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Synthesis, characterization and DNA binding and cleavage properties of copper(II)-tryptophan-tryptophan complex

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Reaction of copper(II) with tryptophan-tryptophan yields a 1:2 chloro bis(trp-trp) cuprate(II) complex. The complex exhibits absorption band at 620 nm (ε = 130 M^{-1} cm^{-1}), which suggests a square pyramidal geometry at Cu(II) as observed for other Cu(II)-peptide complexes. The g_{iso} value of 2.09 for the complex agrees with a Cu(II) environment of distorted square pyramidal geometry. The mononuclear complex binds to calf thymus DNA through moderate intercalative and weak covalent interactions. It converts the supercoiled plasmid pUC19 DNA to the nicked circular form under physiological conditions.

Keywords: Coordination chemistry, Copper, Amino acids, Metallopeptides, Thermal denaturation, DNA quenching, DNA cleavage

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Development of new metallo-peptide complexes which interact with DNA has been the subject matter of intense investigations. This is because they act as simple models for metallo-protein DNA interactions, in addition to their application in biotechnology and medicine. Small bioactive ligands with low molecular weight, which also recognize and interact with DNA, are of chemical, biological, and medicinal significance as potential artificial gene regulators or cancer chemotherapeutic agents. In this regard, metal complexes with different or same ligands have been found to be particularly useful[1-6]. The remarkable biological potencies of these complexes are thought to be a consequence of their ability to bind to cellular DNA and produce single or double strand cleavage with great efficiency.

Incorporation of good intercalators like phen (1, 10-phenanthroline), bpy (2,2′-bipyridine), acridine, etc. cause high affinity between DNA base pairs and their planar structure through "stacking interaction". It has been reported that tryptophan moiety also acts as a good intercalator[7-10].

Keeping this in view, we report herein, the synthesis and characterization of 1:2 K[Cu(II)(trp-trp)$_2$Cl]-(**1**) complex and present its DNA binding and cleavage abilities.

Materials and Methods

Ethidium bromide (EB), Boc-trp-OH, H_2N-trp-Ome, 1-ethyl-3-(3-(dimethyl-amino)-propyl) carbodiimide (EDCI) and 1-hydroxybezotriazole (HOBt) were obtained from Sigma (99.99% purity, USA). $CuCl_2.2H_2O$ (analytical grade) was purchased from Merck. All other chemicals and solvents (spectroscopic grade) were purchased from commercial sources and used without further purification. Doubly distilled water was used to prepare the buffer solutions. Calf thymus DNA (CT-DNA) was obtained from Fluka (Switzerland). pUC 19 DNA and Tris-HCl buffer were obtained from Bangalore Genei (India). Aerated buffer (5 m*M* Tris HCl, 50 m*M* aq.NaCl, *p*H 7.5) was prepared at room temperature.

Elemental analyses were obtained from the micro analytical Heraeus Carlo Elba 1108 elemental analyzer. The molar conductivity was measured on a Digisun digital conductivity bridge (model: DI-909) with a dip type cell, using a 10^{-4} *M* solution of the complex in methanol. Infrared spectra were recorded on a Perkin-Elmer FTIR (1600 series) spectrometer, in KBr pellets in the 4000-200 cm^{-1} range. Magnetic susceptibility for the complex was recorded at room temperature on a Faraday balance (CAHN-7600)

using $Hg[Co(NCS)_4]$ as the standard. Diamagnetic corrections were made by using Pascal's constant[11]. UV-vis spectra of the complex were recorded on a Shimadzu 160A spectrophotometer (800-200 nm). The solid EPR spectrum was recorded on a Jeol (JES-FA200) X-band spectrometer at room temperature. ESI mass spectra of the complex were recorded using a Quattro Lc (Micro mass, Manchester, UK) triple quadrupole mass spectrometer with MassLynx software. The molecular modeling calculations were carried out with semi-emphirical PM3 Hamiltonian as implemented in Hyperchem software programme package.

A concentrated CT-DNA stock solution was prepared in 5 m*M* Tris-HCl/50 m*M* aq.NaCl buffer (*p*H 7.5) and its concentration was determined by UV absorbance at 260 nm. The molar absorption coefficient was taken as 6600 $M^{-1}cm^{-1}$. A solution of CT- DNA in 5 m*M* Tris-HCl/50 m*M* aqueous NaCl gave a ratio of UV absorption at 260 nm and 280 nm (A_{260}/A_{280}) of *ca*. 1.8-1.9, indicating that the DNA was sufficiently free of protein[12]. All stock solutions were stored at 4°C and were used within one week. The concentration of EB was determined spectrophotometrically at 480 nm (ε = 5680 $M^{-1}cm^{-1}$) (ref. 13).

Syntheses of the peptide and complex

The dipeptide (trp-trp) was synthesized by conventional solution phase method[14] using (EDCI) and (HOBt) as coupling agents and dry CH_2Cl_2 as solvent. While *t*-butoxycarbonyl (Boc) group was used for N-protection, the C-terminal was protected as methyl ester (OMe). Deprotection of Boc was achieved using TFA-CH_2Cl_2 (1:1); saponification of the methyl ester required LiOH in THF-MeOH-H_2O (3:1:1).

^{1}H NMR (200 MHz, $CDCl_3$), δ = 2.20 (br, 2H), 2.98-3.20 (m, 4H), 4.27 (m, 1H), 4.70 (m, 1H), 6.82-7.10 (m, 8H), 7.32 (m, 2H), 7.56 (m, 1H), 10.38 (d, 2H); ESI-MS, *m*/*z* 390.

To an aqueous solution of copper(II) chloride dihydrate (170 mg. 1 mmol), aqueous solutions of trp-trp (780 mg. 2 mmol) and KOH (2 ml, 56 mg, 1 mmol) were added at room temperature. The mixture was stirred for 12 hr. The blue colored solid which formed was filtered, washed with cyclohexane and ethanol and air dried (Yield 80%).

Anal.: Calc. for $C_{44}H_{42}N_8O_6ClCu$: C, 60.54; H, 4.73; N,12.7, Found: C, 60.31; H, 4.47; N, 11.20; m. pt: 195°C; Λ_M/Ω^{-1} cm^2 mol^{-1} (10^{-3} *M* methanol)=2.3; UV-vis (MeOH): λ_{max} 620 nm, 280 nm, 310 nm, and 394 nm; IR (KBr cm^{-1}): 3403 br, 2133 m, 1654 vs, 1593 m, 1490 m, 1440 s, 1384 s, 1288 s, 1100 m, 1023 s, 746 s, 651 w, 561 s, 424 s, 260; ESI-MS: *m*/*z* 875 $[M-H]^+$, 876, 877.

DNA binding activity

Absorption spectra were recorded on a Jasco V-530 UV-visible spectrophotometer using 1 cm quartz microcuvettes. Absorption titrations were performed by keeping the concentration of the complex constant (10 μM), and by varying the concentration of CT-DNA from 0-20 μM. The binding constant (K_b), for the binding of the complex with DNA, has been determined from the spectroscopic titration data using the following equation[15]:

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

The 'apparent' extinction coefficient (ε_a) was obtained by calculating A_{obsd}/[Cu]. The terms ε_f and ε_b correspond to the extinction coefficients of free (unbound) and of the fully bound complex, respectively. K_b, the ratio of the slope to the intercept, was obtained from a plot of $[DNA]/(\varepsilon_a-\varepsilon_f)$ versus [DNA], a slope $1/(\varepsilon_b-\varepsilon_f)$ and an intercept $1/K_b$ $(\varepsilon_b-\varepsilon_f)$.

The thermal denaturation studies were performed on a Shimadzu 160A spectrophotometer equipped with a thermostatic cell holder. DNA (12 μM) was treated with complex in a 1:1 ratio in (5 m*M* Tris HCl/50 m*M* NaCl) buffer. The samples were continuously heated at the rate of 1°C min^{-1} with temperature increase, while the absorption changes at 260 nm were monitored. Values for the melting temperature (T_m) and for the melting interval (ΔT_m) were determined according to the reported procedures[16]. Differential melting curves were obtained by numerical differentiation of experimental melting curves.

The competitive binding experiments were performed on a Jasco V-530 UV-visible spectrophotometer using 1 cm quartz microcuvettes. Absorption titrations were carried out by keeping the concentrations of the EB and CT-DNA constant (40 μM), and by varying the complex concentration from 0-20 μM.

Fluorescence spectra were recorded with a SPEX-Fluorolog 0.22 m fluorimeter equipped with a 450 W Xenon lamp. The slit widths were 2×2×2×2 and the

emission spectral range was 550-650 nm. All fluorescence titrations were carried out in 5 m*M* Tris-HCl/50 m*M* NaCl at 25°C. The solution containing DNA and EB was titrated with varying concentrations of the complex**.** The solutions were excited at 540 nm and fluorescence emission, which corresponded to 595 nm, were recorded. The samples were shaken and kept for 2-3 min for equilibrium, and then the spectra were recorded. The concentrations of DNA and EB were maintained at 41 µ*M* and the concentration of complex was in the range of 0-196 µ*M*.

Fluorescence spectra were also utilized to obtain Scatchard plots. For this, titrations of DNA against EB in the absence and presence of the copper complex were performed. The initial concentration of DNA in 5 m*M* Tris-HCl/50 m*M* NaCl was 20 µ*M*, the concentration of the complex was 50 µ*M*. After each addition of EB to the solution containing DNA and the copper complex, the emission spectra were recorded from 550 to 650 nm with 540 nm excitation at 25°C. Corrections were made to the data for the volume changes during the course of titrations. The data were analyzed by the method of Lepecq and Paoletti[17] to obtain bound (c_b) and free (c_f) concentrations of ethidium bromide. Scatchard plots were obtained by plotting r_{EB}/c_f versus r_{EB} (where $r = c_b$ / [DNA]).

DNA cleavage

Agarose-gel electrophoresis experiments were performed with pUC19 DNA at *p*H 7.5 in Tris-HCl buffered solutions. DNA cleavage was monitored by the addition of varying concentrations of the complex (0-120 µ*M*) to 2 µL of pUC19 DNA, and the total volume was increased to 16 µL by adding 5 m*M* Tris HCl/ 5 m*M* NaCl buffer. After mixing, the DNA solutions were incubated at 37°C for 3h. The reaction was quenched by adding 2 µL of 0.25% bromophenal blue. The samples were then electrophoresed for 2h at 60 Von 1% agarose gel using TAE buffer (tris acetic acid EDTA). The gel was stained with 0.5 µg/ml EB and photographed under UV light.

Results and Discussion

Characterization of the Cu(II)-trp-trp complex

The analytical data for the copper (II) complex are in good agreement with the molecular formula of the complex. In the IR spectra, the N-H and C=O stretching vibrations observed at 3396 and 1670 cm^{-1} respectively for free ligand were shifted to 3403 and 1654 cm^{-1} in the complex. This indicates the involvement of N and O in metal coordination. Further, the presence of a υ_{as} (COO^-) and υ_s (COO^-) stretching vibrations for the complex were observed at 1654 cm^{-1} and 1384 cm^{-1}, respectively. The large difference between the two indicates that the carboxylate groups are coordinated to the metal ion in a monodentate fashion[18]. Peaks corresponding to the aromatic ring moiety of the ligand stretching frequencies [υ(C=C)] and [υ(C-N)] at 1576 and 1457 cm^{-1} were shifted to 1593 and 1490 cm^{-1} in the complex indicating the coordination of the ligand to the metal ion. The non ligand peaks observed at 260 cm^{-1} , 424 cm^{-1} and 561 cm^{-1} were assigned to [υ(Cu-Cl)], [υ(Cu-O)] and [υ(Cu-N)] stretching vibrations[19], respectively. Accordingly, the involvement of carboxylate oxygens and amine nitrogens were considered in metal coordination.

The magnetic moment μ_{eff} of 2.04 BM for the complex determined at room temperature is indicative of a monomeric Cu(II) complex.

In the electronic spectra of the complex**,** a broad band observed at 620 nm (130 $M^{-1}cm^{-1}$) was assigned to the *d-d* transition. This suggests a square pyramidal geometry around Cu(II)[20]. In the UV region, three bands observed at 280 nm (5.4×10^4 $M^{-1}cm^{-1}$), 310 nm (3.2×10^4 $M^{-1}cm^{-1}$) and 394 nm (1.6×10^4 $M^{-1}cm^{-1}$) for the complex, may be attributed to the л → л* transition of the coordinated ligands. The EPR spectrum of the Cu(II) complex at room temperature with a g_{iso} value of 2.09 also suggests a square pyramidal geometry. Similar observations have been made earlier[21]. The ESI-mass spectra of the complex K[Cu(II)(trp-trp)$_2$Cl] shows a base peak (molecular ion peak) at *m*/*z* 875 for [M-H]$^-$. The peak at *m*/*z* 876 corresponds to the ^{13}C isotopic signal and *m*/*z* 877 peak to the complex isotopic chloride (^{37}Cl). This suggests that the complex has a [Cu(II)(trp-trp)$_2$Cl]$^-$ stoichiometry.

To obtain the binding geometries, the minimum energy calculations for the corresponding 1:2 copper dipeptide complex were calculated by means of the HYPERCHEM program package. The resulting structure with optimized bond lengths of metal and the interacting N, O and Cl atoms are given in Fig. 1. The bond lengths are within the normal ranges obtained from the crystal structure data[22].

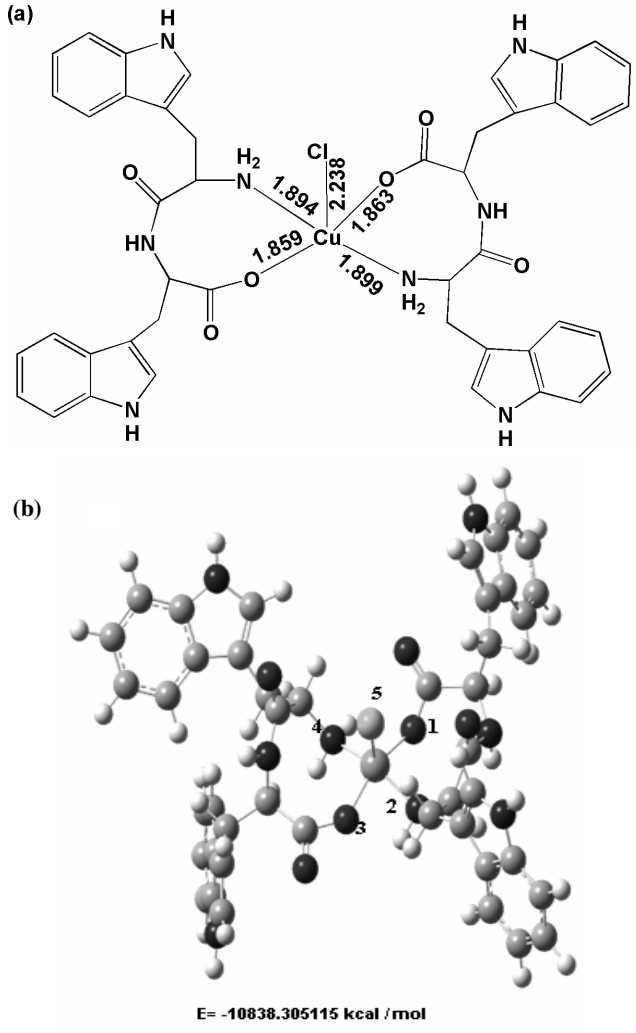

Fig. 1—(a) Proposed geometry, and, (b) energy-minimized molecular structure with energies and bond lengths of (**1**).

DNA binding

The absorption spectra of the complex in the absence and presence of CT DNA are illustrated in Fig. 2. In the presence of DNA, a decrease in absorption intensities (hypochromism) with a slight red shift of the wavelength (bathochromism) was observed. Hypochromism and bathochromism are suggested to arise from the interaction between the electronic state of the intercalating chromophore and that of the DNA bases[23]. These spectral changes are consistent with the intercalation of complex into the DNA base stack. The intrinsic binding constant (K_b) was determined to be $5.6\times10^2\ M^{-1}$.

The thermal denaturation profile of CT-DNA in the absence and presence of the complex is provided in Fig. 3. An increase of 3-4°C was observed in the T_m profile of the complex as compared to that of free

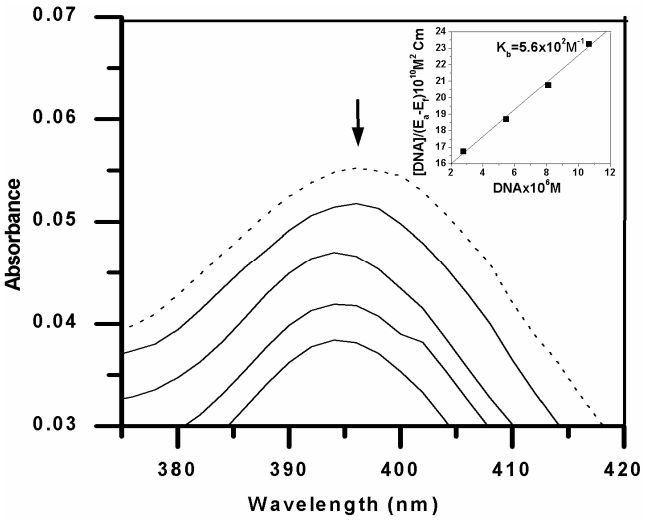

Fig. 2—Absorption spectra of (**1**) in the absence (…….) and presence (—) of increasing amounts of DNA. [[Cu] = 10 μ*M*; [DNA]= 2–10 μ*M*. Arrow (↓) shows the absorbance changes upon increasing DNA concentration. Inset: Linear plot for the calculation of the intrinsic DNA binding constant, K_b].

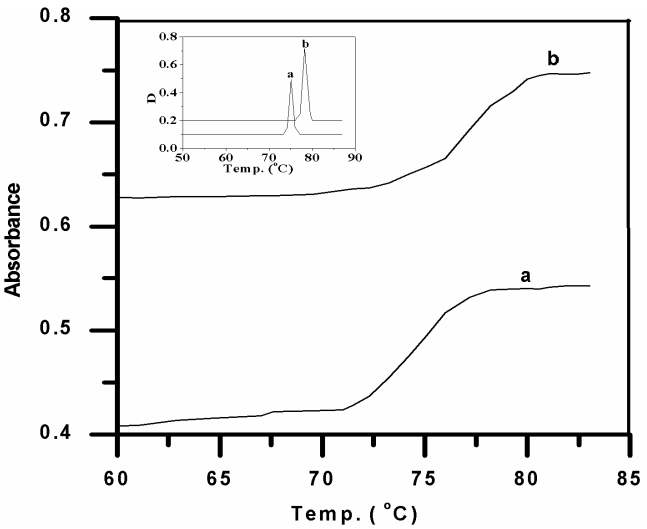

Fig. 3—Thermal denaturation profile of (a) free CT-DNA and (b) after the addition of (**1**). [The concentrations of (**1**) and DNA were 12 μ*M*. Inset: Differential-melting curves].

DNA. It is well known[24,25] that the increase of the T_m value points to an intercalative and/or phosphate binding whereas a decrease is an indicator of non-intercalative DNA binding[26]. These results suggest an intercalative binding of the copper complex with DNA.

Intercalative binding is also demonstrated through competitive binding experiments using ethidium bromide (EB) (Fig. 4), which shows a decrease in absorbance in the presence of CT-DNA. The decrease in absorbance of EB bound to DNA is due to

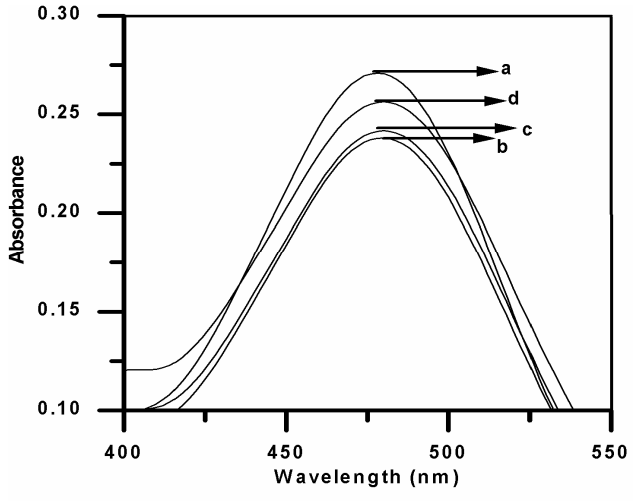

Fig. 4—Absorption spectra of ethidium bromide (EB) in 5 m*M* Tris-HCl/ 50 m*M* NaCl buffer (*p*H 7.5). [(a) in the absence; (b) in the presence of CT DNA; (c) CT DNA + (**1**) (10 μ*M*); (d) CT- DNA + (**1**) (20μ*M*)].

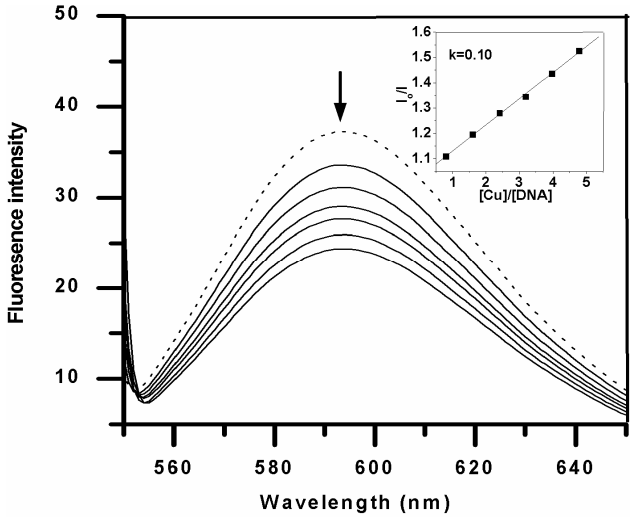

Fig. 5—Emission spectra of EB bound to DNA in the absence (…..) and presence (—) of (**1**). [(**1**) / [DNA] = 0, 0.8, 1.60, 2.41, 3.19, 3.97, 4.78; λ_{ex} = 540 nm; Inset: Stern-Volmer quenching plot].

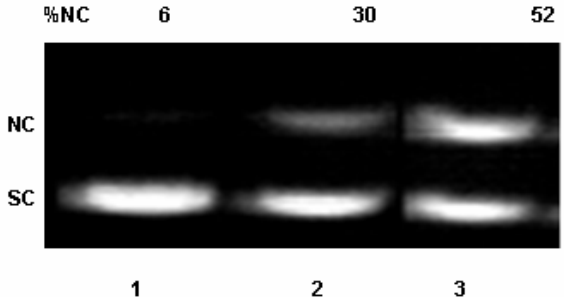

Fig. 6—Agarose gel electrophoresis patterns for the cleavage of pUC19 DNA by (**1**).[Lane 1, DNA control; Lane 2, DNA+(**1**) (60 μ*M*); Lane 3, DNA+(**1**) (120 μ*M*)].

intercalation of the chromophore between two adjacent DNA base pairs. Addition of the complex to CT-DNA bound to EB increases the absorbance to the same extent. This suggests that the complex displaces DNA-bound EB and binds to DNA at the intercalation sites with similar affinity. The spectral results indicate an intercalation of indole rings.

The fluorescence of an ethidium-DNA solution will be diminished in the presence of a competitive intercalating reagent. Figure 5 confirms this trend. The quenching constant was determined according to the Stern-Volmer equation, $I_o/I = 1+K_{sq}\ r$, where I_o and I are the fluorescence intensities in the absence and the presence of the complex, respectively, K_{sq} is linear Stern-Volmer quenching constant, dependent on the ratio of bound concentration of EB to the concentration of DNA, and r is the concentration ratio of the complex to DNA. The quenching plot illustrates that the quenching of EB bound to DNA by the complex is in good agreement with the linear Stern-Volmer equation (Fig. 5. inset). This indicates that the complex competes with EB in binding to DNA. In the plot of I_o/I versus [complex]/[DNA], K_{sq} is given by the ratio of slope to the intercept; the K_{sq} for complex was determined as 0.10.

Further, the binding fits approximately the Scatchard equation[27], $r/c = K_i(n-r)$, where r is the ratio of bound EB to the total nucleotide concentration, c is the concentration of free EB, n is the number of sites per nucleic acid and K_i is the intercalative binding constant for EB. The value of K_i was found to be $4.12\times10^3 M^{-1}$, which is indicative for a moderate intercalation.

In the presence of a competing reagent that can occupy the same site on the DNA duplex as the ethidium cation, the effective binding constant of EB to DNA, measured through the fluorescence intensity and given by the slope of a Scatchard plot, is reduced. If the test reagent binds covalently to the DNA, on altering the nature of the intercalation site, the number of remaining available sites for ethidium bromide, n (given by the coordinate intercept of a Scatchard plot) is lowered[28] as also shown in the present study. At lower concentrations the copper complex competitively inhibits ethidium bromide intercalation. At higher concentrations where the substitutionally labile chloride anion may dissociate, the complex (**1**) covalently binds to the bases, noncompetitively inhibiting ethidium intercalation.

This assay has been used to investigate both covalent and intercalative binding modes of a series of platinum complexes[29]. Though the negative charge on the present Cu(II) complex reduces the affinity for DNA, the specific binding enhances its (complex) interaction with DNA[30].

DNA cleavage

The DNA cleavage activity of the complex was probed by gel electrophoresis using supercoiled plasmid pUC19 DNA in 5 m*M* Tris-HCl/5 m*M* NaCl buffer (*p*H 7.5). When plasmid DNA is subjected to electrophoresis, a relatively fast migration will be observed for the intact supercoil form (SC). If scission occurs on one strand (nicking), the supercoil will relax to generate a slower moving nicked circular form (NC). If both strands are cleaved, a linear form that migrates between SC and NC will be generated[31,32]. At 120 μ*M* concentration of the complex, more than 50% of supercoiled (SC) plasmid DNA was converted into the nicked circular (NC) form in the absence of external agents (Fig. 6). This suggests that the DNA cleavage is hydrolytic in nature. It is important to note here that even at 1.0 m*M* concentration of free Cu(II) and the ligand, there was no significant DNA cleavage activity. The extent of DNA cleavage was quantified via fluoro imaging.

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