DNA Binding Ability and Hydrogen Peroxide Induced Nuclease Activity of a Novel Cu(II) Complex with Malonate as the Primary Ligand and Protonated 2-Amino-4-picoline as the Counterion†

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The DNA binding property of a Cu(II) complex, viz., $[Cu(mal)_2](picH)_2 \cdot 2H_2O$, $(mal)_2$ = malonic acid, picH = protonated 2-amino-4-picoline, has been investigated in this study. The binding of this complex with plasmid and chromosomal DNA has been characterized by different biophysical techniques. From the absorption and fluorescence spectroscopic studies, it has been observed that the said copper complex binds strongly with pUC19 plasmid and CT DNA with a binding affinity of 2.368×10^3 and 4.0×10^3 M^{-1}, respectively, in 10 mM citrate-phosphate buffer, pH 7.4. Spectrofluorimetric studies reveal that the copper complex exhibits partial DNA intercalation as well as partial DNA minor groove binding properties. Consequently, in agarose gel electrophoresis study, it has been observed that the complex alone induces positive supercoiling in plasmid DNA while in the presence of H_2O_2 it exhibits nuclease activity. The induction of the breakage in DNA backbone depends upon the relative concentrations of H_2O_2 and copper complex followed by the time of incubation with DNA. Optical DNA melting study, isothermal titration calorimetry, and absorption spectroscopy have been used to characterize the nuclease activity of this complex in the presence of H_2O_2. Further, ^1H NMR study indicates that Cu(II) in the complex is converted into the Cu(I) state by the reduction of H_2O_2. Finally, agarose gel electrophoresis study with different radical scavengers concludes that the production of both hydroxyl radicals and reactive oxygen species is responsible for this nuclease activity.

Introduction

Recently, the chemistry of binuclear copper complexes involving a variety of bridging ligands has been characterized extensively.[1−5] In this regard, different ligands have been used to produce synthetic Cu(II) complexes. A water-soluble Cu(II) complex, $[Cu(mal)_2](picH)_2 \cdot 2H_2O$, that can easily be prepared from purely aqueous media simply by mixing the reagents in a stoichiometric ratio, draws interest from the crystal engineers for its interesting supramolecular features in the solid state.[6] Malonate has been widely used as a ligand for the synthesis of crystalline coordination materials of diverse architectures, due to its various potential binding modes to transition metals as well as its participation in hydrogen bonding interactions.[7,8] Moreover, in combination with other ligands, malonate has been shown to generate remarkable architectures.[9−13] The use of malonate in association with 2-amino-4-picoline in Cu(II) systems has shown that these two ligands have a preferred recognition pattern through the formation of an $R_2^2(8)$ hydrogen bonding synthon.[6]

The supramolecular materials being interrelated to nanosecond materials have surprising applications not only in chemistry but also in biomedicine.[14] Though the aqueous structures of such supramolecules are not yet clearly known, their interaction with biomolecules is worth exploring. Supramolecule mediated DNA recognition and subsequent stabilization is an active area of contemporary research.[15] A large number of small molecules and some metal complexes have been reported to efficiently stabilize DNA structures. For example, two alkylamine-substituted nickel(II)−salphen complexes were prepared and the interaction with DNA was investigated. Fluorescence resonance energy transfer (FRET) studies have shown that these complexes have a remarkable ability to stabilize G-quadruplex DNA.[16] A family of terpyridine metallo-organic complexes has also been reported to recognize G-quadruplex DNA. It has been proposed that the geometry of the metal center strongly governs the ability of the compounds to discriminate quadruplex from duplex DNA.[17] A novel platinum-quinacridine hybrid-trap preferentially traps the antiparallel structure of an oligonucleotide that mimics

† List of Abbreviations: ITC, isothermal titration calorimetry; NMR, nuclear magnetic resonance; CD, circular dichroism; RF1, replicative form 1; RF2, replicative form 2; RF3, replicative form 3; bp, base pair; ROS, reactive oxygen species; OD, optical density.

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the human telomeric repeats.[18] It has recently been reported that chiral metallo-supramolecular complexes selectively recognize human telomeric G-quadruplex DNA.[19]

Several anticancer agents have been developed with numerous transition metals and screened for their antineoplastic properties. Cu(II) complexes have recently attracted attention, as they can be designed as anticancer agents in an effort to overcome the severe side effects of known drugs like cisplatin, bleomycin, etc.[20−23] Redox active compounds such as Cu(II) can cleave the phosphodiester backbone of DNA molecules and have a wide range of biological activities, and some of them have known antimicrobial,[24,25] antinflammatory,[26] antiviral,[27] and antitumor[28,29] activities. Several other compounds such as bleomycin, pyrrole, thioether, oxime, peptides, and imidazole type ligands, have been complexed with copper ions, exhibit DNA cleavage activities.[30,31]

Among these, some Cu(II) complexes have inherent nucleolytic activity,[32−34] whereas some redox active complexes can induce DNA strand breaks in the presence of either H_2O_2[35,36] or some other reducing agents.[2,37−39] The mechanism of DNA cleavage activity is proposed to be mediated by the production of free hydroxyl radicals during the reaction of reduced Cu(II) complex by H_2O_2.[40] The modification of the ligand for the Cu(II) complex is significant, as it produces subtle to spectacular changes in the properties of the resulting complex. The physical as well as nuclease activity has also been documented for Cu(II) complexes associated with different ligands.[34,41] Among them, 26-membered hexaazamacrocycle, heteroaromatic derivatives, and picoline, polyguanosines, benzimidazole, etc., were used as ligands.[42] Similarly, when nalidixic acid was used as a bridging ligand in a bis copper−phenanthroline complex, DNA binding and mercaptopropionic acid mediated nuclease activities have been documented.[39] A unique Cu(II) complex, comprised of malonate as a primary ligand and protonated 2-amino-4-picoline as a counterion, has been reported recently, focusing mainly on the supramolecular structure in the solid state.[6] The supramolecular structure of the complex is very important for molecular recognition. Though some of the Cu complexes have been reported to have DNA binding and/or cleavage activity, such studies have not been undertaken for a Cu(II) complex showing supramolecular feature in the solid state.

In the present study, we have investigated the DNA binding property of $[Cu(mal)_2](picH)_2 \cdot 2H_2O$ complex with both chromosomal and plasmid DNA. The complex binds with DNA both as a partial intercalator and a partial minor groove binder, as it can replace both bound ethidium bromide and Hoechst 33258 partially from the DNA. Gel retardation study showed that the complex induced positive supercoiling in plasmid DNA. However, in the presence of H_2O_2, it clearly showed nuclease activity under the same experimental conditions. Absorption spectroscopic studies, optical DNA melting profile, and isothermal titration calorimetric studies confirmed the DNA cleavage activity. Moreover, ^1H NMR study revealed that, in the presence of H_2O_2, the oxidation state of Cu(II) was converted into Cu(I). Using different radical scavengers, we confirmed that this nuclease activity of the complex in the presence of H_2O_2 was mediated by the production of hydroxyl radicals and reactive singlet oxygen species.

Experimental Methods

Preparation of $[Cu(mal)_2](picH)_2 \cdot 2H_2O$ Complex and Picoline Solutions. The title Cu(II) complex was prepared following the literature method.[6] The Cu(II) complex is readily soluble in water, while purified 2-amino-4-picoline (henceforth, picoline, Sigma) requires slight warming. They were separately dissolved at a concentration of 10 mM stock in double distilled water and diluted freshly before each experiment.

Preparation of Calf Thymus and Plasmid DNA. Purified calf thymus (CT) DNA (Sigma-Aldrich, USA) and pUC19 plasmid DNA (Bangalore Genei, India) were dissolved separately at a concentration of 6 mg/mL stock in 10 mM citrate-phosphate buffer, pH 7.4, at room temperature. Aliquots were stored at 4 °C and diluted freshly before each set of experiments.

Ethidium Bromide and Hoechst 33258 Stock Solutions. Ethidium bromide (EtBr) dust (Bangalore Genei, India) and Hoechst 33258 (Polysciences, USA) were dissolved in double distilled water at concentrations of 10 mM and 1 mM, respectively. Stocks were stored (at 4 °C in the dark) and diluted freshly before each experiment.

Absorption Spectroscopic Study. Absorption spectroscopic studies were done on a spectrophotometer (model U-2800, HITACHI Ltd., Japan), using either pUC19 plasmid DNA (35 μg/mL) with increasing concentrations of the complex (10−70 μM) or the complex (23 μM) with increasing concentrations of pUC19 plasmid DNA (5.46−27.29 μg/mL). After each addition, the DNA and complex mixtures were incubated at room temperature for 15 min and scanned either from 210 to 305 nm for the DNA or from 210 to 330 nm for the complex. The self-absorption of ligand was eliminated in each set of experiments. Each sample was scanned for a cycle number of 2, cycle time of 5 s at a scan speed of 100 nm/min. Modified Benesi−Hildebrand[43] and Scatchard plots[44] were used for the determination of the ground state binding constant and number of possible binding sites on pUC19 DNA for the Cu(II) complex. The binding constant "*K*" was determined using the following relation:

$$A_0/\Delta A = A_0/\Delta A_{max} + (A_0/\Delta A_{max}) \times 1/K \times 1/L_t$$

where $\Delta A = A_0 - A$, ΔA_{max} = maximum reduced absorbance, A_0 = maximum absorbance of DNA (without any ligand), A = reduced absorbances of DNA (in the presence of ligand), and L_t = ligand (Cu(II) complex) concentration.

To determine the possible spectral shift of the Cu(II) complex (70 μM) in the presence of H_2O_2, increasing concentrations of H_2O_2 (2.67−15 mM) were added to the complex solution and the samples were scanned.

Fluorescence Spectroscopic Studies of Cu(II) Complex with Plasmid DNA. Fluorescence spectroscopic study of Cu(II) complex (100 μM) with varying concentrations of pUC19 plasmid DNA (up to 50 μg/mL) was done on a spectrofluorimeter (Hitachi F3010, excitation and emission band-pass 5 nm each, scan speed 60 nm/min, and spectral response 2 s). The complex naturally fluoresces at excitation wavelengths of 232, 287, and 609 nm, with emission maxima at 353, 356, and 700 nm, respectively. At an excitation wavelength of 609 nm, the binding constant of the complex and plasmid DNA was calculated by using modified the Benesi−Hildebrand (BH) equation.

In another experiment, EtBr solution was gradually added to 120 μg/mL CT DNA and at each time the fluorescence pattern was scanned from 550 to 640 nm at an excitation wavelength of 546 nm[44] using a spectral response of 2 s at a scan speed of 60 nm/min. The fluorescence intensity of DNA-bound EtBr was saturated at a 100 μM concentration of EtBr. At this level, the said complex was added gradually (up to 120 μM) until the fluorescence pattern decreased and reached saturation. The displacement experiment was repeated with Hoechst 33258 instead of EtBr. For Hoechst 33258, the excitation wavelength

was 351 nm and emission spectra were scanned from 400 to 550 nm.[44] Saturation of fluorescence intensity for 30 μg/mL DNA was observed at a concentration of 4.8 μM Hoechst 33258, and then, the complex was added gradually up to 100 μM, until a saturation level was achieved.

Circular Dichroic Study. A JASCO J815 spectropolarimeter (Jasco International Co. Ltd., Tokyo, Japan) equipped with a Jasco temperature controller (model PTC 343) and controlled by a PC was used for all circular dichroic measurements at 20 $\pm$ 0.5 °C.[45] A rectangular stain-free quartz cell of 1 cm path length was used. Each spectrum was averaged from five successive accumulations at a scan rate of 50 nm/min, keeping a bandwidth of 1.0 nm at a sensitivity of 100 millidegree, baseline corrected and smoothed within permissible limits using the inbuilt software of the unit. The molar ellipticity values (θ) are expressed in terms of per DNA nucleotide (220−400 nm regions). The CD unit was routinely calibrated using an aqueous solution of d-10 ammonium camphor sulfonate.

Study of Binding and Nuclease Activity of the Cu(II) Complex by Agarose Gel Electrophoresis. pUC19 plasmid DNA and CT DNA stock solutions were used in these experiments. pUC19 plasmid DNA (500 ng) was incubated with different concentrations of the complex (0.08−0.82 mM) at 37 °C for 1 h, and then, the samples were run in 2% agarose gel in 1× TASE buffer (pH 7.4) at 2 V/cm for 12 h.

In another set of experiments, varying concentrations of the said complex (41.67−625 μM) were preincubated with a fixed concentration of H_2O_2 (1.6 M) for 30 min at room temperature. Then, these treated complex aliquots were incubated with either chromosomal CT DNA (1000 ng) or pUC19 plasmid DNA (500 ng) for 15 min and finally subjected to 1.5 and 1.2% agarose gel electrophoresis, respectively, in an applied electric field of 3 V/cm. Gels were stained with EtBr solution (500 μg/mL), and photographs were taken with a gel documentation system (Transilluminator, UVPRO).

Sodium azide (50 mM), potassium iodide (50 mM), and catalase (Sigma, 500 U/mL) were used separately as different radical scavengers.

Optical Melting Study with CT DNA. Absorbance versus temperature profiles (melting curves) of CT DNA were measured on the Shimadzu Pharmaspec 1700 unit equipped with the Peltier controlled TMSPC-8 model accessory (Shimadzu Corporation, Kyoto, Japan). In a typical experiment, a 50 μg/mL DNA sample was mixed with either the complex (1:1 molar ratio) or with the H_2O_2 pretreated complex (1:1 molar ratio), incubated for 15 min, and diluted into the desired degassed buffer in an eight-cell micro-optical cuvette of 1 cm path length. The temperature of the microcell accessory was raised at a heating rate of 0.5 °C/min, slit width of 2 nm, and spectral response of 0.2 nm, and the corresponding absorbance changes at 260 nm were monitored continuously.[46] Melting curves allowed an estimation of melting temperature (T_m), the midpoint temperature of the unfolding process. The van't Hoff enthalpy, ΔH_{VH}, was calculated using the standard equation described previously[46]

$$\Delta H_{VH} = (2 + 2n)RT_m^2(\delta\alpha/\delta T)_{T=T_m}$$

where α is the fraction of single-stranded DNA in the duplex state.

Isothermal Titration Calorimetric Study with CT DNA. Isothermal titration calorimetry (ITC) experiments were performed on a highly sensitive Microcal VP-ITC microcalorimeter (MicroCal, Inc., Northampton, MA, USA). Origin software was used for acquisition and analysis. Prior to use, all of the solutions were thoroughly degassed by stirring under a vacuum (140 mbar, 8 min) on the Microcal's Thermovac unit to eliminate air bubbles. In each experiment, the ITC cell was filled with CT DNA solution (1 mM) and titrated with either mock treated Cu complex (20 μM) or H_2O_2 pretreated Cu complex solution (in 1:1 molar ratio) from the syringe. Binding enthalpies for the interaction in each case were determined from the heat of reactions obtained after each injection using a model free ITC protocol[47] to obtain multiple estimates of ΔH without any fitting bias. The dilution heats, determined for injecting DNA into the buffer alone, were subtracted from the corresponding ΔH values determined for the titration of DNA with ligands to obtain the correct values of binding induced enthalpy change.

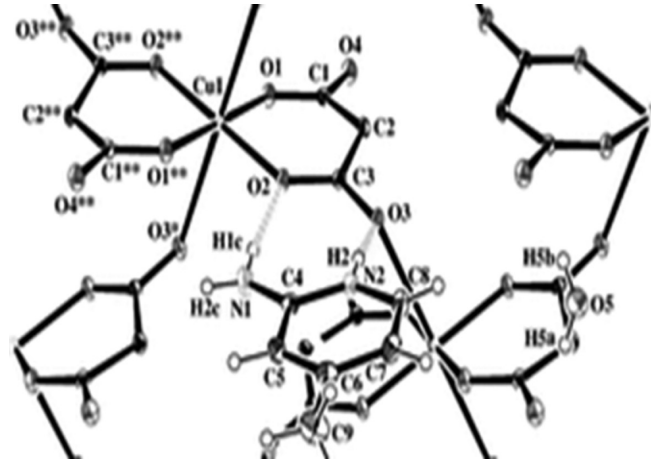

Figure 1. Chemical structure of $[Cu(mal)_2](picH)_2 \cdot 2H_2O$.

Nuclear Magnetic Resonance Spectroscopy of H_2O_2 Treated Cu(II) Complex. A 5.2 mg portion of the Cu(II) complex was dissolved in 500 μL of distilled water and treated with 30 μL of 8.3 M H_2O_2. The treated complex was then desiccated completely in a vacuum. Finally, the product was dissolved in 500 μL of D_2O and subjected to ^1H NMR study (300 MHz, Bruker NMR spectrophotometer). The data was analyzed in paramagnetic scale.[48]

Results

The interaction of a novel Cu(II) complex, $[Cu(mal)_2](picH)_2 \cdot 2H_2O$ (Figure 1), with both chromosomal and plasmid DNA has been investigated in this report. At first, spectrophotometric method was used to determine the possible interaction of the said Cu(II) complex with pUC19 plasmid DNA. Gradual addition of the complex (10−70 μM) to the DNA solution (35 μg/mL) resulted in a hypochromic effect in the absorption spectrum of the DNA with a small red shift (Figure 2a) at the wavelength maximum. Conversely, the characteristic absorption spectrum (peaks at 232 and 294 nm) of the Cu(II) complex showed similar hypochromicity on the gradual addition of the DNA (Figure 2b). The binding constant and the number of binding sites of the Cu(II) complex with DNA were calculated to be 2.368×10^3 M^{-1} (Figure 2c) and 0.45 per bp (calculation not shown), respectively. We found no change in the absorption pattern of the DNA (35 μg/mL) when the purified ligand, picoline (10−70 μM), was added separately as control, indicating the binding between the Cu(II) complex and DNA was not directly involved with the picoline moiety of the complex (Figure 2d). On the other hand, the binding constant of purified Cu−malonate (without picoline) with DNA was calculated to

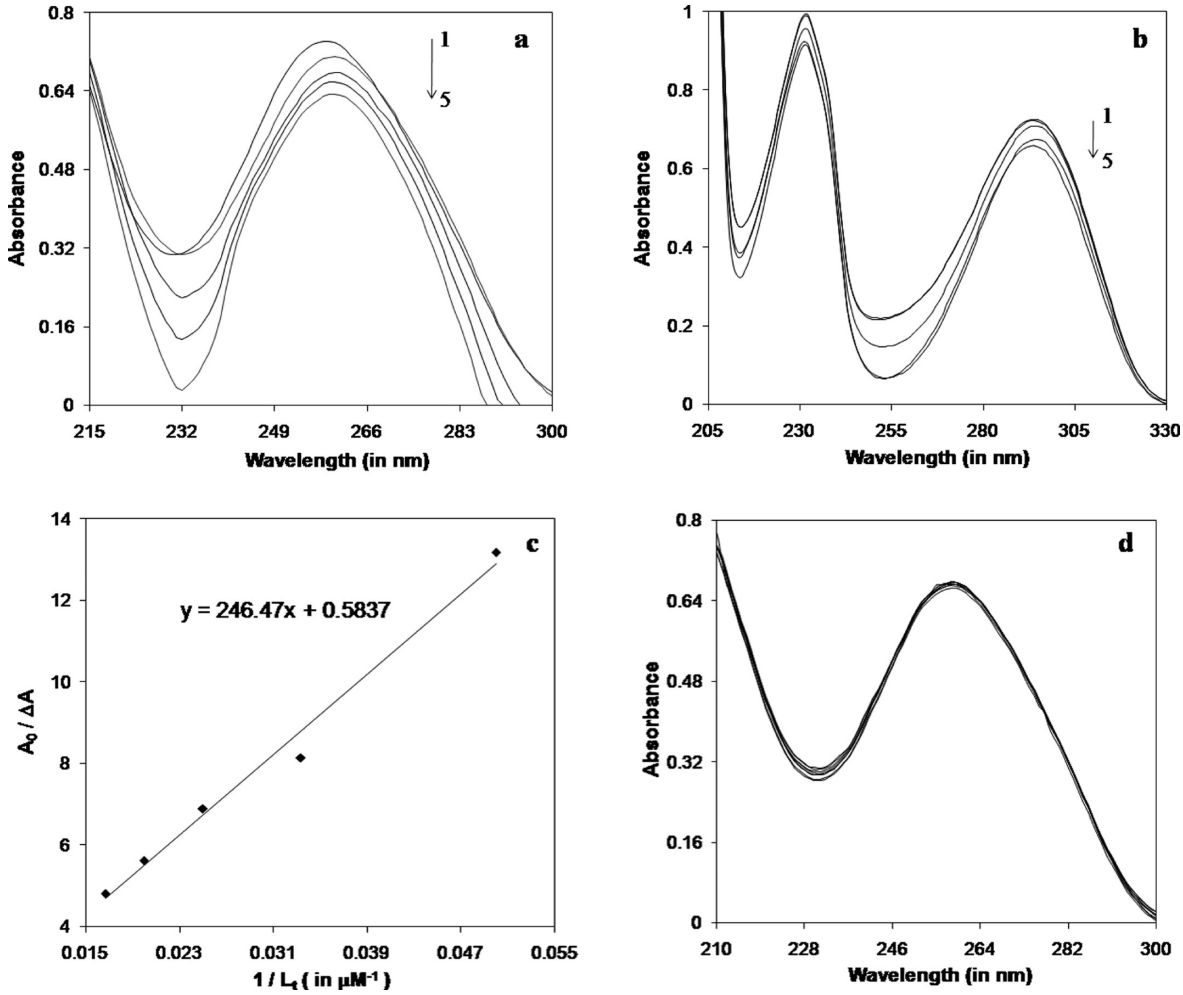

Figure 2. (a) Absorption spectra of pUC19 plasmid DNA (35 μg/mL) with varying concentrations of Cu(II) complex, 0, 10, 20, 40, and 70 μM (1 → 5), respectively. (b) Absorption spectra of Cu(II) complex (23 μM) with increasing concentrations of pUC19 plasmid DNA, 0, 5.46, 10.92, 21.84, and 27.29 μg/mL (1 → 5), respectively. Part c represents a modified Benesi−Hildebrand plot for the determination of the ground state binding constant between pUC19 DNA and the Cu(II) complex (see text for details). Part d represents absorption spectra of pUC19 DNA with increasing concentrations of purified ligand, picoline in the range of concentrations as used for the Cu(II) complex in part a (see text for details).

be nearly 10-fold less than that of the Cu−malonate−picoline complex (data not shown).

Being a natural fluorophore, the complex has three characteristic excitation maxima at wavelengths of 232, 287, and 609 nm. We used this intrinsic property of the complex to further evaluate the specific binding of the complex with DNA. At all the three excitation wavelengths, the fluorescence intensities of the complex (100 μM) were quenched gradually (Figure 3a−c) on addition of DNA (up to 50 μg/mL). At an excitation wavelength of 609 nm, the amount of the fluorescence quenching was much less and reached saturation faster than the other two wavelengths. At this emission maximum, we calculated the binding constant between the complex and DNA by using a modified Benesi−Hildebrand (BH) plot (Figure 3d). The binding constant was estimated to be 4.0×10^3 M^{-1}, which was in good agreement with the binding constant evaluated from the data of absorption spectroscopic analysis. To determine the nature of binding, we increased the ionic strength of the buffer by adding either monovalent ion (up to 100 mM NaCl) or divalent ion (up to 100 mM $CaCl_2$) in the solutions containing DNA saturated with the complex, but no significant change was observed in the fluorescence pattern, indicating the binding was nonionic in nature (data not shown).

To determine the possible mode of binding of this Cu(II) complex with DNA, we performed experiments to see whether the complex can displace some strong known DNA binding agents. We gradually added ethidium bromide, a well-known DNA intercalator, up to 100 μM to a fixed concentration of CT DNA (120 μg/mL), and at each point, the characteristics fluorescence spectrum of the DNA bound ethidium bromide was taken until a saturation in the fluorescence intensity was achieved (Figure 4a). At this saturation level, Cu(II) complex was added gradually (up to 120 μM) to the solution and the corresponding fluorescence spectrum was taken after each addition. As seen in Figure 4b, the fluorescence intensity of the solution decreased but did not reach the basal level, indicating the partial removal of ethidium bromide from DNA molecules by the complex. Similar experiments were done by monitoring the DNA-bound fluorescence property of Hoechst 33258, a classical DNA minor groove binder (Figure 4c). Here also it was observed that the complex partially removed the bound Hoechst from DNA (Figure 4d). Thus, the said Cu(II) complex may be a partial intercalator as well as a partial minor groove binder of DNA.

We next observed the effect of this complex on plasmid DNA by agarose gel electrophoresis study. pUC19 plasmid DNA (500 ng) was incubated for an hour with different concentrations of the complex (0.08−0.82 mM) at room temperature and then subjected to 2% agarose gel electrophoresis at a low potential gradient (2 V/cm). After staining with ethidium bromide (Figure 5), it was observed that the complex induced positive super-

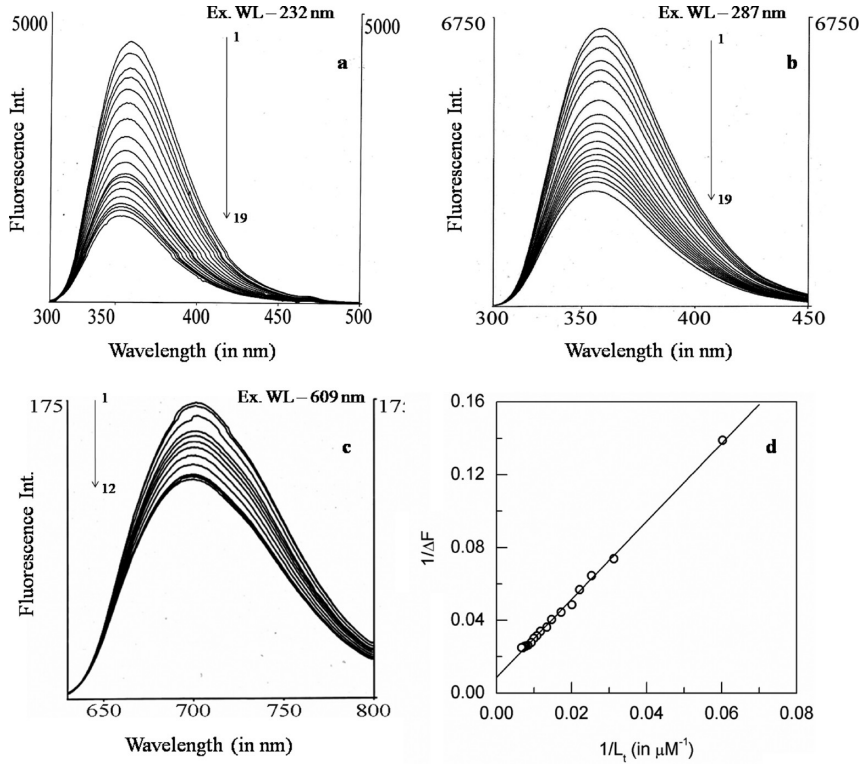

Figure 3. Intrinsic fluorescence spectra of Cu(II) complex (100 μM) with increasing concentrations of CT DNA. In parts a and b, spectra 1 → 19 represent fluorescence quenching of the said complex with the increasing concentrations of DNA (0 → 50 μg/mL), whereas, in part c, spectra 1 → 12 represent the same for 0−40 μg/mL concentrations of DNA. Part d represents a modified Benesi−Hildebrand plot for the determination of the binding constant between Cu(II) complex and chromosomal CT DNA.

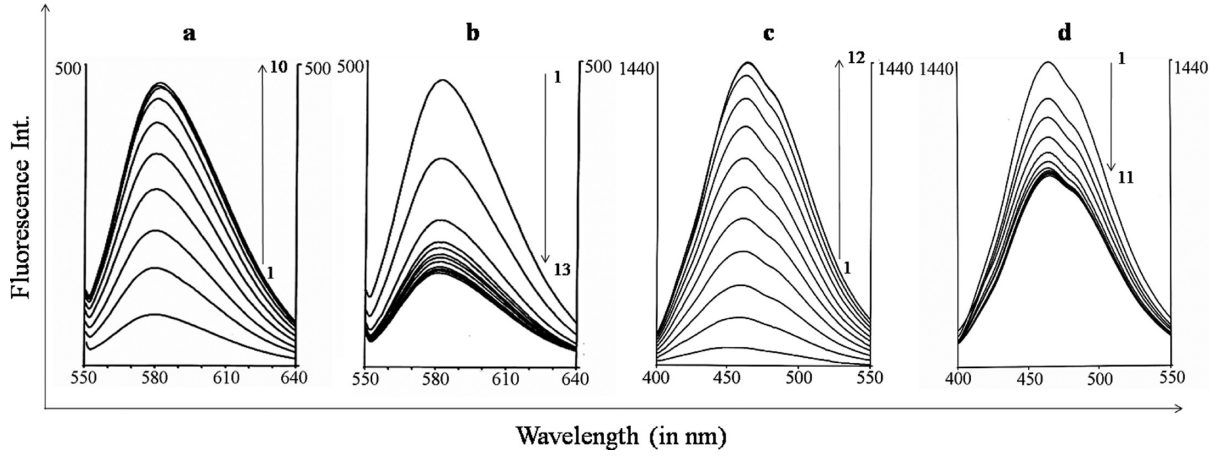

Figure 4. Extrinsic fluorescence spectra of EtBr bound (a, b) and Hoechst bound (c, d) CT DNA with increasing concentrations of Cu(II) complex. Part a represents the mode of binding of EtBr with 120 μg/mL DNA. The concentrations of EtBr were 10, 20, 30, 40, 50, 70, 90, 100, 110, and 120 μM (1 → 10, respectively). The maximum fluorescence intensity of EtBr−DNA complex was observed at a minimum concentration of 100 μM EtBr. At this point of saturation, the said complex was added with increasing concentrations of 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120 μM (1 → 13, respectively), which are represented in part b. Similarly, part c represents the mode of Hoechst saturation (up to 4.8 μM represented in 1 → 12) with 30 μg/mL CT DNA. At this point of saturation, Cu(II) complex was added gradually (up to 100 μM), which is represented in spectra 1 → 11 of part d.

coiling in the plasmid DNA, resulting in the faster migration of the supercoiled form (RF1). Thus, the complex can efficiently interact with DNA and induces conformational change, resulting in a more compact configuration in the DNA.[44] However, the complex has no DNA cleavage activity in the concentration range we have tested.

It was reported earlier that some Cu(II) complexes induced DNA cleavage activity in the presence of reducing agents like H_2O_2.[36] Thus, we explored the possible nuclease activity of our complex in the presence of H_2O_2 too. We observed that H_2O_2 (1.6 M) alone was unable to induce DNA cleavage (Figure 6a, lane 8) in pUC19 DNA (500 ng), but when different concentrations (41.67−625 μM) of the said Cu(II) complex were pretreated with the indicated concentration of H_2O_2, DNA cleavage activity was observed with the increasing concentrations of the activated complex (compare lane 2 to 7 in Figure 6a). Here, the plasmid DNA was completely fragmented at a maximum concentration of 625 μM of the complex, which was less than the concentration of the same (820 μM) used in the gel electrophoresis experiment, shown earlier in Figure 5. It was also evident from the same figure that, with the increasing concentrations of H_2O_2-activated complex, form 1 (RF1) was

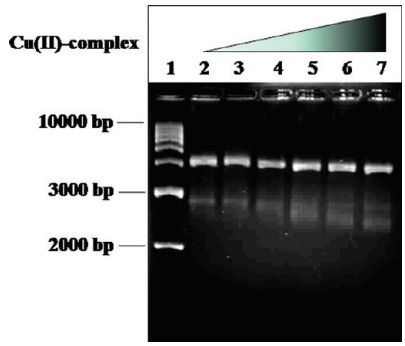

Figure 5. Agarose gel (2%) electrophoresis study of pUC19 plasmid DNA (500 ng) with increasing concentrations of Cu(II) complex. Purified pUC19 plasmid DNA was treated with increasing concentrations of the said complex (0, 0.08, 0.2, 0.4, 0.61, and 0.82 mM, in lanes 2 → 7, respectively) for 1 h and was run in a low potential gradient (2 V/cm) for 6 h. A low range DNA ruler (2−10 kbp) was used as a DNA marker (lane 1).

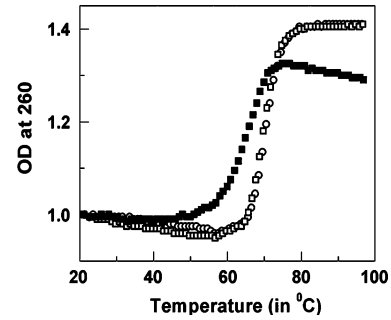

Figure 7. Optical melting profile of mock treated 50 μg/mL CT DNA (□) in the presence of either Cu(II) complex (○) only or peroxide pretreated complex (■) in a 1:1 molar ratio, over the range of temperatures from 20 to 98 °C (see text for details).

gradually converted into nicked (RF2) followed by the complete linearization (RF3) in the plasmid DNA. At higher concentrations (lanes 6 and 7), the DNA was degraded into much smaller fragments and ran out of the gel (Figure 6a). A similar result was obtained when the pUC19 DNA (500 ng) was incubated with a single concentration of the said Cu(II) complex (417 μM) pretreated with increasing concentrations of H_2O_2 (up to 138.5 mM) (Figure 6b). The nuclease activity of the complex pretreated with H_2O_2 was also observed in the case of calf thymus DNA (1000 ng) (Figure 6c, lanes 1−6). The kinetics of DNA degradation by H_2O_2 pretreated Cu(II) complex was also performed, where a single concentration of the activated complex (420 μM Cu complex:55 mM H_2O_2) was incubated with pUC19 DNA (500 ng) for 0 min to 60 min. As seen in Figure 6d, the amount of DNA degradation increased gradually with increasing time of incubation. Thus, from gel electrophoresis studies, it was evident that the H_2O_2 pretreated Cu(II) complex degraded the DNA in smaller fragments. Consequently, the absorption spectrum of the pUC19 plasmid DNA was increased by the H_2O_2 pretreated Cu(II) complex (data not shown), though the same was decreased by the Cu(II) complex treatment alone (Figure 2a).

On the contrary, an increment in the absorption spectrum of the DNA occurred either by induction of single-strandedness (DNA melting) from double-stranded DNA or by the degradation of the DNA molecules due to the nuclease activity of peroxide pretreated Cu(II) complex or partially by both at different portions in DNA. To explore that, we performed an optical melting study of the DNA either in the presence of Cu(II) complex alone or in the presence of H_2O_2 activated complex. It was observed that the melting point of DNA did not change much (Figure 7) due to the presence of Cu(II) complex alone (68.7 °C) compared to that of H_2O_2 activated complex (65 °C). From these data, we calculated the van't Hoff enthalpy for DNA melting in all of the cases and it was 21.72 kcal/mol for DNA

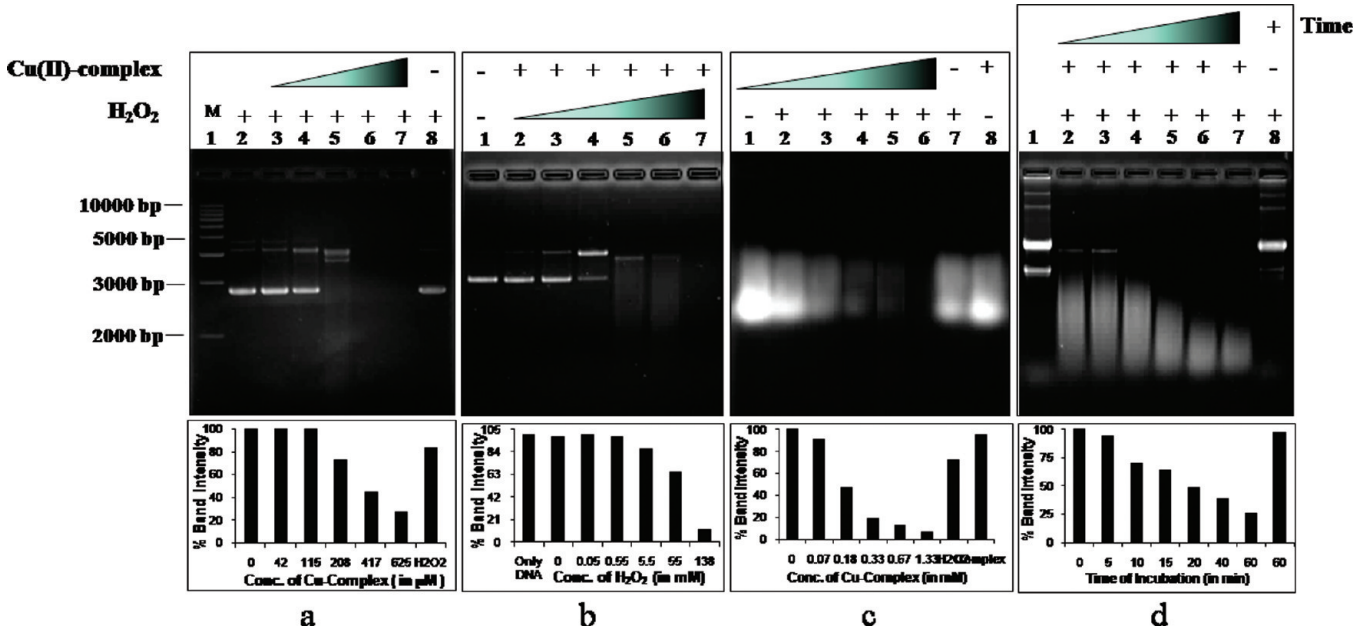

Figure 6. Agarose gel (1%) electrophoresis study of pUC19 plasmid (parts a, b, and d) DNA (500 ng) or chromosomal (part c) CT DNA (1000 ng) with H_2O_2 pretreated Cu(II) complex. (a) In aliquots, concentrations of Cu(II) complex were gradually increased (up to 625 μM) in the presence of a fixed concentration of H_2O_2 (1.6 M). (b) In aliquots, a fixed concentration of Cu(II) complex (417 μM) was preincubated with varying concentrations of H_2O_2 (up to 138 mM). (c) Concentrations of Cu(II) complex were gradually increased (up to 1.33 mM) in the presence of a fixed concentration of H_2O_2 (1.6 M) for CT DNA. (d) Temporal kinetics of plasmid DNA cleavage in the presence of Cu(II) complex (420 μM) pretreated with H_2O_2 (55 mM). The bar diagram below each agarose gel represents the percentage intensity of total DNA present in each lane with respect to the corresponding control.

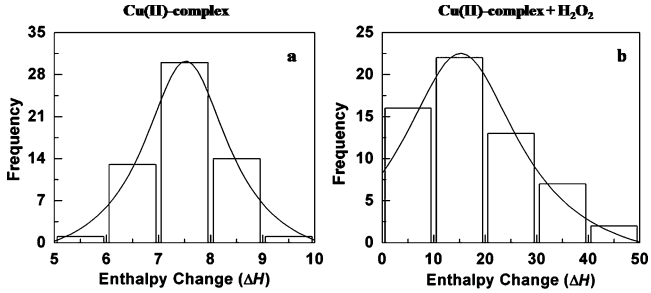

Figure 8. Isothermal titration calorimetric study of CT DNA (1 mM) with either Cu(II) complex alone (20 μM, part a) or H_2O_2 (2.6 mM) pretreated complex (part b). Distributions of endothermic enthalpy values after each injection of the ligand (complex or peroxide treated complex) to the DNA were obtained from two separate sets of experiments. A Gaussian fit for each set of enthalpy values is shown by the solid line (see text for details).

TABLE 1: Binding and Energetics of the Interaction between CT DNA and Cu(II) Complex, Pretreated with and without H_2O_2

sample	$K^a \times 10^4$ (M^{-1})	ΔG^b (kcal/mol)	ΔH^c (kcal/mol)	$T\Delta S^d$ (kcal/mol)
Cu(II) complex	0.40	−4.86	7.51	12.37
Cu(II) complex + H_2O_2	6.94	−6.53	15.27	21.80

[a] Affinity values were obtained from spectroscopic titration and fitting to a Benesi−Hildebrand plot. [b] Obtained from the equation $\Delta G = -RT \ln K$. [c] ΔH was elucidated from model free fitting of ITC data. [d] $T\Delta S$ was from the equation $T\Delta S = \Delta H - \Delta G$.

alone, 21.85 kcal/mol for DNA treated with Cu(II) complex, and 17.35 kcal/mol for the case of DNA incubated with H_2O_2 pretreated complex. These data indicated that the peroxide activated complex might unwind DNA slightly, though it could not induce complete denaturation.

The nuclease activity of the H_2O_2 activated Cu(II) complex was also thermodynamically established. Here, we estimated the energetics of the reaction in isothermal titration calorimetry (ITC). Single injection modes (model free titration) were performed in both Cu(II) complex−DNA and H_2O_2 activated Cu(II) complex−DNA systems. Individual addition of the complex or peroxide activated complex with CT DNA were found to be endothermic in nature, but binding of the activated complex with DNA was observed to be more endothermic. When the complex was titrated with DNA alone, heat was found to evolve, and after some time, the curve sloped down, indicating the termination of binding (data not shown). On the other hand, when the H_2O_2 activated Cu complex was titrated with DNA, a similar endothermic curve was obtained first, which eventually sloped in the upward direction, indicating some extra endothermic heat consumption due to the possible breakages in the DNA backbone (data not shown). We have plotted the change in enthalpy vs frequency of occurrence in bar diagrams, where it is evident that, in the case of H_2O_2 pretreated complex, the maximum frequency of ΔH was observed in the range 10−20 kcal/mol (Figure 8b) compared to 7−8 kcal/mol (Figure 8a) in the case of Cu(II) complex present with DNA alone. With the help of binding constants obtained from spectrofluorimetric data, thermodynamical parameters like ΔG, ΔS, and ΔH were estimated and are depicted in Table 1.

To explore the mechanism of activation of the Cu(II) complex in the presence of H_2O_2, we performed spectrophotometric study. As seen in Figure 9a, it was evident that the characteristic absorption spectrum of the Cu(II) complex was gradually deformed in the presence of H_2O_2. Moreover, the characteristic peak of the complex at about 294 nm gradually vanished with the increasing concentrations of H_2O_2. Thus, there might be an alteration in the chemical structure of the Cu(II) complex in the presence of H_2O_2. To explore this further, we performed a nuclear magnetic resonance study of the H_2O_2 activated Cu(II) complex. The characteristic peaks (Figure 9b) in the paramagnetic scale (positive axis) of the NMR spectrum clearly demonstrated that the oxidation state of copper in the complex was converted from Cu(II) to Cu(I) by the possible reduction mechanism of H_2O_2. Finally, agarose gel electrophoresis with pUC19 plasmid DNA (500 ng) was performed (Figure 10) in the presence of different radical scavengers, sodium azide, potassium iodide, and catalase to confirm the production of reactive oxygen species in the reaction between Cu(II) complex (417 μM) and H_2O_2 (550 μM). Sodium azide is a well-known singlet oxygen scavenger; potassium iodide can eliminate hydroxyl radicals,[49] and catalase can neutralize reactive hydrogen peroxide efficiently. As seen in Figure 10a, the reduced Cu(II) complex mediated plasmid DNA cleavages were completely protected in the presence of either of the two scavenging agents, KI and NaN_3 (compare the RF1 band intensities in lanes 4 and 5 with respect to lane 3). Similarly, incubation of catalase with the peroxide pretreated Cu(II) complex (Figure 10b) also proficiently protected the DNA molecules from nicking (compare the RF1 band intensities in lanes 4 and 5 with respect to lane 3).

Discussion

In this report, we have evaluated the DNA binding ability of a ternary Cu(II) complex consisting of malonate and protonated picoline as bridging ligands with metalated Cu(II) atom in the central position. The resulting structure of this complex is interesting from the crystallographic point of view, as it produces a supramolecular assembly in the solid state.[6] Among all of the transitional elements, several Cu(II) complexes are being synthesized presently because of their potential implications in chemotherapy, specifically to overcome the possible side effects of the known chemotherapeutic drugs.[50] Generally, the chemotherapeutic agents are tested for their ability to bind with DNA, as DNA is the potential target in the biological systems. Thus, the in vitro effect of the titled Cu(II) complex on DNA was aimed for in this study.

The solid state structure of the Cu(II) complex reveals that the Cu(II)−malonate units are hydrogen bonded with the protonated picoline moieties through the oxygen atoms.[51,52] In aqueous solution, the speciation would thus be $[Cu(mal)_2]^{2-}$ and $picH^+$ (both hydrated). The protonation status of these two species however depends on the pH of the media. Under the condition of DNA binding and nuclease activity experiments in citrate−phosphate (CP) buffer, pH 7.4, ligand exchange to generate Cu(II)−citrate or Cu(II)−phosphate complexes is not at all possible because of the much lower stability constants for the formation of the latter two species in comparison to that for the Cu(II)−malonate complex.[53] It is further supported by the fact that, under our experimental conditions, formation of any trace amount of insoluble Cu(II)−phosphate was not visualized.

Our spectrophotometric data suggest that the said complex binds with DNA, exhibiting a hypochromic effect with a slight red shift (258−261 nm) in the absorption spectrum (Figure 2a). The hypochromicity is due to the fact that this complex may bind to DNA in such a manner that the overall vibrational fidelity of the bases in the DNA is reduced. The extrinsic competitive fluorescence data indicate that the complex binds

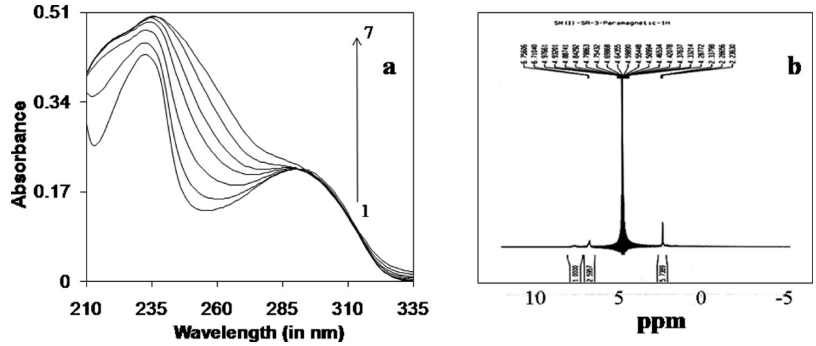

Figure 9. (a) Absorption spectra of Cu(II) complex (70 μM) with increasing concentrations of H_2O_2 (up to 15 mM represented in 1 → 7). (b) ^{1}H NMR spectrum of H_2O_2 pretreated Cu(II) complex in the paramagnetic scale (see text for details).

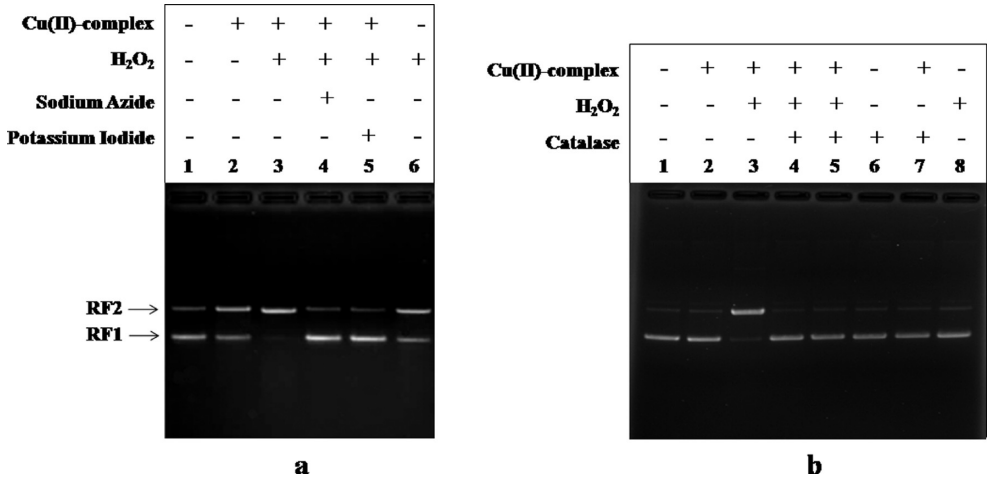

Figure 10. Agarose gel (1%) electrophoresis study of pUC19 plasmid (parts a and b) DNA (500 ng) with H_2O_2 (550 μM) pretreated Cu(II) complex (417 μM). (a) Sodium azide (50 mM) and potassium iodide (50 mM) were separately used as radical scavengers. After subsequent incubation with DNA, the mixture was run and shown in lanes 4 and 5, respectively (see text for details). (b) In a similar experiment, catalase (500 U/mL) was used as a hydrogen peroxide scavenger. The said enzyme was treated in aliquots before (lane 4) or soon after (lane 5) H_2O_2 addition with the complex and followed by incubation with DNA (see text for details).

to DNA both as a partial intercalator and a partial minor groove binder (Figure 4b,d). To understand whether the complex induces any conformational changes in the DNA, we have studied gel mobility shift assay with plasmid DNA. The induction of positive supercoiling can be explained by the faster migration of the supercoiled plasmid DNA (Figure 5) with the increasing concentrations of the complex. The ring structure of the malonate moiety in the complex may be responsible for this DNA binding ability. Since malonate rings in the complex are not completely planar, they can selectively interact with the minor groove and/or intercalate between the bases of the DNA. Hence, a saturated DNA groove binding or intercalation is not possible for the complex. On the other hand, from the absorption spectrophotometric study of purified Cu−malonate with DNA, the binding constant was estimated to be nearly 10-fold less than that of our Cu−malonate−picoline complex with DNA. The probable explanation of the greater binding constant in this case compared to that of only Cu−malonate is due to the fact that the negative charges in the Cu−malonate moiety for our complex are neutralized by the protonated picoline moiety, rendering the higher binding affinity toward DNA.

In the presence of H_2O_2, the complex might possibly induce conformational changes in the plasmid DNA by nicking and therefore by converting the supercoiled form to the relaxed form (Figure 6a). Moreover, with increasing either the concentration of the complex (Figure 6a,c) or H_2O_2 (Figure 6b) or the time of incubation (Figure 6d), the amount of DNA degradation was increased. These data strongly indicate that the production of reactive intermediates upon addition of H_2O_2 with the Cu(II) complex are responsible for the DNA cleavage activity.

It has been reported previously that some of the Cu(II) complexes can induce DNA cleavage activity directly or some redox active Cu(II) complexes can induce DNA cleavage in the presence of some reducing agents.[32−40] Although in our study the Cu(II) complex or H_2O_2 alone cannot induce DNA cleavage at the concentration range we have tested, in combination, they can cleave DNA efficiently. H_2O_2 mediated change in the redox potential of the Cu(II) in the complex may be responsible for the production of the intermediate free radicals, which eventually cleave the DNA. Binding of some complexes with DNA followed by the treatment with H_2O_2 has been reported earlier to induce nucleolytic activities. It has been proposed that the $(Phen)_2Cu(I)$ complex in the presence of hydrogen peroxide efficiently cleaves double-stranded DNA by oxidative attacks on C1′ and C4′ of 2-deoxyribose hydrogens by interacting within the minor groove, while the Phen−Cu(I) complex alone is less efficient.[54,55] The mechanisms of 1,10-phenanthroline−cuprous complex $[(OP)_2Cu+]$ with hydrogen peroxide possessing an artificial DNase activity that rapidly cleaves double-stranded DNA have been proposed to proceed via the oxidation of the deoxyribose to a resonance-stabilized furan derivative by hydroxyl radical production from the hydrogen peroxide oxidation of $[(OP)_2Cu+]$ intercalated in the double-stranded DNA.[56] Interestingly, we have also documented that our Cu(II) complex interacts with the DNA minor groove by partially removing Hoechst from the DNA as well as intercalating by partially

replacing ethidium bromide from the DNA. The partial nature of groove binding and/or intercalation may be due to the bulky structure of the said complex, where groove binding or intercalation of one unit of the complex prevents the binding or intercalation of the next unit to the subsequent DNA minor groove or base pairs, respectively.

Induction of either DNA denaturation or even cleavage can result in increase in the absorption pattern of the DNA.[57,58] Both of these two possibilities are partially true in our case, as the melting profile of DNA either alone or in the presence of Cu(II) complex did not change much in terms of both the melting point and van't Hoff enthalpy for DNA melting. However, in the presence of H_2O_2, the complex induced slight unwinding of the DNA, resulting in a slight decrement of the DNA melting point (Figure 7). On the contrary, circular dichroic spectroscopic study showed that Cu(II) complex either alone or in the presence of H_2O_2 did not change the spectral character of DNA at all (data not shown). Taken together, our data suggest that the complex itself binds with DNA mainly by hydrophobic and/or weak electrostatic interaction, whereas the H_2O_2 treated Cu complex does not produce significant unwinding during its DNA cleavage activity. Again, from gel electrophoresis study, we have provided clear evidence about the nuclease activity of the Cu(II) complex in the presence of H_2O_2. Thus, it seems likely that the complex in the presence of H_2O_2 acquires nuclease activity and in turn induces slight unwinding of DNA, which is firmly supported by the DNA melting analysis as described in the previous section. As a matter of fact, our data is in excellent agreement with an earlier study, where it has been reported that a reduced form of metronidazole breaks calf thymus DNA, where it also decreases the melting temperature of the DNA slightly.[59]

One step further, the DNA binding and nuclease activity of the H_2O_2 activated Cu complex was established thermodynamically by the isothermal titration calorimetric study. Any breakage in the 3′,5′-phosphodiester backbone of DNA is an endothermic process.[60] Consistent with that, a more negative Gibb's free energy, a high value in enthalpy of reaction, and a more positive entropy (Table 1) clearly demonstrate the DNA cleavage activity in the case of CT DNA treated with peroxide activated Cu(II) complex compared to that of Cu(II) complex alone. More entropy signifies (Table 1, fifth column) more randomness in a system with fragmented DNA molecules in the case of DNA incubated with H_2O_2 treated complex. It has already been reported that enzymatically any template directed DNA polymerization requires a range of thermal energy between 9.8 and 16 kcal/mol bp.[61] Our data is also consistent with that range of values (a central tendency of 15.27 kcal/mol bp in Table 1) for the DNA cleavage activity.

Thus, the nuclease activity of the peroxide pretreated Cu complex was characterized extensively; moreover, the mechanism of cleavage by this activated complex in terms of the possible changes in the redox status of copper were analyzed further. Earlier, in a separate fluorimetric analysis (spectra not shown), we observed that the binding affinity of this activated Cu(II) complex toward DNA was increased up to ~17-fold compared with that of the Cu(II) complex alone (comparing the binding constants in Table 1). The absorption spectrum of the complex itself was distorted with the gradual addition of H_2O_2, indicating the possible change in the coordination number of copper atom in the complex after the addition of this reducing agent (Figure 9a). Visually, the faint blue coloration of the complex turned into yellow on gradual addition of H_2O_2, indicating the possible conversion of Cu(II) to Cu(I), and subsequently our NMR data confirmed this conversion (peaks in paramagnetic scale of Figure 9b).

Several reports suggest that the possible reduction of Cu(II) to Cu(I) and followed by the redox cycling of the metal ion gives rise to the formation of reactive oxygen species, particularly production of either hydroxyl radical or singlet oxygen species in this process, are responsible for the DNA cleavage activity.[37,38,49,62] Similarly, in our experiment, when the hydroxyl radicals and singlet oxygen species were scavenged by potassium iodide and sodium azide, respectively, the nucleolytic activity of the peroxide treated complex in the plasmid DNA was completely abolished (Figure 10a). Superoxide radicals have generally a shorter half-life. In our experimental conditions, at room temperature (~30 °C) and physiological pH in the buffer system, when the complex was preincubated with H_2O_2 for 30 min, we eliminated the possibility of superoxide radicals exposure to the DNA. Similarly, addition of catalase in the said reaction before the possible reduction of Cu(II) in the complex by H_2O_2 was shown to inhibit the nuclease activity compared to that of the peroxide treated complex alone (Figure 10b). It is in good agreement with a previous study, where it has been demonstrated that, under reductive conditions (induced by MPA), the Cu-phen-nal complex behaves as a powerful nuclease, which is partially inhibited by catalase.[38]

Moreover, when we reduced the said complex by sodium citrate and hydrazine sulfate separately instead of using H_2O_2, no significant cleavage in the plasmid DNA was observed (data not shown). It clearly indicates that these reducing agents (sodium citrate and hydrazine sulfate) were unable to produce any free radicals for the degradation of DNA molecules. Thus, taken together, our data suggest that the generation of radicals by H_2O_2 in the redox cycling of copper in the complex is solely responsible for the nuclease activity.

Furthermore, as Cu(I) has a higher affinity than that of Cu(II) toward DNA (comparative fluorescence data in Table 1),[63,64] it can also be possible that the strong association of Cu(I) toward DNA makes the latter more accessible for the reactive oxygen species, resulting in the DNA cleavage activity.[65] A possible reaction mechanism of DNA cleavage involving Fenton type reaction[66,67] can be represented as follows:

$$\text{Cu(II) complex} + H_2O_2\ (\text{reducing agent}) \rightarrow \text{Cu(I) complex} + O_2$$

$$\text{Cu(I) complex} + \text{DNA} \rightarrow \text{Cu(I)}-\text{DNA complex (high affinity)}$$

$$\text{Cu(I) complex} + H_2O_2\ (\text{oxidizing agent}) \rightarrow \text{Cu(II) complex} + OH^- + HO^\bullet$$

$$\text{Cu(I)}-\text{DNA complex or free DNA} + HO^\bullet \rightarrow \text{DNA cleavage (weak)}$$

$$HO^\bullet + HO^\bullet \rightarrow H_2O + \text{ROS}$$

$$\text{Cu(I)}-\text{DNA complex or free DNA} + \text{ROS} \rightarrow \text{DNA cleavage}$$

Moreover, the crystal structure of the complex[6] clearly reveals that two axial Cu−O bonds are weak due to Jahn−Teller effects.[68] Being labile, these bonds may react with H_2O_2 and/or

OH radicals, forming reactive Cu−peroxide, which possibly has nucleolytic activity as reported earlier.[69] Thus, the unique nuclease activity of this Cu(II) complex in the presence of H_2O_2 needs to be explored further to investigate whether this cleavage in the DNA backbone is sequence specific or structure specific.

Conclusion

A novel Cu(II) complex, $[Cu(mal)_2](picH)_2 \cdot 2H_2O$, has been studied for its potential DNA binding and nuclease activity. The said complex binds to DNA as a partial intercalator as well as a partial DNA minor groove binder. The binding constant between the DNA and Cu(II) complex is in the order of 10^3 M^{-1}. The nuclease activity of the complex is achieved when it is pretreated with H_2O_2. Interestingly, when other agents like sodium citrate or hydrazine sulfate reduced the complex, no DNA cleavage was observed. In fact, the redox cycling of copper in the presence of H_2O_2 results in the production of ROS which is responsible for this DNA cleavage activity. Thus, the additions of radical scavengers inhibit this nuclease activity. A possible mechanism of DNA binding and peroxide mediated DNA cleavage activity by this complex has been discussed.

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