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RESEARCH ARTICLE

Surfactant Complex Binding to DNA Interaction Study: Controlling Hydrophobicity in β-Cyclodextrin–DNA Binding Reactions

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Abstract: The interaction of cis-$[Co(phen)_2(TA)_2](ClO_4)_3$, a cationic surfactant complex (phen = 1-10 phenanthroline, TA= Tetradecylamine), with calf thymus DNA has been studied by physici-chemical techniques. The spectroscopic studies together with cyclic voltammetry and viscosity experiments support that the surfactant-cobalt(III) complex binds to calf thymus DNA (CT DNA) by intercalation through the aliphatic chain present in the complex into the base pairs of DNA. The presence of phenanthroline ligand with larger π-frame work may also enhance intercalation. Besides the effect of binding of surfactant cobalt(III) complex to DNA in presence of β-cyclodextrin has also studied. In presence of β-cyclodextrin the binding occur through surface and (or) groove binding. The complex was investigated as one of the potential selective anticancer prodrugs. The complex was tested in vitro on human monolayer tumour cell lines: HepG2 (Human hepatocellular liver carcinoma) also.
Keywords: Surfactant cobalt(III) complex; DNA binding; Intercalation; electrostatic modes; 1,10-Phenanthroline; Hydrophobic interaction; β-cyclodextrin; Tetradecylamine; Calf thymus DNA.

1. INTRODUCTION

Deoxyribonucleic acid plays an important role in the life process, because it bears heritage information and instructs the biological synthesis of proteins and enzymes through the replication and transcription of genetic information in living cells. DNA is a particularly good target for metal complexes as it offers a wide variety of potential metal binding sites (S. Chan *et al.* 1995, G.J. Prativel *et al.* 1998). Such sites include the electron rich DNA bases or phosphate groups that are available for direct covalent coordination to the metal center. There are non-covalent binding modes as well, such as hydrogen bonding and electrostatic binding to grooved regions of the DNA and intercalation of planar aromatic ligands into the stacked base pairs. The interaction of transition metal complexes with DNA has been extensively studied in the past decades. Due to unusual binding properties and general photoactivity, these coordination compounds are suitable candidates as DNA secondary structure probes, photo cleavers and antitumor drugs (F. Liang *et al.* 1901). Transition metal complexes can interact non-covalently with DNA by intercalation, groove binding or external electrostatic binding. Many useful applications of these complexes require that the complex binds to DNA through an intercalative mode. However, the majority of such studies have been focused on complexes of ruthenium, and to a far lesser extent, on other metal complexes. Much attention has been paid to the complexes containing symmetric aromatic ligands such as 1, 10-phenanthroline and its derivatives. Some of these complexes exhibit interesting properties upon binding to DNA (R.J. Morgan *et al.* 1991, X.H. Zou *et al.* 2000, H. Chao *et al.* 2002). Aromatic ring stacking between nucleobases is considered to be a major driving force for these bindings, which depends on the size and electron density of the interacting aromatic rings, as well as on the combined effect of hydrophobic and hydrophilic interactions.

Studies of complex formation between DNA and a cationic amphiphilic molecule have implications for gene therapy, a conceptually new approach for the treatment of human disease, (J.O. Radler *et al.* 1997) which is rapidly progressing from basic research clinical application (W.F. Anderson. 1998). In gene-therapy, DNA-cationic surfactant complexes are very useful. Due to the polyanionic nature of DNA, its diffusion through the eukaryotic cell is difficult. To remove such difficulties, various synthetic gene transfer vectors, e.g. cationic lipids and related surfactants, have been recently developed (T. Akao *et al.* 1996, D.L. Reimer, *et al.* 1995, N. Dan. 1997). Properties such as structure, thermodynamics, and morphology of cationic lipid-DNA complexes have been widely investigated by a variety of methods (P.C.A. Barreleiro *et al.* 2000, S.M. Mel'nikov *et al.* 1995, S.M. Mel'nikov *et al.* 1995, D.D. Lasic *et al.* 1995, D. Matulis. 1992). These studies show that variations in length, degree of unsaturation, flexibility, and chemical structure of the lipid chain and the nature of the counter ion can have large effects on the interaction process and transfection efficiency. A change or replacement of the surfactant with another amphiphile having different molecular structural features may bring back high transfection efficiency [R. Leventis *et al.* 1990]. The elucidation of the optimal transfection recipe for any given cell line therefore appears to require a prior knowledge of the surfactant. This in turn necessitates clear understanding of the nature of interaction between various surfactants and DNA. Many surfactants used for this purpose by them also show interesting properties. For example, double chain cationic surfactant such as dimethyldialkyalammonium bromide shows interesting immuno adjuvant properties (M.D. Prager *et al.* 1980, C.L. Huyck. 1944, F.M. Menger *et al.* 1997, F.M. Menger *et al.* 2000). Cetylpyridinium chloride is another single chain cationic surfactant with well-known antibacterial properties (R. Zana. 2002, T. Zana. 2002). Several surfactants also find utility in the purification of DNA through precipitation [J. Hamaguchi *et al.* 1962, S. Z. Bathaie *et al.* 1999, Y. F. Sasaki *et al.* 1994). Recently, cationic surfactants are shown to induce coil to globular transition of DNA and this has been demonstrated by using fluorescence

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microscopy (D. Matulis *et al.* 2000). Modeling of electrostatic, hydrophobic, and hydration forces in such interaction has also been widely studied (V.M., Meidan *et al.* 2000).

A variety of mixed ligand cobalt(III) complexes having phenanthroline ligand and its derivatives serve as bioinorganic model compounds (M.J. Waring. 1997). They exhibit antiviral activities by their interaction with nucleic acid templates and inhibit proviral DNA synthesis. Ji et al. have synthesized some novel cis-$[Co(phen)_2(pdtb)]^{3+}$ complexes, and their binding properties to calf thymus DNA indicating that the size and shape of the intercalated ligand pdtb had a marked effect on the binding affinity of the complexes to DNA (Y. Fang *et al.* 1977). The complexes may approach closely to and intercalate into the base pairs of DNA, when the size, shape and chirality of the complexes are fit to the DNA structure. We have been interested in the synthesis and reactions involving surfactant-cobalt(III) complexes which contain one or two ligands with long aliphatic chains (R. Senthil kumar *et al.* 2008, R. Senthil kumar *et al.* 2009, K. Sasikala *et al.* 2010). Studies on the chemistry of transition metal complex surfactants have received a sustained high level of attention due to their relevance in various redox processes in biological systems; they are considered to be promising agents for the development of new antitumor drugs. These surfactant metal complexes are a very special type of surfactants where the metal complex part with coordinated ligands acts as the head group and the tail and head parts are connected through coordinate bond. Like any other organic surfactant these surfactant-metal complexes can also form micelles above their CMC values. In this work, an attempt has been made to determine the binding affinity of one of the such surfactant cobalt(III) complex, containing two long aliphatic amines coordinated to the cobalt centre, with DNA. We have made use of electronic absorption, fluorescence, circular dichroism spectroscopy techniques as well as cyclic voltammetry and viscosity methods to probe the interaction of this surfactant-cobalt(III) complex containing hydrophobic ligands with DNA.

2. Materials and methods

2.1 Materials

Calf Thymus (CT) DNA was obtained from Sigma-Aldrich, Germany, and used as such. Cobalt(II) chloride hexahydrate and β-cyclodextrin were purchased from Merck, and 1,10-phenanthroline, Tetradecylamine and sodium perchlorate were obtained from Loba, India. The surfactant-cobalt(III) complex was synthesized as reported in the literature (K. Sasikala *et al.* 2010). A solution of calf thymus DNA in the buffer gave a ratio of UV absorbance at 260 and 280 nm of ~1.8-1.9:1, indicating that the DNA was sufficiently free from protein (J. Marmur. 1961).

2.2. Physical Measurements

The DNA concentration per nucleotide was determined by absorption spectroscopy using the known molar extinction coefficient value of 6600 mol^{-1} dm^3 cm^{-1} at 260 nm (M.D. Selm *et al.* 2007). Milli-Q water was used to prepare phosphate buffer. Absorption spectra were recorded on a Shimadzu UV-3101PC spectrophotometer, The absorption titration was performed by using a fixed cobalt(III) surfactant complex concentration to which increasing amount of the DNA solution was added. Surfactant cobalt(III)-DNA solutions were allowed to incubate for 20 min before the absorption spectra were recorded.

Emission studies were done using ethidium bromide as the emitting agent. Emission spectra were recorded on a JASCO FP 770 Spectrofluorimeter. Fluorescence quenching experiments were done by using DNA pretreated with ethidium bromide (EB) and kept for 30 minutes for incubation. The cobalt(III) surfactant metal complex was then added as a second molecule to this EB-DNA mixture and the effect on emission intensities were measured. Samples were excited at 450 nm and emission was observed between 500 and 700 nm.

All voltammetric experiments were performed in a single compartment cell with a three-electrode configuration on a EG&G PAR 273 potentiostat equipped with a personal computer. The working electrode was a glassy carbon and the reference electrode was standard calomel electrode. A platinum wire was used as the counter electrode. The supporting electrolyte was phosphate buffer at pH 7.1. Solutions were deoxygenated by purging with nitrogen gas for 15 min prior to measurements; during measurements a stream of nitrogen gas was passed over the solution

Viscosity experiments were carried out using an Ubbelodhe type viscometer maintained at a constant temperature of 25°C. Calf thymus DNA sample solutions were prepared by sonication in order to minimize complexities arising from DNA flexibility. Flow time was measured with a digital stopwatch and each sample was measured at least for three times. Data are presented as $(\eta/\eta_0)^{1/3}$ versus ratio of [Co]/[DNA] (G. Cohen *et al.* 1965), where η is the relative viscosity of DNA in the presence of the complex, and η_0 is the viscosity of DNA alone. The relative viscosity was calculated according to the relation $\eta = (t-t_0)/t_0$, where t_0 is the flow time for the buffer and t is the observed flow time for DNA in the presence and absence of the complex.

Circular dichroic experiments were recorded on a JASCO J-716 spectropolarimeter (220-320 nm) were obtained at 25°C using a quartz cell of 1cm path length. Each CD spectrum was collected after averaging over at least 4 accumulations using a scan speed of 100 nm min^{-1} and 1s response time. The region between 220nm and 320 nm was scanned for each sample. The spectra was recorded in the absence and in the presence of surfactant-cobalt(III) complex.

2.3. Cytotoxicity assay

The cytotoxicity of the surfactant cobalt(III) complex was measured in the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl tetrazolium bromide) assay as described earlier (J. Mosmann et al. 1983). 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 5×10^4 HepG-2 liver cancer cells/well into 96-well plates. After 24 h, the cells were treated with surfactant cobalt (III) complex at various concentrations (31, 62, 125, 250 µg/ml) and incubated for 24, 48 and 72 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.

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

Acridine orange and ethidium bromide staining was performed as described by Spector et al. (D.L. Spector *et al.* 1998). 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,

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nuclear morphology and membrane integrity, and percentages of apoptotic and necrotic cells were then calculated. Morphological changes were also observed and photographed.

2.3.2. 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.

2.3.3. 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.

2.3.4. Hoechst 33258 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◦C 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.

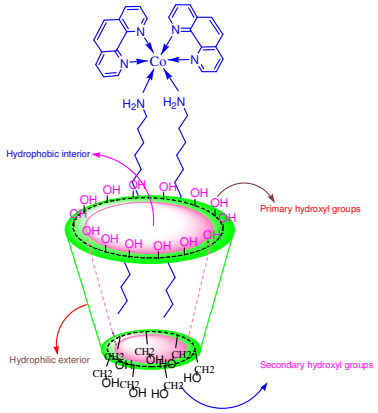
3. Results and discussion

3.1. Electronic absorption spectral studies

Electronic absorption spectroscopy is often employed to ascertain the binding of metal complexes with DNA (M.J. Waring. 1977, M.J. Waring. 1965, J.B. Chaires *et al.* 1982, C.L. liu *et al.* 1999, J. Liu *et al.* 2003). The absorption spectra of the surfactant-cobalt(III) complex of the present study in the presence of calf thymus DNA (both below and above CMC) are shown in Fig. 2A and 2B. With increase in concentration of calf thymus DNA, the absorption bands of the complex show hypochromism (41% of above and 28% of below CMC) which can be attributed to the strong stacking interaction between long aliphatic double chains present in the cobalt(III) complex and the base pairs of DNA due to the hydrophobic attraction and also presence of a π-system in phenanthroline ligand which can enhance the intercalation. The extent of the hypochromism is commonly consistent with the strength of intercalative interaction (T.M. Kelly *et al.* 1985, M. Sethuraman *et al.* 1998). Cationic metal complexes possessing planar aromatic ligands also may bind to DNA by intercalation (B. Coyle *et al.* 2003). When a monocationic surfactant like tetradecylamine is combined with DNA in phosphate buffered solution, the hydrophilic heads are attracted by the DNA through electrostatic interactions with the polyanionic scaffold (Coulomb forces), whereas the hydrophobic tails of the surfactants interact among each other through van der Waals forces (M. Cardenas *et al.* 2011, R.S. Dias *et al.* 20028).

3.2. Effect of β-cyclodextrin

β-cyclodextrin has the ability to form complex with host molecules that the complex forms when a suitable hydrophobic molecule displaces water from the cavity (A. Arslantas *et al.* 2006, S. Osinsky *et al.* 2004). The effects of presence of cyclodextrin in the medium on DNA binding of the surfactant cobalt(III) complex of the present study have also been investigated. The effect of β-cyclodextrin on the absorption spectra of the surfactant-cobalt(III) complex in the absence and the presence of calf thymus DNA (both below and above CMC) are shown in Fig. 1A and 1B. In the presence of cyclodextrin the surfactant cobalt(III) complex shows hyperchromism (33% of above and 27% of below CMC) on addition of increasing concentration of CT DNA. It is well known that in β-cyclodextrin media the long-chain aliphatic ligand of surfactant metal complex will be buried in the cavity (Scheme 1). The results suggest that in presence of cyclodextrin the intercalation of surfactant cobalt(III) complex will be difficult due to decrease of hydrophobic interaction.



Scheme 1. Surfactant chain buried into the cavity of β-Cyclodextrin

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In order to compare the binding strength of the complex with DNA, the intrinsic binding constant, K_b was determined by using the equation (S. Bhattacharya *et al.* 1977, J.M. Brown. 1998).

$[DNA]/(\varepsilon_a - \varepsilon_b) = [DNA]/(\varepsilon_0 - \varepsilon_f) + 1/K_b(\varepsilon_0 - \varepsilon_f)$

where [DNA] is the concentration of DNA base pairs, ε_a, ε_f and ε_0 correspond to A_{obsd} [Co], the extinction coefficient of the free cobalt complex and the extinction coefficient of the complex in the fully bound form, respectively, and K_b is the intrinsic binding constant. The ratio of slope to intercept in the plot of [DNA]/ (ε_a–ε_f) versus [DNA] gives the value of K_b. The intrinsic binding constant thus obtained for the surfactant cobalt(III) complex in presence and absence of β-cyclodextrin are shown in Table 1. Besides K_b values obtained for our surfactant-cobalt(III) complex inpresence of β-cyclodextrin are lower of cyclodextrin media. Besides the binding constant at below CMC values are lower than that at above the CMC values. Also the K_b of surfactant-cobalt(III) complex is very much higher than that for the ordinary metal complexes, like $[Co(bpy)_3]^{3+}$ (K_b, 9.3 $\times 10^3$ M^{-1}) (M.T. Carter *et al.* 1989), $[Co(bpy)_2(imp)]^{3+}$ (K_b, 1.1 $\times 10^4$ M^{-1}) (S. Satyanarayana *et al.* 1992), $[Co(bpy)_3(BHBMe)]^{2+}$ (K_b, 1.23 $\times 10^4$ M^{-1}), $[Co\text{-}(bpy)_2(BHBNO_2)]^{2+}$ (K_b, 2.06 $\times 10^4$ M^{-1}) (J. Olmsted et al. 1977). The trends observed in K_b values of our surfactant-cobalt(III) complex indicate that in the binding between our surfactant-cobalt(III) complexes and DNA hydrophobic effects play an important role.

3.3. Competitive binding studies

The surfactant-cobalt(III) complexes are non-fluorescent on excitation in the visible region. So the binding of surfactant-cobalt(III) complex with DNA could not be directly determined by the emission spectra. It is well known that ethidium bromide (EB) is shown to emit intense fluorescence light in the presence of DNA, due to its strong intercalation between the adjacent DNA base pairs (B.C. Baguley *et al.* 1984). It is also known that the fluorescence light could be quenched by the addition of a second molecule (J.R. Lakowicz *et al.* 1984). The quenching of fluorescence of EB binding to DNA is used to determine the extent of binding between the second molecule and DNA. So we have followed the quenching of emission DNA-bound-EB by our surfactant cobalt(III) complex. The addition of the complex to DNA pre-treated with EB causes appreciable reduction in the emission intensity. One form of classical Stern-Volmer equation (S. Bhattacharya *et al.* 1997) is,

$$Io/I = 1 + Kr$$

where I_o and I are the fluorescence intensities in the absence and the presence of complex, respectively. K is a linear Stern-Volmer quenching constant dependent on the ratio of r_{be} (the ratio of the bound concentration of EB to the concentration DNA), r is the ratio of the total concentration of complex to that of DNA. The emission quenching spectra illustrate that the quenching of EB bound to DNA by the surfactant-cobalt(III) complex is in good agreement with the linear Stern-Volmer equation, which also indicates that the complexes bind to DNA. In the plot of Io/I versus [complex]/[DNA], K is given by the ratio of the slope to intercept (Fig. 4). The K values thus obtained for surfactant-cobalt(III) complex are shown in Table 3. Also, the percentage of fluorescence intensity has been determined by plot of fluorescence intensity vs. concentration of surfactant (in mM). It is generally agreed that strong fluorescence enhancement accompanies due to a gradual release of the free EB out of the EB-DNA complex. This must be due to the surfactant induced perturbation of DNA organization leading to dissociation of the EB from the EB-DNA systems (S. Satyanarayana *et al.* 1992). It is well known that strong fluorescence enhancement accompanies intercalation of the surfactant chain of the complexes into the double helix conformation of the DNA which is facilitated by the hydrophobic interaction between long chain amines and DNA. In the presence of cyclodextrin in the medium also the binding of the surfactant cobalt(III) complex of the present study with DNA has been investigated. The effect of β-cyclodextrin on the emission spectra of the surfactant-cobalt(III) complex in the presence of calf thymus DNA is shown is Fig. 3B. The emission quenching spectra in presence of cyclodextrin (Fig. 3A) also illustrates that the value of K_{sv} less also observed through absorption studies and the values are shown in Table 2.

3.4. Viscosity Studies

Further clarification on the nature of the interactions between the surfactant cobalt(III) complex in presence and absence of β-cyclodextrin was carried out by viscosity measurements. Optical and photophysical probes provide necessary, but not sufficient clues to support a binding model. So the binding modes of the surfactant-cobalt(III) complexes were further investigated by viscosity measurements. In the absence of crystallographic structure data, hydrodynamic methods which are sensitive to DNA length increases are regarded as the least ambiguous and the most critical tests of binding in solution [S. Satyanaryana *et al.* 1993, D.H. Johnston *et al.* 1995). A classical intercalation model results in lengthens the DNA helix, as base pairs are separated to accommodate the binding ligand, leading to the increase of DNA viscosity. However, a partial and /or non-classical intercalation of ligand may bend (or Kink) DNA helix, resulting in the decrease of its effective length and, concomitantly, its viscosity (S. Satyanaryana *et al.* 1993, D.H. Johnston *et al.* 1995). The effects of the surfactant-cobalt(III) complex $[Co(phen)_2(TA)_2]^{3+}$ in the presence and absence of β-cyclodextrin and EB on the viscosity of rod-like DNA are shown in Fig. 7. The intercalator EB significantly increased the relative specific viscosity of DNA as expected for the lengthening of the DNA double helix resultant from well-characterised intercalation. In contrast, the binding of surfactant-cobalt(III) complex to DNA in the presence of β-cyclodextrin the complex slight decreased the relative specific viscosity of DNA while in the absence of cyclodextrin the complex increased the DNA viscosity. This is due to decrease of hydrophobic interaction in cyclodextrin media which decreases the intercalative formation of the surfactant cobalt(III) complex. These experiments unequivocally confirm the intercalative nature of binding of our complex with DNA.

3.5. Electrochemical Studies

Cyclic voltammetry techniques have been employed to study the interaction of the redox active surfactant-cobalt(III) complexes with DNA in order to further testing the DNA-binding modes assessed from the spectral and viscosity studies [M.T. Carter *et al.* 1987, S. Arunaguiri *et al.* 1996). The cyclic voltammogram (CV) of complex in phosphate buffer exhibit one redox couple in the potential range, +1500 to -1000 mV. On the basis of the available electrochemical data of $Co(phen)_3]^{3+}$, under our experimental conditions the observed redox couple in presence and absence of DNA voltammetric results of surfactant complex are given in Table 4 and are assigned to metal centred redox reactions (N. Maki *et al.* 1975). In the absence of DNA, the cathodic peak (Epc) around 577.5 mV can be assigned to Co^{3+}/Co^{2+} reduction and the corresponding oxidation Co^{2+}/Co^{3+} occurs in the -656.5 mV range and the separation of the anodic and cathodic peak potentials, ΔEp = -1234 mV. It indicates that ΔEp is high and the ratio of ipa/ipc is greater than one shows quasi-reversible redox process. The formal potential $E_{1/2}$, taken as the average of E_{pc} and E_{pa}, are 71.5 mV. The presence of DNA in the solution at the same concentration of complex

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caused a considerable decrease in the voltammetric current in the scan rate. This indicates that binding between surfactant complex bind to DNA takes place by intercalation due to its long hydrophobic alkyl chain into the base pairs of DNA. Therefore, the net negative charge on the electrode surface increases and also complex containing phenanthroline ligand enhances the electrostatic repulsion between the negative probe and the electrode, resulting in i_P decrease. From Fig. 5B, it is seen that the potentials shifted positively after adding DNA. Bard and co-workers (M.T. Carter *et al.* 1989) had pointed out that the electro chemical potential of small molecule would shift positively when they intercalate into DNA double helix, and if bound to DNA by electrostatic interaction, the potential would shift in a negative direction. So, the positive shift of our surfactant-cobalt(III) complex indicate that the complex bind to DNA by intercalation.

Similarly the effect of β-cyclodextrin on the electrochemical behaviour of surfactant cobalt(III) complex in presence and absence of DNA has also been studied. Fig. 5A shows that the influence of β-cyclodextrin on the cyclic voltammetric behaviours of surfactant cobalt(III) complex using phosphate buffer as the supporting electrolyte. It was observed that the electrochemical behaviour of the complex was changed greatly in cyclodextrin medium. In the presence of cyclodextrin medium the surfactant cobalt(III) complex had shown one redox couple in presence and absence of DNA. Table 3 summarizes the voltammetry results. The cathodic peak (Epc) around -1256 mV can be assigned to Co^{3+}/ Co^{2+} reduction and the corresponding oxidation Co^{2+}/ Co^{3+} occurs in the -1068 mV range. In the absence of DNA, the separation of the anodic and cathodic peak potentials, ΔEp = 188 mV and the formal potential $E_{1/2}$, taken as the average of E_{pc} and E_{pa}, are -1162 mV indicates a irreversible redox process. The presence of DNA in the solution at the same concentration of complex caused a considerable decrease in the voltammetric current with highly shifted negatively in the entire scan rate. This indicates that surfactant complex bind to DNA by strong electrostatic interaction due to inclusion of long hydrophobic alkyl chain into the cavity of cyclodextrin. Therefore, the hydrophobic interaction is completely arrested; however the complex containing phenanthroline ligand enhances the electrostatic repulsion between the negative probe and the electrode, resulting in i_P decrease. As shown in Fig. 11, it was observed that the potentials shifted negatively after adding DNA. So, the negative shift in our surfactant-cobalt(III) complex indicates that our system bind to DNA by strong electrostatic interaction in presence of β-cyclodextrin.

The drop of the voltammetric current of the metal complex in the presence of CT DNA can be attributed to diffusion of the metal complex bound to the large, slowly diffusing DNA molecule. The more pronounced decrease of the peak currents upon addition of CT DNA, in β-cyclodextrin, may indicate the binding affinity of the former to DNA is higher than that of the latter. The results parallel the spectroscopic and viscosity data of surfactant complex in the presence of DNA.

3.6. Circular Dichroism Studies

Circular dichroism studies were conducted in order to determine the extent of change in conformation of DNA upon binding of complex $[Co(phen)_2(TA)_2]^{3+}$. The B form conformation of DNA shows two conservative CD bands in the UV region, a positive band at 278 nm due to base stacking and a negative band at 246 nm due to poly nucleotide helicity as seen in Fig 6. The CD spectrum of CT DNA was monitored in the presence of increasing amounts of complex $[Co(phen)_2(TA)_2]^{3+.}$ The positive band showed increase in molar ellipticity with a red shift of the band maxima when the complex concentration was progressively increased. This increase in intensity with a red shift in positive bands suggests that surfactant cobalt(III) complex binds to DNA via intercalation due to strong hydrophobic interaction. It is also evident from the CD spectrum that binding of complexes does not lead to any significant change in the conformation of CT DNA. So these data suggest that the interaction of our surfactant complex with DNA followed the same trend as has been observed through viscosity measurements.

4. Cytotoxity Studies

4.1.MTT assay

We examined the cytotoxicity of the effects of the surfactant cobalt(III) complex on cultured HepG2 liver cancer cells by exposing cells for 24 and 48 h to the medium containing the complex at 31-250μg/mL concentration. *In vitro* antitumor activity of these compounds 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 results of these experiments are summarized in Table 5. As shown in table increasing the concentration of surfactant cobalt(III) complex was accompanied by progressive increase in the NVC %. This is due to the fact that by increasing the concentration of cationic surfactant complexes 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 cobalt(III) complex is the most active one at a concentration of 250 μg/ml, the NVC % reaching up to 90.8%. This means that the drug at this concentration causes the death of most of the tumor cells, while at concentration 125 μg/ml the percentage reached 86.5%. At a concentration of 62 μg/ml the NVC % reached 47.7%. 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 9.2-250μg/mL, whereas for the 48 h treatment groups the IC_{50} value fell in the range of 7. 1-250μg/mL (Fig. 8). 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 cobalt(III) complex show excellent cytotoxic activity against tumor cell lines and, at very low concentrations, reduces the survival to 50%. This is due to the fact that cobalt complexes have a capacity to reduce the energy status in tumors as well as to enhance tumor hypoxia, which also influences their antitumor activities. It may be also concluded that the level of cellular damage inflicted by these complex depends on the nature of their axial ligands. It is known that phenanthroline-containing metal complexes have a wide range of biological activities such as antitumor, antifungal, apoptosis (D. Baskic *et al.* 2006, B. Thati *et al.* 2007, B. Coyle *et al.* 2003, B. Coyle *et al.* 2004) 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 redox-active cobalt complexes upon tumors is due to their specific reactivity· (S. Osinsky et al. 2004). From these results, surfactant cobalt(III) 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 (J.M. Brown. 1998).

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4.2. Fluorescence Microscopic Analysis of apoptotic cell death (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 cobalt(III) complex for 24 h. In this study, we used acridine orange/ethidium bromide (AO/EB) double staining assay (D. Baskic *et al.* 2006). 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. 8A, 8B). Our results indicate that surfactant cobalt(III) 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.

4.3. 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 cobalt(III) complex, morphological changes of apoptosis was performed in the treated cells by Hoechst 33258 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 cobalt(III) complex (10-40 µg/mL) for 24 and 48 h the cells were observed for cytological changes adopting Hoechst 33258 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. 9A, 9C) whereas untreated cells did not show such changes (Fig. 9B, 9D). 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 polymer-copper(II) complex resulted in remarkable chromatin condensation and nuclear fragmentation in HepG2 liver cancer cells.

5. Conclusions

The binding of our cationic surfactant complex, cis-$[Co(phen)_2(TA)_2](ClO_4)_3$ with DNA was studied through uv-visible absorption, emission spectroscopy, viscosity and electrochemical methods both in presence and absence of β-cyclodextrin. Electronic absorption study has shown that the surfactant-cobalt(III) complex binds to CT DNA by intercalation into the base pairs of DNA via the long aliphatic chain present in the complex. The presence of phenanthroline ligand may also enhance this intercalation due to the involvement in partial insertion of the extended phen ring in between the DNA base pairs. The cyclic voltammograms and viscosity studies of the complex in the presence of CT DNA solution have also shown that the complex binds to CT DNA by the intercalative binding mode. Competitive binding studies with ethidium bromide (EB) through uv-visible emission studies indicate that the complex can displace the DNA-bound EB suggesting strong competition with DNA. In presence of β-cyclodextrin the intercalative mode of binding of the complex cis-$[Co(phen)_2(TA)_2](ClO_4)_3$ to DNA is prevented due to the incorporation of long aliphatic chain into the cavity of β-cyclodextrin. The binding strength of our complex with DNA is higher than that of most of the ordinary metal complexes. The complex also shows anticancer properties on human tumour cell lines.

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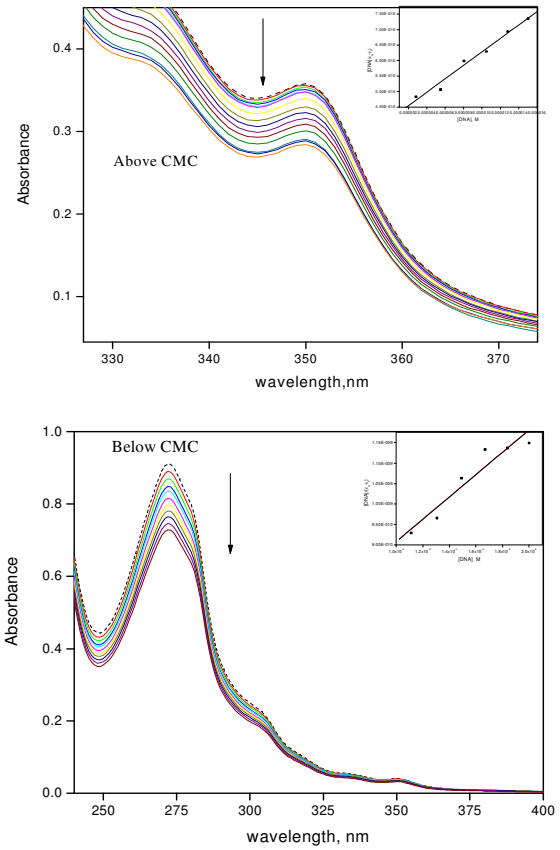

Fig. 1. Absorption spectra of complex (Presence of β-cyclodextrin) in the absence (dotted lines) and in the presence of increasing amounts of DNA (solid lines), [Complex] = 1× 10^{-5} M (below CMC), 5× 10^{-4} M (above CMC), [DNA] = 0-6.67 × 10^{-5} M. β-cyclodextrin = 1×10^{-3} M. Arrow shows the absorbance changes upon increasing DNA concentrations. Inset: plot of [DNA]/ ($\varepsilon_a - \varepsilon_f$) versus [DNA].

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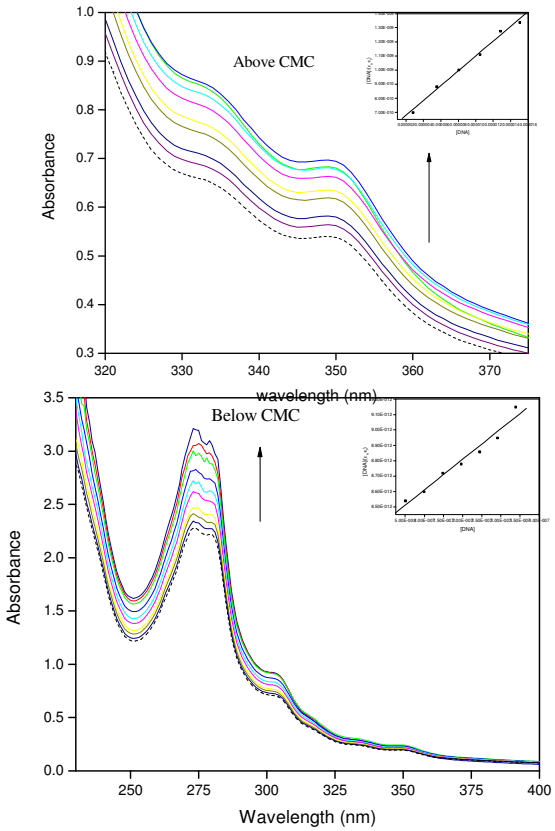

Fig. 2. Absorption spectra of complex (Absence of β-cyclodextrin) in the absence (dotted lines) and in the presence of increasing amounts of DNA (solid lines), [Complex] = 1× 10^{-5} M (below CMC), 5× 10^{-4} M (above CMC), [DNA] = 0–6.67 × 10^{-5} M. Arrow shows the absorbance changes upon increasing DNA concentrations. Inset: plot of [DNA]/ ($\varepsilon_a - \varepsilon_f$) versus [DNA].

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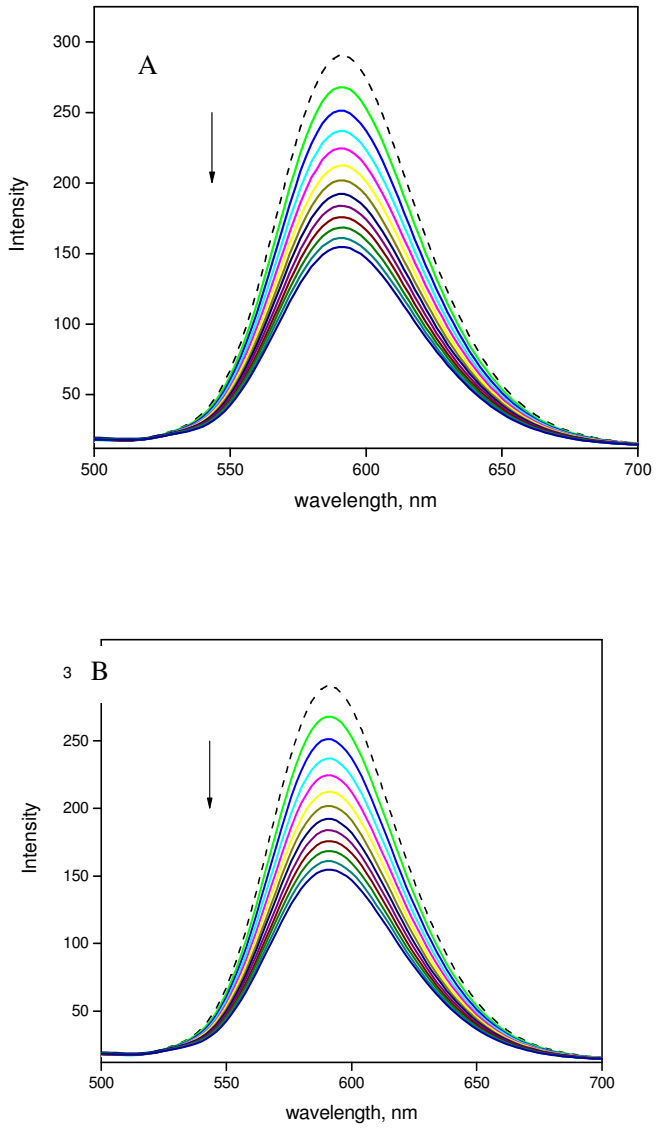

Fig. 3. Emission spectra of EB bound to DNA in the absence (- - -) and in the presence (—) of complex (A: presence of β-cyclodextrin) and (B: absence of β-CD) [EB] = 2×10^{-5} M, [DNA] = 1×10^{-4} M, [Complex] = 0-1.43×10^{-6} and β-cyclodextrin = 1×10^{-3} M. Arrow shows intensity changes upon increasing the concentration of the complexes.

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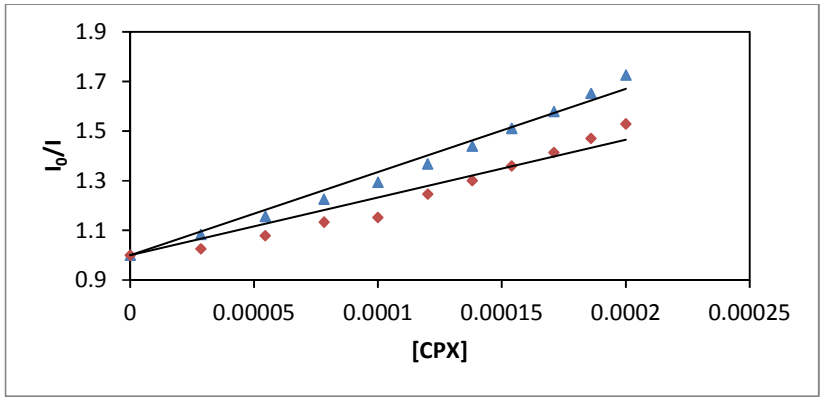

Fig. 4. Fluorescence quenching curves of EB bound to DNA by surfactant–cobalt(III) complex presence of β-CD (▲) and absence of β-CD (♦)

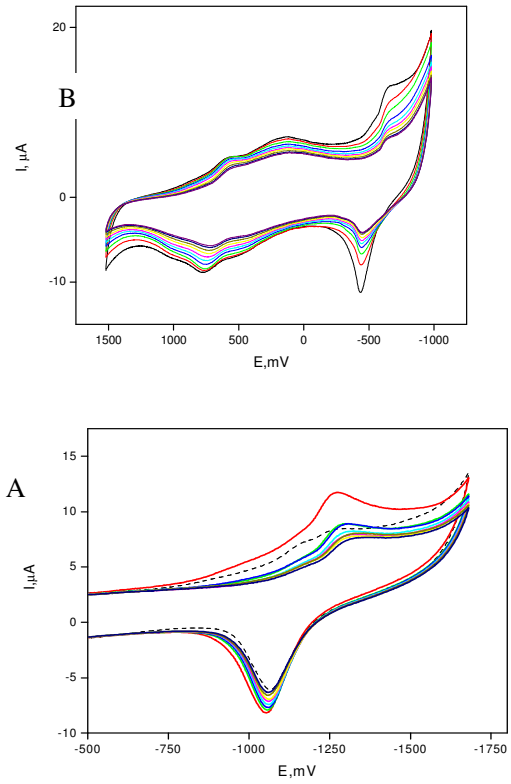

Fig. 5. CV spectra of Surfactant-cobalt(III) complex in the absence (dotted line) and in the presence (solid line) of ct DNA; (A: presence of β-cyclodextrin) and (B: absence of β-cyclodextrin) [complex]= 1×10^{-3} M; [DNA] =0 – 2.68×10^{-5} M and β-cyclodextrin = 1×10^{-3} M.

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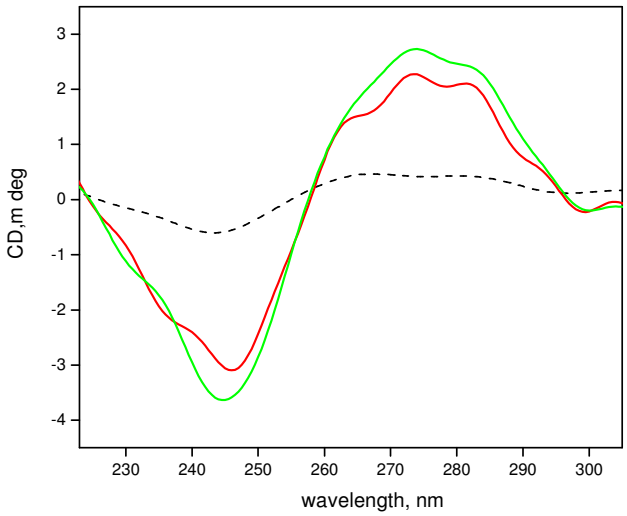

Fig. 6. Circular dichroism spectra in the absence(dotted line) and in the presence of surfactant-cobalt(III) (solid line).[complex] = 1×10^{-5}M and 2×10^{-5}M; [DNA] = 1×10^{-5} M.

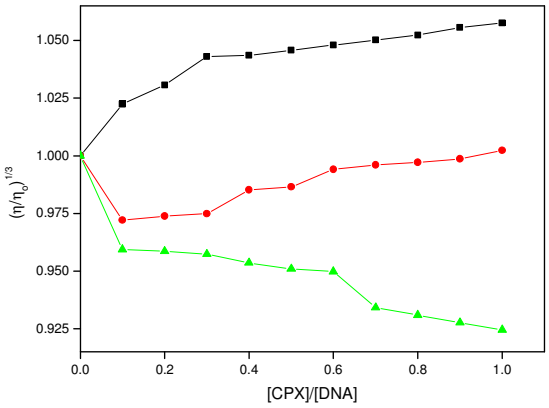

Fig. 7. Effects of increasing amounts of complex presence(•)and absence of β-cyclodextrin (▲) and EB (■)on the relative viscosities of calf thymus DNA at 29.0 (±0.1) °C.

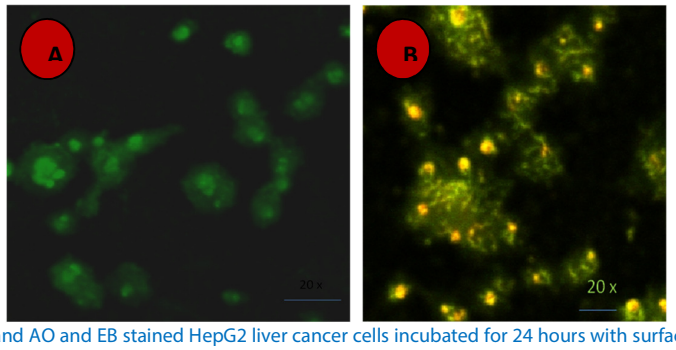

Fig. 8. Photomicrographs of control and AO and EB stained HepG2 liver cancer cells incubated for 24 hours with surfactant cobalt(III) complex. A. Untreated control cells. B. surfactant cobalt(III) complex treated control cells. ; Viable (light green), early apoptotic (bright green fluorescing), late apoptosis (red to orange fluorescing) and necrosis (red fluorescing) cells were observed. Magnification at 200x.

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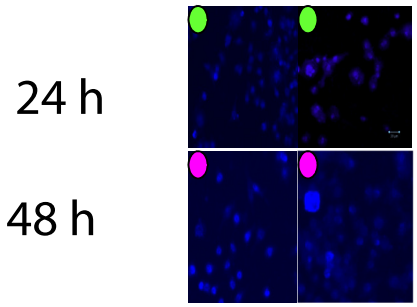

Fig. 9. Surfactant cobalt(III) complex induces apoptosis in HepG2 liver cancer cells. Representative fluorescent micrographs of HepG2 liver cancer cells stained with Hoechst 33258 fluorescent dye after the compound exposure for 24 and 48 hours. A, B Untreated control cells; C, D surfactant cobalt(III) complex treated control cells. A, B – 24 hours; C, D - 48 hours. Magnifications at 200X.

Table 1. The binding constant (K_b) of $[(Co(phen)_2(TA)_2]^{3+}$, with DNA (Presence and absence of β-cyclodextrin)

Complex	K_b (M^{-1})		Hyperchromism %		Hypochromism %	
$[(Co(phen)_2(TA)_2]^{3+}$ presence of βCD	Above cmc	Below cmc	Above cmc	Below cmc	Above cmc	Below cmc
	6.19×10^5	5.54×10^4	32.94	27.59	-	-
$[(Co(phen)_2(TA)_2]^{3+}$ Absence of βCD	4.86×10^6	2.77×10^5	-	-	41.40	28.99

Table 2. Fluoresence data of $[(Co(phen)_2(TA)_2]^{3+}$ of K_{sv} and % of EB fluorescence (In presence and absence of β-cyclodextrin) using Phosphate buffer.

Complex	K_{sv}	% of EB fluorescence (% I/Io)
$[(Co(phen)_2(TA)_2]^{3+}$(In presence of β-CD)	2.9×10^3	26.11
$[(Co(phen)_2(TA)_2]^{3+}$(Absence of β-CD)	3.5×10^3	28.48

Table 3. Cyclic voltammetric data (mV) of $[Co(phen)_2(TA)_2]^{3+}$, (Absence of β-cyclodextrin) a scan rate of 100 mV/s with phosphate buffer as supporting electrolyte

Complex (Absence of β-cyclodextrin)	Epc (mV)	Epa (mV)	ΔEp (mV)	E1/2 (mV)	i_{pa}/i_{pc}
$[Co(phen)_2(TA)_2]^{3+}$	577.5	-656.5	-1234	71.5	2.3
$[Co(phen)_2(HA)_2]^{3+}$ + DNA	561.5	-636.5	-1012	57.5	1.2

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Table 4. Cyclic voltammetric data (mV) of $[Co(phen)_2(TA)_2]^{3+}$ (Presence of β-cyclodextrin) a scan rate of 100 mV/s with phosphate buffer as supporting electrolyte

Complex (Presence of β-Cyclodextrin)	Epc (mV)	Epa (mV)	ΔEp (mV)	E1/2 (mV)	i_{pa}/i_{pc}
$[Co(phen)_2(TA)_2]^{3+}$	-1256.5	-1068.5	188	-1162.5	0.74
$[Co(phen)_2(TA)_2]^{3+}$ + DNA	-1320.5	-1062.5	448	-1190.5	0.91

Table 5. Cell viability assay by MTT3-(4-5 dimethylthiozol-2-yl) 2-5 diphenyl-tetrazolium bromide method.

Complex	Concentration (µg/mL)	Cell viability (VC %)	Non cell viability (NVC %)	Cell viability (VC %)	Non cell viability (NVC %)
		(24 hours)		(48 hours)	
$[Co(Phen)_2(TA)_2]^{3+}$	31	61.45	38.55	54.17	45.83
	62	52.3	47.7	41.68	58.32
	125	13.5	86.5	10.51	89.49
	250	9.2	90.8	7.1	92.9

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CONFLICTS OF INTEREST
"The authors declare no conflict of interest".