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DNA-Binding and Cleavage Studies of Zinc(II) Mixed-polypyridyl Complex

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The complex of Zn[(phen)(dione)Cl]ClO_4•H_2O (where phen is 1,10-phenanthroline and dione is 1,10-phenanthroline-5,6-dione) has been synthesized and characterized. The interaction of the complex with DNA was investigated using UV absorption, fluorescence spectroscopy and electrophoresis measurements. The results show that the complex mainly binds to the double helix of DNA with intercalation mode and the binding constant K is 2.4×10^4 mol^{-1}•L. Moreover, the complex can efficiently cleave plasmid DNA at physiological pH and temperature. The cleavage occurs via a hydrolysis mechanism, which is showed by adding radical scavengers, rigorously anaerobic experiments, analysis for malondialdehyde-like products, and the hydrolysis experiment of BDNPP with a rate constant k_{obs} of 5.3×10^{-6} s^{-1}.

Keywords zinc(II) complex, DNA, binding, hydrolytic cleavage

Introduction

Recently, the investigations on simple and efficient reagents, which can cleave nucleic acids under mild conditions, have been attracted considerable attentions.[1,2] Generally, in order to eliminate the possibility of significant cytotoxic side effect of reactive oxygen species, pathways that result in DNA cleavage by hydrolysis mechanisms are preferable.[3,4] As far, lanthanide ions and their complexes are primary hydrolysis cleavage reagents.[5,6] However, the catalytic center of many natural nucleases does not contain lanthanide ions, but contain some transition metal ions. So the studies on transition metal complexes as cleavage reagents have great application value in biological project, molecular biologic treatment and related fields. And more importantly, the studies can help us understand and clarify the role of metal ions in natural nucleases. Complexes composed of transition metals like copper, cobalt, nickel, ruthenium *etc.*, and bidentate polypyridyl ligands like 1,10-phenanthroline have been studied extensively for numerous applications including their biological activity as artificial nucleases and their rich photochemical and photophysical behavior,[2,7-10] and most studies of nucleic acid cleavage by the small molecules have been focused on photocleavage and oxidative cleavage.[11-16] In recent years, the studies on DNA hydrolysis cleavage by zinc complex have been attracting great interest in the filed of artificial metallonuclease[17-21] because zinc ion has been found at the catalytic sites of many natural nucleases. However, the nuclease activity of zinc polypyridyl complexes has less been investigated. We report here the synthesis, characterization, and properties of a new complex of Zn(II) with phen and dione. The results show that the interaction of the complex with DNA performs mainly in intercalative mode and the cleavage reaction of DNA in the presence of the title compound shows a different behavior from the copper(II)-1,10-phenanthroline system which is able to break the DNA chain in the presence of H_2O_2 by a mechanism similar to that observed in the Fenton reaction.[22,23] The zinc complex can efficiently cleave pBR322 DNA without addition of external agents and the pseudo- first-order rate constant of pBR322 DNA hydrolysis at pH 8.1 and 37 ℃ is estimated to be 0.21 h^{-1}.

Experimental

Materials and instruments

All chemicals were of reagent grade and were used without further purification unless otherwise noted. Calfthymus (CT) DNA and plasmid pBR322DNA were purchased from Sino-American Biotechnology. Agarose was purchased from Promega. Ethidium bromide was obtained from Fluka. Bis(2,4-dinitrophate) phosphate (BDNPP) was synthesized according to the reference method.[24] CT DNA was purified by standard procedure. A UV-Vis spectrophotometer was employed to check DNA purity (A_{260} : A_{280} > 1.9) and concentration (ε = 6600 dm^3•mol^{-1}•cm^{-1} at 260 nm). 1,10-Phenanthroline-5,6-dione and Zn(phen)Cl_2 were prepared from Refs. 25—26. All solution were prepared using double distill water.

Electrophoresis experiments were performed with

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pBR322DNA in fresh sterilized polypropylene tubes. Reactions with pBR322DNA were performed using 0.8 μg/μL pBR322DNA and varying concentrations of zinc complex in 5 mmol•L^{-1} Tris-HCl (pH＝7.26, containing 3.1 mmol•L^{-1} NaCl) buffer. The DNA solutions were incubated at 37 ℃. At the appropriate times, the reactions were quenched by addition of EDTA and bromphenol blue and the mixtures were analyzed by gel electrophoresis. Anaerobic experiments were performed in a nitrogen-filled glove bag. All buffer solution and water used in the experiment were equilibrated with nitrogen to aid the deoxygenation process followed. Reaction mixtures were prepared in a nitrogen-filled glove bag using the deoxygenated buffer solution and water and incubated in the glove bag.

Other measurements were conducted using solutions of the complex in Tris-HCl buffer (pH 7.16) containing 5 mmol•L^{-1} Tris and 50 mmol•L^{-1} NaCl.

UV-Vis spectra were recorded on a Hewlett Packard HP-843 chemstation spectrophotometer. Fluorescence experiments were performed with a Perkin-Elmer LS-50B spectrofluorometer. ^{1}H NMR spectra were recorded on a Bruker DRX 300 mHz NMR spectro-meter. Cleavage products were quantitated and analyzed with a Genesnap 4.0 complete gel documentation & analysis system.

The intrinsic binding constant K was determined according to Eq. (1),[27] where [DNA] is the total concentration of base pairs, ε_A, ε_F and ε_B correspond to the extinction coefficients for the absolutely bound zinc complex, free zinc complex, and the actually bound zinc complex, respectively. K was obtained from the ratio of the slope to the Y-intercept.

$$[DNA]/(\varepsilon_A-\varepsilon_F)=[DNA]/(\varepsilon_B-\varepsilon_F)+1/K(\varepsilon_B-\varepsilon_F) \quad (1)$$

The pseudo-first order rate constant of hydrolysis of BDNPP k (s^{-1}) was calculated from the slope of the linear plot of $\ln[a/(a-x)]$ versus time (t),[28] which a is the initial concentration of BDNPP and x is the concentration of BDNPP at t time.

Synthesis and characterization of Zn[(phen)(dione)Cl]ClO_4•H_2O

1,10-Phenanthroline-5,6-dione[25] (0.208 g, 1.00 mmol) and Zn(phen)Cl_2[26] (0.316 g, 1.00 mmol) were suspended in 20 mL of water and stirred for 25 h at room temperature, and the solution turned gradually to dark yellow, filtrated. 30% $NaClO_4$ solution was added. Dark yellow precipitate was distilled, washed with methanol and iced water twice, and dried in vacuum. The dark yellow product was recrystallized from MeCN/Et_2O (yield 44.1%). UV-Vis (MeCN) λ_{max}(log ε): 203 (4.94), 224 (4.72), 267 (4.58) nm; ^{1}H NMR (DMSO) δ: 8.9585 (dd, J＝8.23 Hz, 2H), 8.7382 (dd, J＝8.23 Hz, 2H), 8.5405 (s, 2H), 8.3469 (dd, J＝7.68 Hz, 2H), 8.0879 (dd, J＝7.68 Hz, 2H), 7.9364 (dd, J＝5.67 Hz, 2H), 7.6593 (dd, J＝5.67 Hz, 2H); IR (KBr) v: 3456 (b), 2725 (b), 1672 (s), 1508 (ms), 1423 (ms), 1137 (w), 1082 (w), 860 (s), 727 (s) cm^{-1}; molar conductivity: (DMF): 86.9 ohm^{-1}•cm^2•mol^{-1}. Anal. calcd for $C_{24}H_{16}N_4O_7Cl_2Zn$: C 47.39, N 9.22, H 2.65; found C 47.66, N 9.26, H 2.73.

Results and discussion

Binding studies of calf thymus DNA by the zinc complex

The electronic absorption spectra of complex (1×10^{-5} mol•L^{-1}) in the case of increasing amounts of DNA showed strong decreases in the peak intensities (Figure 1). Hypochromism was suggested to be due to a strong interaction between the electronic state of the intercalating chromophore and that of the DNA bases.[29] In addition to the decrease in intensity, a small red shift was also observed in the spectra. These spectral changes are consistent with the intercalation of the zinc complex into the DNA base stack. The plot of the absorption titration data according to Eq. (1) gives a linear plot and results in an intrinsic binding constant (K) of 2.4×10^4 mol^{-1}•L.

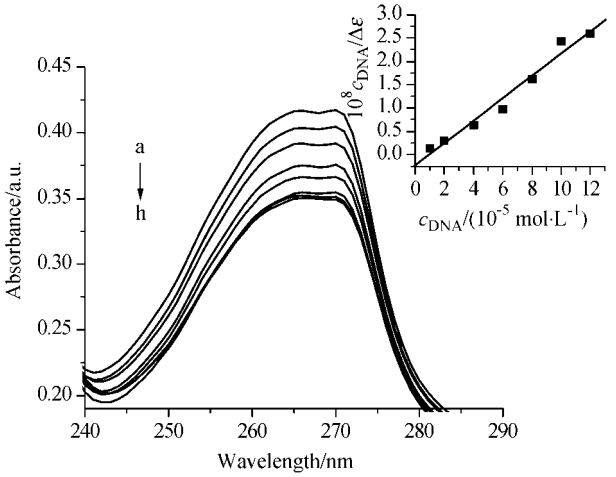

Figure 1 UV absorption spectra of complex in the presence of different molar ratio of $c_{DNA}/c_{complex}$ and plot of binding constant a—h (R_t): 0, 1.0, 2.0, 4.0, 6.0, 8.0, 10.0, 12.0.

To further clarify the interaction of the complex with DNA, the emission spectra of DNA-EB ($c_{DNA}=3.5\times10^{-5}$ mol•L^{-1}, $c_{EB}=2.5$ μg/mL) adduct have been measured upon the increase of the complex concentration. Ethidium bromide (EB) has long been used as a probe of DNA secondary structure as it can be intercalated into the double-stranded DNA and enhanced the sensitivity of fluorescence.[30] If the duplex of DNA decreases, the fluorescence of DNA-EB complex is quenched evidently. The fluorescence emission spectra of the zinc complex interaction with calf thymus DNA-EB with various R_t ($c_{DNA}/c_{complex}$) are shown in Figure 2. The fluorescence intensity of DNA-EB adducts decreases with the increasing concentrations of

the complex. This may be because the intercalation of zinc complex inhibits EB from binding to DNA.

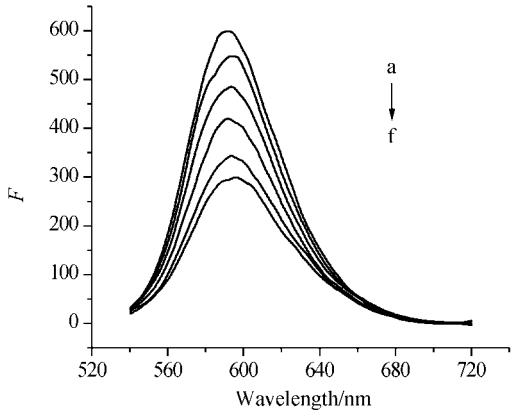

Figure 2 Influence of different molar ratio of $c_{complex}/c_{DNA}$ on fluorescence intensity of DNA-EB. a—f (R_t): 0, 2.2, 11.4, 29.8, 62.3, 88.2.

As a means of further exploring the interaction between complex and DNA (1.43×10^{-4} mol•L^{-1}), viscosity study was carried out. A classical intercalation model results in the lengthening of the DNA helix as the base pairs are separated to accommodate the binding ligand, leading to an increase in DNA viscosity.[31] Figure 3 shows that with an increasing amount of complex, the relative viscosity of DNA increases steadily, which suggests that the complex can bind to DNA by the classical intercalation.

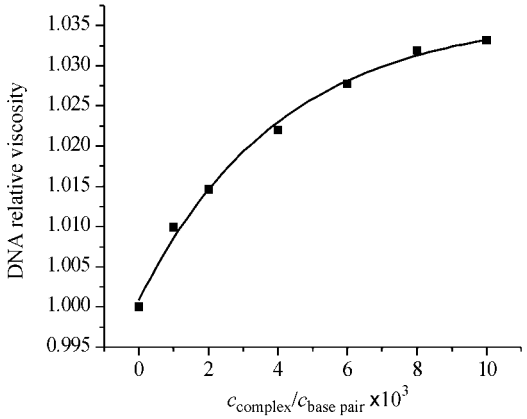

Figure 3 The effect of the complex on the relative viscosity of DNA.

Other strong evidence in support of the above interaction comes from thermal denaturation measurement. Interaction of small molecules into nucleic base pairs can stabilize the double helix, increasing the melting point of DNA.[32] The DNA melting curves in the absence and presence of zinc complex are presented in Figure 4. The increase in the melting temperature lends strong support for the intercalation of zinc complex into the helix.

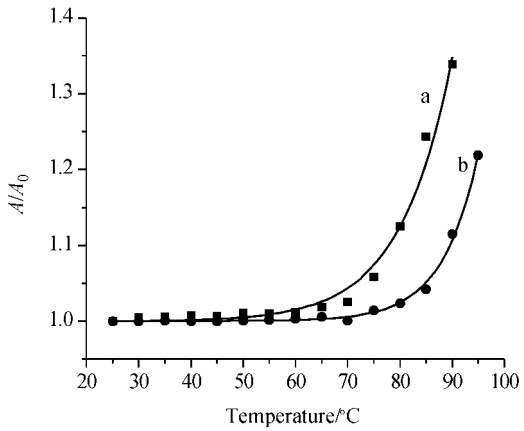

Figure 4 UV melting curves recorded at 260 nm for DNA in the absence (a) and presence (b) of zinc complex. c_{DNA} 90 μmol•L^{-1} and $c_{complex}$ 90 μmol•L^{-1}.

Cleavage studies of double-stranded pBR322 DNA by the zinc complex

The DNA-cleavage ability of zinc complex was examined under physiological pH and temperature. When plasmid pBR322DNA was incubated with zinc complex, the supercoiled DNA (form I) was transited to the open circular form II and then slowly to the linearized form III. Figure 5 shows agarose gel electrophoresis patterns for the cleavage of plasmid pBR322DNA after being treated with the complex of various concentrations at pH 7.26 (5 mmol•L^{-1} Tris-HCL, 3.1 mmol•L^{-1} NaCl) and 37 ℃ for 4 h. The initial concentration of DNA was set at 7.1 μmol•L^{-1}, and the concentration of the complex was varied from 0 to 5 mmol•L^{-1}. The conversions of form I to form II and to form III were observed with increases in the concentration of the complex. Form III began to appear in the presence of 0.1 mmol•L^{-1} complex (lane 4) and Form I was barely observable in the presence of 3 mmol•L^{-1}complex (lane 7).

Figure 5 Agarose gel electrophoresis patterns for the cleavage of DNA by various concentrations complex. Lane 1 DNA; lane 2—8 [$c_{complex}$/(mol•L^{-1})]: 10^{-5}, 5×10^{-5}, 10^{-4}, 5×10^{-4}, 10^{-3}, 3×10^{-3}, 5×10^{-3}.

In addition, a time course of a gel electrophoresis pattern of pBR322DNA cleavage during a reaction in the presence of 3 mmol•L^{-1} complex at pH 8.1 and 37 ℃ is shown in Figure 6. The reaction time was varied from 0—8 h. The conversion of form I to form II and to form III was observed with the increase of the reaction time, and the formation of form III began to appear after 2 h (lane 4). Form I was hardly observed after 6 h (lane

6). With reaction time increase, the amount of form III increased and form I was completely disappeared. The results show that the complex can effectively cleave the double-stranded DNA without addition of external agents. And the cleavage of DNA by the complex has strong dependence on the concentration of complex and reaction time.

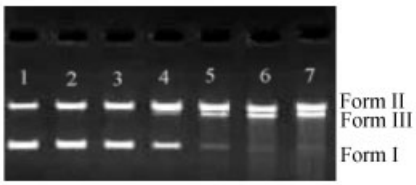

Figure 6 Agarose gel electrophoresis patterns for the cleavage of DNA by 3 mmol•L^{-1} complex in various reaction time. Lane 1: DNA 8 h; lane 2—7: 30 min, 1 h, 2 h, 4 h, 6 , 8 h.

The chemistry of DNA strand scission by zinc complex has been kinetically characterized by quantitation of supercoiled, nicked and linear DNA. The observed distribution of supercoiled, nicked and linear DNA in an agarose gel provides a measure of the extent of cleavage in each plasmid DNA, and we used these data to perform simple kinetic analyses. Figure 7 shows the mass fractions of DNA species present during a reaction under physiological conditions.

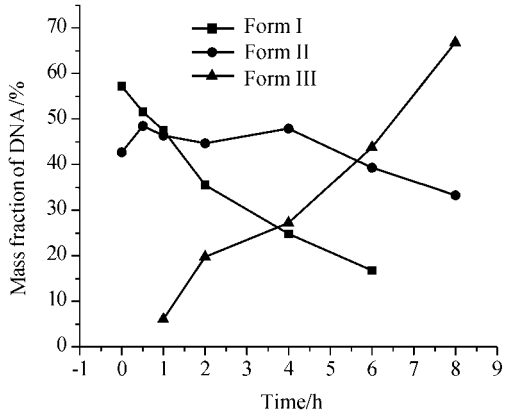

Figure 7 Mass fraction of DNA species during the cleavage reaction of pBR322DNA with 3 mmol•L^{-1} complex.

As shown, the decrease of form I fits well to a single-exponential decay curve. A straight line was obtained in log plots of form I fraction vs. time, indicating that the cleavage follows pseudo first-order kinetics, and a rate constant (k_{obs}) of 0.21 h^{-1}(Figure 8) was determined for the cleavage at 37 ℃, pH 8.1 and 3 mmol•L^{-1} complex.

It is well known that the activity of natural enzyme is strongly influenced by the pH of the reaction mixture. DNA scission vs. pH was also investigated over a pH range of 4—11. As can be seen from Figure 9, the pH-rate profile of k_{obs} of DNA cleavage shows a bell-shaped curve having maximum around pH 8, with an ascending leg from pH 4.95 and a descending leg beyond pH 10.95. The results indicate that the efficiency of DNA cleavage by complex is high in the neutral and weakly alkaline mediums. However, the cleavage is not obvious in the acidic or strongly alkaline mediums.

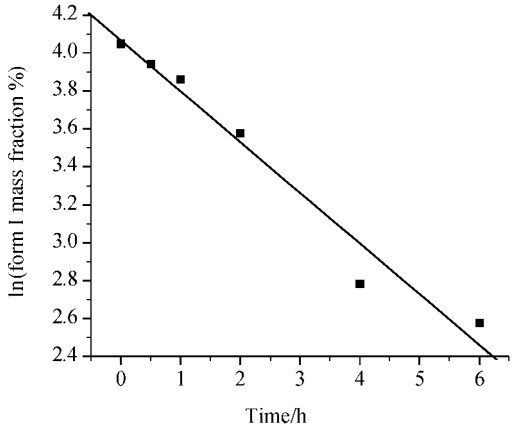

Figure 8 Kinetics plot for the decrease of form I DNA under the above conditions.

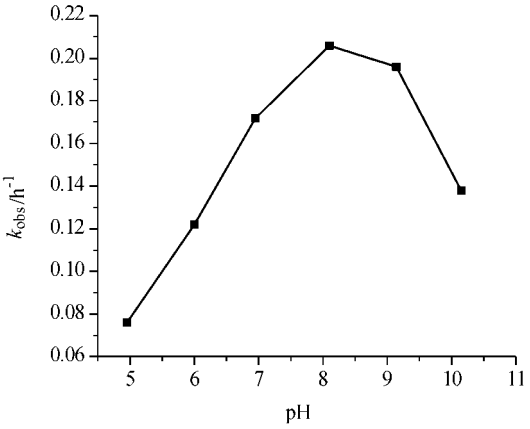

Figure 9 pH dependence of DNA cleavage.

Under the same condition (at pH 7.26 and 37 ℃ for 4 h), other analogous phen complex of Zn(II) (such as $Zn(phen)_3(ClO_4)_2$,[33] $Zn(phen)Cl_2$), free ligand phen and dione were also tested for their ability to cleave pBR322 DNA (Figure 10). At a concentration of 3 mmol•L^{-1}, only title complex generated form III and increased the fraction of form II DNA, while the other complexes more weakly cleaved DNA and ligands hardly cleaved DNA. The results suggest that the title complex is the most potent complex for the degradation of DNA.

Mechanism studies of double-strand scission chemistry

Although the complex cleavage system does not require addition of external agents, we were keen to discount the possibility that DNA cleavage was occurred via a hydroxyl radical-based depurination pathway. HO• scavengers such as DMSO, glycerol or methanol were introduced to afford a final concentration of 0.4 mol•L^{-1}

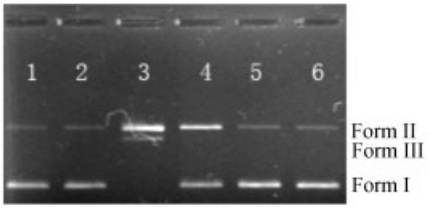

Figure 10 Agarose gel electrophoresis patterns for the cleavage of DNA by 3×10^{-3} mol•L^{-1} various complexes. Lane 1: DNA; lane 2: $Zn(phen)_3(ClO_4)_2$; lane 3: $Zn[(phen)(dione)Cl]ClO_4•H_2O$; lane 4: $Zn(phen)Cl_2$; lane 5: phen; lane 6: dione.

or 2.5 mol•L^{-1} before complex addition. Figure 11 shows that these radical scavengers have hardly influence on the efficiency of DNA cleavage. These results suggest that in the cleavage of DNA by complex diffusible hydroxyl radicals were not produced via the Fenton reaction.

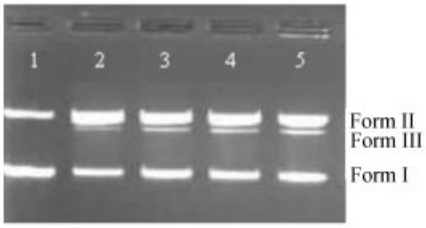

Figure 11 DNA strand scission by complex in the presence of HO• scavengers. Lane 1: DNA; lane 2: complex; lane 3: complex +0.4 mol•L^{-1} DMSO; lane 4: complex+0.4 mol•L^{-1} glycerol; lane 5: complex+2.5 mol•L^{-1} methanol.

Furthermore, in order to examine if oxidizing reducing reagents were required during the course of cleavage of DNA, H_2O_2 and ascorbic acid were added to the reaction mixtures respectively. The experiment of DNA cleavage under anaerobic conditions was also performed. The results show that the addition of oxidizing reducing reagents and oxygen hardly had influence on the efficiency of DNA cleavage (Figure 12).

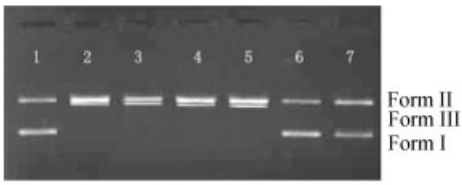

Figure 12 DNA strand scission by complex in the presence of oxidizing reducing reagents. Lane 1: DNA; lane 2: complex; lane 3: complex+5 mmol•L^{-1} H_2O_2; lane 4: complex+0.5 mmol•L^{-1} ascorbic acid; lane 5: complex under anaerobic conditions; lane 6: 0.5 mmol•L^{-1} H_2O_2; lane 7: 0.5 mmol•L^{-1}ascorbic acid.

It has been proved that oxidative cleavage of CT DNA yields malondiadehyde-like product, which reacts with 2-thiobarbituric acids to produce pink adducts absorbing at 532 nm on UV-Vis spectroscopy.[34,35] The analysis for malondiadehyde-like products was formed by adding 0.6% 2-thiobarbituric acid (TBA) to this reaction system. After calf thymus DNA had been treated with zinc complex at 37 ℃ for 24 h and analyzing the sample on spectrophotometer at 532 nm, malondiadehyde-like products were not determined. The observation indicates that the ring of ribose of CT DNA were not destroyed. However, under the same experimental conditions, after the zinc complex was substituted by the oxidative cleavage reagent Fe(EDTA)$^{2-}$/DTT, the absorbing at 532 nm was determined on UV-vis spectroscopy (Figure 13). Taken together, these data suggest that the double-stranded DNA cleavage mediated by zinc complex does not occur by an oxidative mechanism.

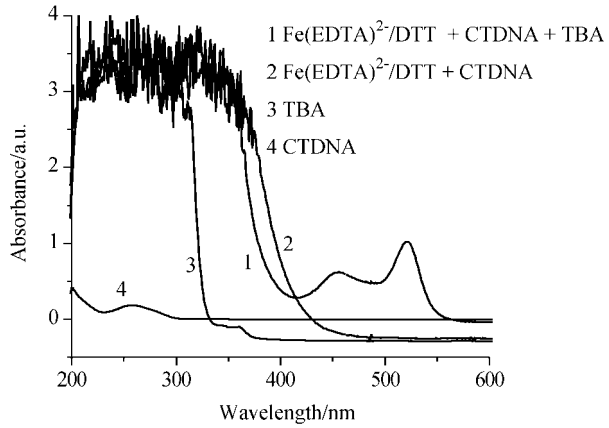

Figure 13a UV absorption spectra of interaction of Fe(EDTA)$^{2-}$/DTT and CT DNA in the presence of TBA.

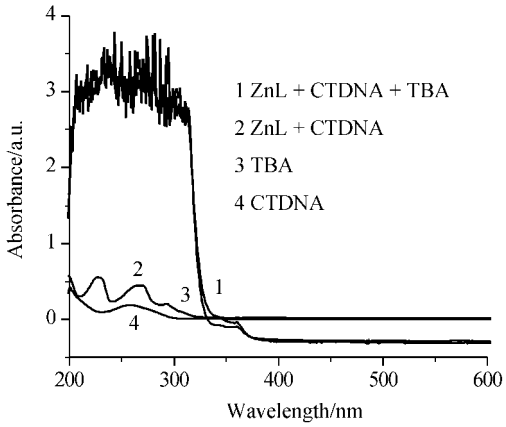

Figure 13b UV absorption spectra of interaction of Zn complexand CT DNA in the presence of TBA.

Hydrolysis studies of BDNPP by the zinc complex and proposed mechanism of phosphate-diester hydrolysis

In order to investigate the ability of complex to promote the hydrolysis of phosphate diester, we employed an activated phosphate diester with good leaving groups, bis(2,4-dinitrophate)phosphate (BDNPP), as a

model substrate for biologically relevant phosphate diester presented in nucleic acids. The rate of phosphate diester hydrolysis was studied at room temperature and pH 7.26. The concentrations of BDNPP and the complex were set at 5×10^{-4} and 5×10^{-3} mol•L^{-1} respectively. Figure 14 shows that two new peaks at 365 and 400 nm were more obvious with reaction time increase. As shown, phosphate diester of BDNPP was hydrolyzed by zinc complex.

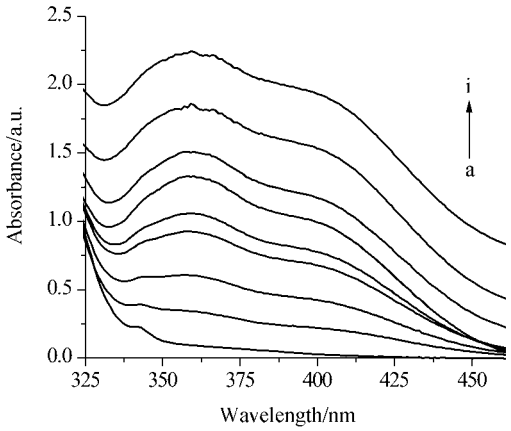

Figure 14 Absorbance of BDNPP in different reaction time. a —i: 0, 5, 12, 22, 28, 44, 58, 85, 110 h.

Moreover, the kinetic studies of BDNPP hydrolysis show that its rate constant is 5.3×10^{-6} s^{-1} and its half-life is 36.5 h in the presence of the complex (Figure 15). It is reported that the rate constant of hydrolysis of BDNPP is 1.8×10^{-7} s^{-1} and its half-life is 1.5 months at 25 ℃.[36] Thus results show thatcomplex largely promoted the hydrolysis of phosphate diester of BDNPP.

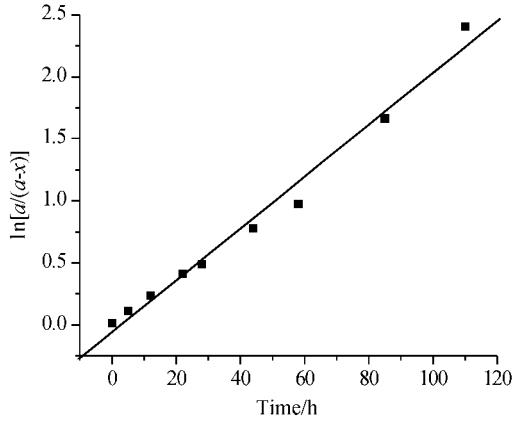

Figure 15 Kinetic plot of reaction of complex and DNA.

Hydrolytic degradation of DNA by nuclease is an important biochemical reaction in living cells. Divalent metal zinc ion has been confirmed to be the catalytic centers for many natural nucleases. We here propose the simple mechanism for zinc complex-catalyzed DNA phosphate-diester hydrolysis as follows.[37-39] It appears plausible that the Zn^{2+}-bound chlorin ion of zinc complex may be substituted by hydroxide ion (or water) in solution;[40] then the oxygen atom of hydroxide ion (or water) attacks the phosphorus atom and Zn^{2+} ion binds O^- ion of the phosphorus by electrostatic interactions to form a pentacovalent phosphorane intermediate, ultimately leading to P—O bond cleavage. Furthermore, phosphate-water and water-dione H-bonding could help to stabilize the intermediate (Figure 16). However, the hydrolysis mechanism may be much more complex than proposed; further research is warranted.

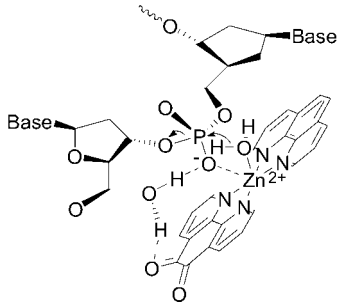

Figure 16 Proposed simple mechanism for the hydrolysis of DNA by the zinc complex.

Conclusion

In summary, we have showed that Zn[(phen)(dione)-Cl]ClO_4•H_2O mainly binds to DNA with intercalation mode and more importantly, the complex can effectively promote cleavage of plasmid DNA without addition of external agents at pH 7.26 and 37 ℃. The cleavage of double-stranded DNA by the zinc complex has strong dependance on the concentration of complex and reaction time. As mechanism studies of DNA cleavage shown, the cleavage of DNA by complex did not produced diffusible hydroxyl radicals and the addition of oxidizing reducing reagents and oxygen hardly had influence on the efficiency of DNA cleavage. Furthermore, interaction studies of the zinc complex and BDNPP indicate that the complex can hydrolyze phosphate diester present in nucleic acids. The cleavage reaction is proposed to proceed via a hydrolytic mechanism. Our finding may give a new application and open a new way in the design of more effective and useful zinc complex catalysts for DNA hydrolysis. However, the detailed mechanisms of zinc complex-mediated DNA hydrolysis warrant further study.

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