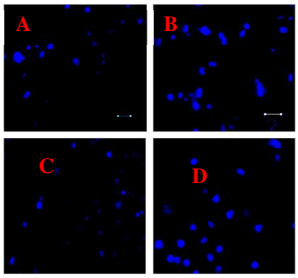


Figure 5. Photomicrographs of control and AO and EB stained HepG2 liver cancer cells incubated for 24 hours with surfactant copper(II) complex. A. Untreated control cells. B. surfactant copper(II) complex treated control cells (90 µg/mL). ; Viable (light green), early apoptotic (bright green fluorescing), late apoptosis (red to orange fluorescing) and necrosis (red fluorescing) cells were observed. Magnification at 200x.

3.5.3. Apoptosis Detection Hoechst 33342 DNA Staining

It is possible to perform apoptosis detection assay with Hoechst 33342 (Sigma B-2262), but the increase in fluorescence seen in the apoptotic cells may be less dramatic. Hoechst dyes can also be obtained from Molecular Probes. H342 is a "vital" DNA stain that binds preferentially to A-T base-pairs. The cells require no permeabilization for labeling, but do require physiologic conditions since the dye internalization is an active transport process. This condition typically varies among cell types (Stander et al., 2009). The procedure for Staining and analysis of cells using Hoechst 33342 (H342): To investigate if HepG2 liver cancer cells were triggered to undergo apoptosis due to the exposure of surfactant copper(II) complex, morphological changes of apoptosis was performed in the treated cells by Hoechst 33342 staining. Apoptosis is one of the major pathways that lead to the process of cell death. After the cells were treated with IC_{50} concentrations of surfactant copper(II) complex (90 µg/mL) for 24 and 48 h the cells were observed for cytological changes adopting Hoechst 33342 staining. The observations revealed that the complex brought about cytological changes such as chromatin fragmentation, binucleation, cytoplasmic vacuolation, nuclear swelling, cytoplasmic blabbing and late apoptosis indication of dot-like chromatin and condensation (Fig. 6) whereas untreated cells did not show such changes. Data collected from the manual counting of cells with normal and abnormal nuclear features. Both apoptotic and necrotic cells increased in dose-dependent manner. These data clearly indicated that higher doses of surfactant copper(II) complex resulted in remarkable chromatin condensation and nuclear fragmentation in HepG2 liver cancer cells.

24 hour



48 hour

Figure 6. Surfactant copper(II) complex induces apoptosis in HepG2 liver cancer cells. Representative fluorescent micrographs of HepG2 liver cancer cells stained with Hoechst 33342 fluorescent dye after the complex exposure for 24 and 48 hours. A, C Untreated control cells; B, D surfactant copper(II) complex treated control cells (90 µg/mL). A, B – 24 hours; C, D - 48 hours. Magnifications at 200X

4. Conclusions

In this work, we have reported the synthesis, characterization and cmc value of a new surfactant copper(II) complex containing a substituted phenanthroline ligand with extended aromaticity and its binding with DNA and RNA. The critical micelle concentration value of this surfactant copper(II) complex is very low and the complex has more capacity to associate forming aggregates, compared to those of ordinary synthetic organic surfactants. The interaction of this complex with nucleic acids has been studied with electronic spectroscopy revealing its ability to bind to nucleic acid. Competitive binding study with EB has also been performed by fluorescence spectroscopy. All these experiments show that the interaction between the nucleic acids and the surfactant copper(II) complex took place via the intercalative mode. Viscosity measurements of nucleic acids solutions in presence of our complex have confirmed that this intercalation as the most possible binding mode. Cyclic voltammetric studies have also established the intercalating binding nature of our surfactant copper(II) complex to nucleic acids. This intercalation is due to the presence of more extended aromaticity of dppz ligand and besides the presence of long aliphatic chain in the complex enhanced this intercalation. The complex also shows anticancer properties on liver carcinoma human tumour cell lines.

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