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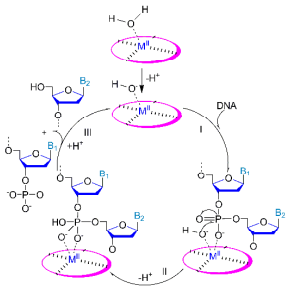
Graphical abstract

Copper and zinc complexes of diaza-crown ether serving as artificial nucleases exhibited high nuclease activities towards hydrolytic cleavage of DNA.



Copper and zinc complexes of diaza-crown ether as artificial nucleases for efficient hydrolytic cleavage of DNA

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Artificial nucleases are a kind of new non-enzymic breakage tool enzyme, which mimic the function of restricted enzyme and can catalyze nucleic acid cleavage highly efficiently and selectively. Some metal complexes are highly efficient cleavage agents for DNA. In this report, a compound of diaza-crown ether (L) was synthesized and its copper and zinc complexes (CuL and ZnL) were prepared to serve as artificial nucleases. The compositions of two metal complexes were confirmed by LC-MS analysis, and nuclease activity of the complexes towards pUC19 DNA cleavage was studied by agarose gel electrophoresis. The results indicate that two metal complexes can accelerated the breakage of DNA from supercoiled form (form I) to nicked form (form II) under the appropriate conditions; the optimal pH values of DNA catalytic cleavage are 8.01 for both CuL and ZnL; the complex ZnL was better catalyst in the DNA cleavage process than the complex CuL at low concentration; and unreactive μ-hydroxo dimers were formed to hamper the DNA cleavage process at high concentration. Under the presence of free radical scavengers, supercoiled plasmid DNA cleavage catalyzed by the complexes has been performed and their catalytic mechanisms have been investigated.

Introduction

Artificial nucleases have attracted extensive attention for their potential applications not only in molecular biology but also in the development of new drugs.[1-10] Due to their diversity in structure and reactivity, transition metal complexes as artificial nucleic acid cleaving agents, have been widely investigated.[2, 4, 6, 11-15] Generally, DNA cleavage reactions proceed *via* two major pathways: [16, 17] one is by an oxidative pathway targeting its basic constituents like base and/or sugar; the other one is by a hydrolytic pathway involving the phosphate group. And lots of transition metal complexes have been reported in the last years, and these complexes can cleave nucleic acids or phosphate esters not only by hydrolytic pathway [2, 4, 13] but also by oxidative pathway.[14, 18, 19] At present, although many domestic and international scholars focus on investigating the nucleic acid cleavage of the oxidative

pathway, the hydrolysis of nucleic acids has much more advantages,[20] such as basic constituents like base and/or sugar are not destroyed, free radicals are not produced and accessory factors are not needed in the cleavage reactions. Up to now, the activity of the metal complexes as artificial nucleases is still much lower than that of the corresponding enzymes.[21] Therefore, the research of nucleic acid cleavage by a hydrolytic pathway by artificial nucleases has significant research value and is a challenging topic.

Aza-crown ethers are known to be a kind of good complexing agents for transition-metal ions.[3, 22, 23] And some aza-crown ether transition metal complexes as artificial nucleases were found to promote nucleic acid or model phosphate esters hydrolytic cleavage effectively. For example, the dizinc(II) complexes of two aza-crown ethers, 1,4,7,16,19,22-hexaaza-10,13,25,28-tetraoxacyclo-triacontane and 4-(2-hydroxyethyl)-1,4,7,16,19,22-hexaaza-10,13,25,28-tetraoxacyclotriacontane, designed by Bianchi and co-workers[24, 25] increase the rate of phosphodiester hydrolysis of bis(*p*-nitrophenyl) phosphate (BNPP). Lu et al. [26] designed the copper(II) complexes of 1,7-dioxa-4,10-diazacyclododecane with double aminoethyl or guanidinioethyl side arms as the "bifunctional catalyst", which exhibit powerful efficiency for promoting the cleavage of the plasmid pUC19 DNA. Therefore, the complex of transition metal ion centre and the aza-crown ether might construct an effective catalytic model as artificial nucleases to promote DNA cleavage. But up to now, the research of aza-crown ether transition metal complexes serving as artificial nucleases was rarely reported due to the expensive and time-consuming synthesis of the aza-crown ether ligands and their complexes. In addition, in the matter of these reported works of aza-crown ether metal complexes as artificial nucleases for nucleic acids or model phosphate esters cleavage, it is a pity that the effect of the different branch chains of the ligand, the effect of the metal ionic radius of the complex, and the effect of different metal ions of the complex were rarely investigated and discussed.

In order to design efficient nuclease models, and study the effect of different metal ions for the DNA cleavage, two transition metal complexes (CuL, ZnL) of a diaza-crown ether, 1,4,10,13-tetraoxa-7, 16-diazacyclooctadecane (L), were designed as artificial nucleases to promote DNA cleavage. We report the synthesis of 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane (L) and its transition metal complexes CuL and ZnL. Moreover, we investigated the promoting effect of these diaza-crown ether transition metal complexes for the plasmid pUC19 DNA cleavage with agarose gel electrophoresis. We also studied the effects of pH, reaction time and the complex concentration on the hydrolysis of DNA. The mechanism of DNA cleavage was also studied and discussed in this work.

Experimental

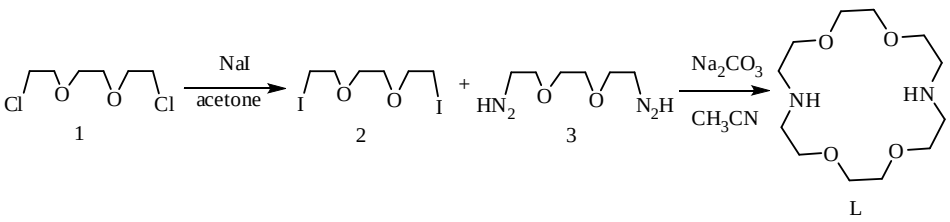
Chemicals and instruments

The mass spectrum of the ligand was run on an Agilent 1100 LC-MS (Agilent Technologies Co. USA.). The NMR spectrum of the ligand L was recorded on a Bruker 400MHz NMR Spectrometer (Bruker Co. Switzerland). The pH of the solution was determined by using a Sartorius PB-10 pH meter (Sartorius Scientific Instrument (Beijing) Co., Ltd., China). Melting points were determined on a SGW X-4A micro-melting point apparatus (Shanghai Shenguang Instrument Co., Ltd., China). DNA cleavage was analyzed by gel electrophoresis on DYY-12 electrophoresis meter (Beijing) and gel image analyzing system (Vilber Lourmat BIO-1D, France).

Plasmid pUC19 DNA, 50×TAE, 6×loading buffer, goldview dye, and agarose were purchased from Beijing Changsheng Biotechnology Co., Ltd. Trishydroxymethylaminomethane (Tris), HCl, NaCl, and NaOH were analytical grade products and used as supplied. 1,2-bis(2-chloroethoxy)ethane (1), 1,2-bis(2-diazenylethoxy)ethane (3) and NaI were purchased from Sigma Chemical Co.. All other chemicals purchased from Chongqing chemical Co., unless otherwise indicated, were of analytical grade. The water used for experiments was doubly distilled water.

Synthesis of the 1,4,10,13-tetraoxa-7,16-diazacyclooctadecane (L) [27]

The mixture containing 1 (19.0g) and NaI (33.3g) in acetone (50mL) was refluxed for 70h and then filtered and evaporated. The residue was dissolved in ether (160mL) and washed three times with water solution (240mL) of sodium thiosulfate (60.3g). The ether phase was evaporated to give 2 (32.5g). Then the solution of 3 (33.3g) in acetonitrile (100ml) was added slowly to the mixture of 2 (32.5g) in acetonitrile (400mL) and Na_2CO_3 (125.4g), after which the mixture was refluxed for 18h and then filtered and evaporated. Then the residue dissolved in the mixture solution of dioxane (50mL) and acetone (50mL), refluxed for 30min. After cooling and standing overnight, the crystals were precipitated, filtered and then dissolved in water (100mL). The solution was extracted with chloroform (350mL) for 18h. Evaporation of the chloroform phase gave a crude product, which was recrystallized with heptane for four times giving white solid L, Yield 1.89g (8.2%), m.p.: 111.0-112.3^{o}C; LC-MS: *m/z* 263.2 (M^+, 68%); ^{1}H NMR (ppm, $CDCl_3$) δ_H: 3.59~3.61 (m, 16H, CH_2O), 2.80 (t, J =9.2 Hz, 8H, CH_2N).



Scheme 1 Synthesis of compound L

Synthesis of the metal complexes

Synthesis of the complex $CuL(NO_3)_2 \cdot H_2O$ (CuL). 0.1208g (0.5mmol) of compound L and an equal amount of $Cu(NO_3)_2 \cdot 3H_2O$ was dissolved in 5mL ethyl acetate, respectively. Then 15 drops of triethyl orthoformate was added to the ethyl acetate solution and then was refluxed for 5h. After cooling, the ethyl acetate solution of L was added slowly to the ethyl acetate solution of $Cu(NO_3)_2 \cdot 3H_2O$, the blue precipitate was observed immediately. Stirring for 2h continually, then the blue precipitate was filtered, washed with a small amount of ethyl acetate, dried in vacuum, to give the metal complex. Yield 0.1608g (68.2%); LC-MS: *m/z* 468.4 (M^+); calcd for ($CuL(NO_3)_2 \cdot H_2O$): 467.1.

Synthesis of the complex $ZnL(NO_3)_2 \cdot H_2O$ (ZnL). 0.1482g (0.5mmol) of compound L and an equal amount of $Zn(NO_3)_2 \cdot 6H_2O$ were treated as in the previous CuL complex synthesis. Yield 0.1482g (80.2%); LC-MS: *m/z* 468.4 (M^+); calcd for ($ZnL(NO_3)_2 \cdot H_2O$): 468.1.

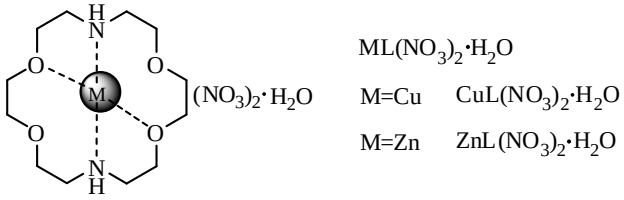


Fig. 1 Structures of metal complexes $CuL(NO_3)_2 \cdot H_2O$ and $ZnL(NO_3)_2 \cdot H_2O$

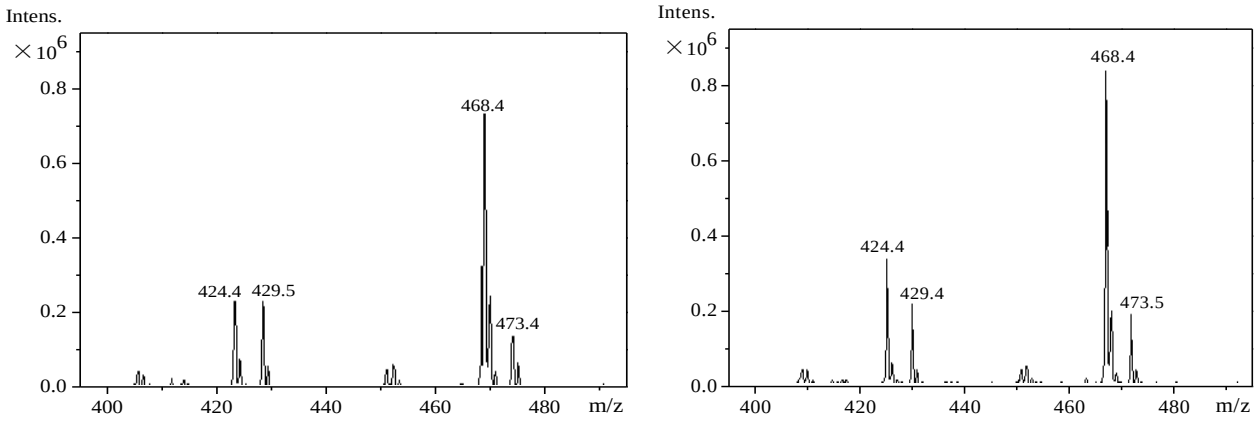


Fig. 2 MS spectra of metal complex (left, CuL; right, ZnL) in aqueous solution

Cleavage of plasmid DNA

The plasmid pUC19 DNA (25μg·mL^{-1}) in buffer (5 mM Tris-HCl/10 mM NaCl) at a certain pH was treated with increasing amount of the complex CuL or ZnL and then diluted with the buffer to a total volume of 13μL. The mixed solution was incubated at 37^{o}C for certain time. While for the DNA cleavage experiment in the presence of the typical radical scavengers, the incubation was carried out in the same buffer but in the presence of singlet oxygen (NaN_3), superoxide (KI), and hydroxyl

radical (DMSO and *t*-BuOH) scavengers; All reactions were quenched by the addition of 6×loading buffer (2μL) and then the resulting solutions were loaded on a 0.8 % agarose gel containing gold view dye. Gel electrophoresis was carried out at 60V for 1h in 1×TAE buffer, and then the plasmid bands were visualized by a transilluminator and quantified using Vilber Lourmat BIO-1D software.

Results and discussions

Stoichiometry of metal complex in solution

In order to verify the structure and stoichiometry of the complex formed by Cu(II)/Zn(II) ion and ligand L in the solution, the Cu(II) and Zn(II) complexes of ligand L were also synthesized. LC-MS analysis of these metal complexes was carried out at pH 7.0 aqueous solution (Figure 2). Form this figure, it is can be seen that: to Cu(II) complex, the peak at *m/z* 468.4 shows the $CuL(NO_3)_2 \cdot H_2O$ signal (calcd. 467.1); to Zn(II) complex, the peak at *m/z* 468.4 shows the $ZnL(NO_3)_2 \cdot H_2O$ signal (calcd. 468.1). These results assesses without doubt that these complexes are [M]/[L]=1:1 (M=Cu, Zn). LC-MS analysis indicate that the complex formed by Cu(II)/Zn(II) ion and ligand L is [M]/[L]=1:1 (M=Cu, Zn) metal complex. Therefore, the stock metal complex solutions for DNA cleavage assays were prepared by mixing equimolar amounts of ligand L and metal nitrate in buffer.

DNA cleavage activity

Agarose gel electrophoresis is a common method to analyze the DNA molecule. Upon this method, the locus of DNA can be confirmed in the agarose gel, further to confirm the molecular weight of DNA molecule and its fragments, to observe the conformational change. Therefore, the DNA cleavage promoted by the metal complex can be investigated by agarose gel electrophoresis.

The pUC19 plasmid exists in a compact supercoiled conformation (Form I). The supercoiled form of DNA can convert into the nicked circular form (form II) following the single strand cleavage of the supercoiled DNA, and can convert into the linear form (form III) following the double chain cleavage of the supercoiled DNA. In gel electrophoresis, form I usually moves faster toward the anode than that of form II, and the migration rate of form III is between form I and form II under the same conditions. [11, 12]

pH-dependence of the plasmid DNA cleavage

The acidity of the system is one of the important factors for catalytic reaction. Generally, the spatial conformation of the complex (as artificial nuclease) and the ionic state on the surface of the complex, which are interrelated to the catalytic activity of the artificial nucleases, are controlled by the acidity of the reaction system. The spatial conformation and the ionic state should been transformed, and the

complex catalytic activity should been changed as the changes of the acidity of the reaction system. Moreover, the stability of the intermediate formed from the substrate and the active species can be also changed with the change of the acidity of the buffer solution. Therefore, the acidity of the reaction system was strictly controlled in the catalytic reactions.

Figure 3 shows the pH-dependent profiles for DNA cleavage by metal complexes CuL and ZnL. From this figure, it can be seen that the pH-rate curve presents a ‘‘bell-shape’’ profile with the acidity change of the catalytic system, and nicked circular form (Form II) produced in pH range 6.06~9.03. When pH=8.01, the yield of form II achieves largest. Such phenomenon is similar to the works reported by Mao et al.[28] and Chin et al.[15] The reason explained is the deprotonation of the metal-coordinated water molecule leads to the formation of the metal-bound hydroxide acting as an active nucleophile. However, when pH value is higher than 8.01, the yield of form II decreases rapidly. Such behavior observed may be due to the second deprotonation forms an anionic species, which binds to the metal ion more tightly than the conjugated acidic precursor.[15] As a consequence, the available coordination sites on the metal ion are saturated leaving little or no chance for the coordination of the substrate. Hence, for all other DNA cleavage assays in this work were performed in Tris-HCl buffer with a pH of 8.01.

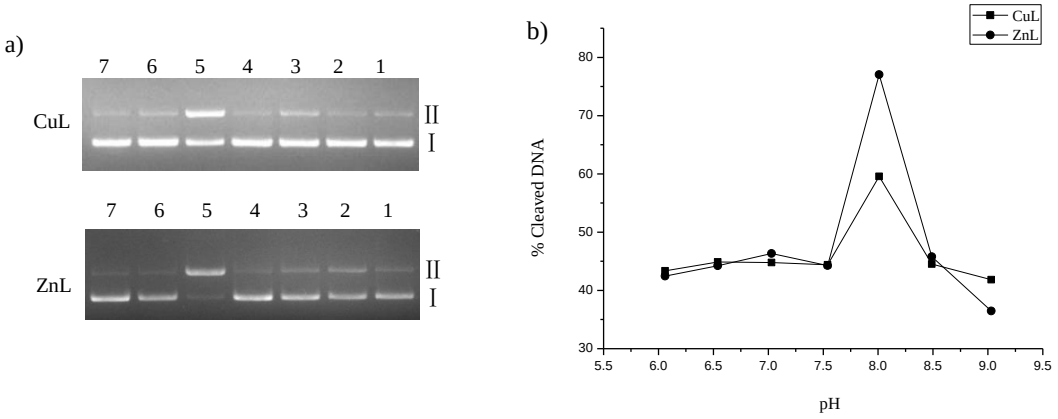


Fig. 3 Profiles of the acid effect for pUC19 plasmid DNA (0.025μg·μL^{-1}) cleavage promoted by $CuL(NO_3)_2$ H_2O (CuL, 1.54×10^{-5}mol Ł$^{-1}$) and $ZnL(NO_3)_2$ H_2O (ZnL, 1.54×10^{-5}mol Ł$^{-1}$) in buffers (5 mM Tris-HCl/10 mM NaCl) of different pH at 37^{o}C for 9h. Lanes 1-7, pH 6.06, 6.54, 7.03,7.54, 8.01, 8.49, 9.03, respectively.

Concentration dependence assays of DNA cleavage

Figure 4 shows agarose gel electrophoresis of pUC19 plasmid DNA cleavage by different concentrations of the complexes CuL and ZnL. From this figure, it is can be observed that the amount of the cleaved DNA (Form II) increases as the concentrations of metal complex in range from 0 to 7.69×10^{-5} mol Ł$^{-1}$, but when the concentration of metal complex further increases, the amount of the cleaved DNA decreases. Such unusual behavior is similar to the works reported by Lu

et al.[2] and Palumbo et al..[14] In their reports, they suggested that the increase can be attributed to a very high affinity of the metal complex for the DNA backbone and then the decrease can be ascribed to the formation of unreactive μ-hydroxo dimers, which hampers the transformation of supercoiled form (Form I) to nicked circular form (Form II).

Generally speaking, Lewis acidity of metal cation can not only produce polarization effect through activating phosphate ester bonds in the nucleophilic attack, but also can provide the hydroxyl anion attached to the metal in neutral pH. Therefore, the catalytic efficiency of Cu complex is generally higher than that of Zn complex because of Lewis acidity of Cu^{2+} is higher than that of Zn^{2+}.[29] However, from Figure 4, it can be seen that when the concentrations of metal complex is in the range from 0 to 7.69×10^{-5} mol L^{-1}, the catalytic efficiency of the complex ZnL is higher than that of the complex CuL. Zn^{2+} is a Lewis acid of the moderate soft-hardness,[30] and the ionic radius of Zn ion is larger than that of Cu ion, which should provide the more available coordination space to the ligand and the substrate, and is beneficial to combination with DNA molecule to form the intermediate. This may be the reason that the catalytic activity of the complex ZnL is higher than that of the complex CuL.

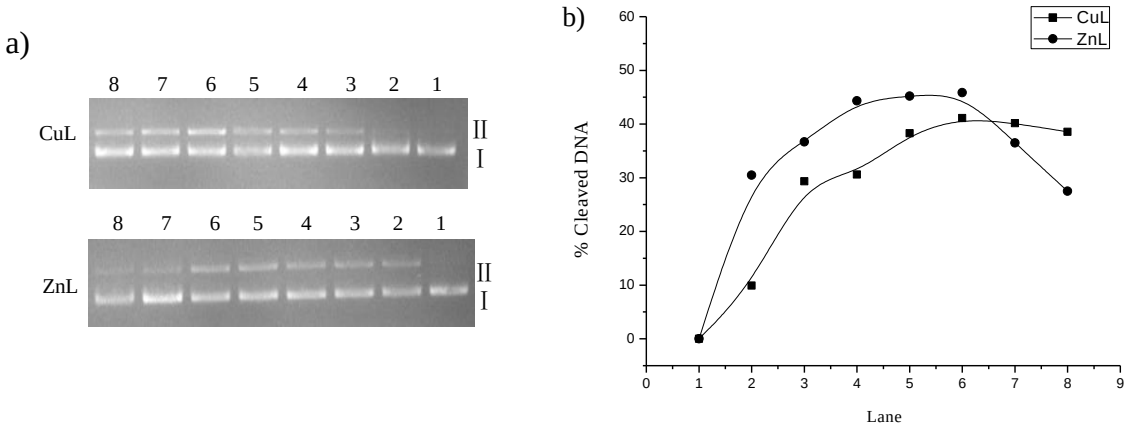


Fig. 4 Agarose gel electrophoresis of pUC19 plasmid DNA ($0.025\mu g\cdot\mu L^{-1}$) cleavage by different concentrations of $CuL(NO_3)_2\cdot H_2O$ (CuL) and $ZnL(NO_3)_2\cdot H_2O$ (ZnL) metal complexes in pH=8.01 buffer(5 mM Tris-HCl/10 mM NaCl) at 37°C for 9h; Lanes 1-8: 0, 7.69×10^{-7}, 3.85×10^{-6}, 7.69×10^{-6}, 3.85×10^{-5}, 7.69×10^{-5}, 3.85×10^{-4}, 6.15×10^{-4}mol L^{-1}, respectively.

Figure 4 also show that when the concentration higher than 7.69×10^{-5} mol L^{-1}, the amount of the cleaved DNA decreases sharply by the complex ZnL while the amount of the cleaved DNA decreases slowly by the complex CuL, and the cleavage efficiency of the complex CuL higher than that of the complex ZnL. Such behavior may be ascribed to the ionic radius of Zn ion is larger than that of Cu ion, and Zn ion is more easily form unreactive μ-hydroxo dimer than Cu ion, thus hampers the transformation of supercoiled form (Form I) to nicked circular form.

Time course of pUC19 DNA cleavage

Figure 5 shows time course of pUC19 DNA cleavage promoted by complexes CuL and ZnL. It reveals that the longer the reaction time at 37°C, the more transformation of plasmid DNA from Form I to Form II. However, the linear DNA (Form III) was not observed in this work. This result is in agreement with the works obtained by Lu et al.[2] using Cu^{2+} complex of cyclen containing pyridine subunit and by Mao et al.[31] using Zn^{2+} complexes of disubstituted 2,2′-bipyridine with ammonium groups as DNA cleaving agents. In addition, it is also can be seen that the catalytic efficiency of the complex ZnL is higher than that of the complex CuL.

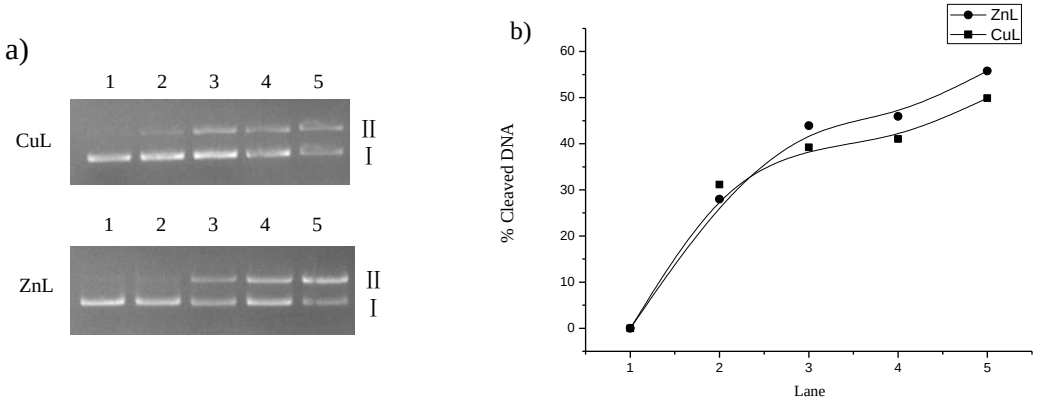


Fig. 5 (a)Time course of pUC19 DNA (0.025μg·μL^{-1}) cleavage promoted by $CuL(NO_3)_2 \cdot H_2O$ (CuL, 1.54×10^{-5} mol·L^{-1}) and $ZnL(NO_3)_2 \cdot H_2O$ (ZnL, 1.54×10^{-5}mol·L^{-1}) in 8.01 buffers (5 mM Tris-HCl/10 mM NaCl)at 37°C; lanes 1-5: reaction time 0, 1, 5, 10, 20h, respectively. (b)Quantification of pUC19 DNA forms.

The comparison of catalytic activity between the ligand, metal ions and metal complexes

Based on the pH-dependence, concentration dependence and time course assays of DNA cleavage, the catalytic efficiency of the complex ZnL is higher than that of the complex CuL in low concentrations of metal complexes at pH 8.01. Such result is in agreement with the works reported by Jang et al.[32] using $[M(2,2'\text{-dipyridylamine})_2(NO_3)_n]^{x+}$(M = Cd, Cu, Ni, Zn, n = 1,2, x = 0,1) complexes and by Song et al.[33] using metal(II) benzoates (M=Co, Ni, Cu, Zn, Mn, and Cd) with 4,4′-bipyridine as DNA cleavage agents. But the result is contrary to the work reported by Yu and coworkers[34] using mononuclear macrocyclic polyamine Zn(II), Cu(II), Co(II) complexes linking with uracil as DNA cleavage agents. Therefore, besides the characteristics of metal ions, the structure of the ligand is also an important factor influencing the activities of the artificial nuclease.

From the experimental results, we can see that both CuL and ZnL exhibited high nuclease activities towards the cleavage of supercoiled plasmid DNA. But there are some reports showed that some metal-free cleaving reagents also exhibited high nuclease activities in cleaving DNA.[35, 36] In order to confirm that if the diaza-crown ether as nuclease alone can also shows the high nuclease activity, the pUC19 plasmid DNA cleavage assay by diaza-crown ether and metallic ions as nuclease

alone was performed in this work. The experimental results are shown in figure 6. From figure 6, it can be seen that the diaza-crown ether alone hardly cleave the DNA, and the metallic ions slightly cleave DNA from supercoiled form to nicked form. But the metal complexes CuL and ZnL exhibited high nuclease activities, and the catalytic efficiency of the metal complexes is much higher than that of the metal ions. These results prove that CuL and ZnL are the active species for DNA cleavage in the complex solution.

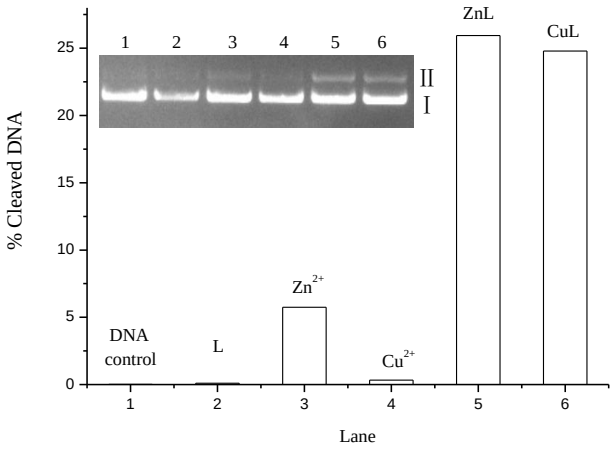


Fig. 6 Agarose gel electrophoresis of pUC19 plasmid DNA (0.025μg·μL^{-1}) cleavage by $Cu(NO_3)_2$ $3H_2O$ (Cu^{2+}, 1.54×10^{-5}mol Ł$^{-1}$), $Zn(NO_3)_2$ $6H_2O$ (Zn^{2+}, 1.54×10^{-5}mol Ł$^{-1}$), L(1.54×10^{-5}mol Ł$^{-1}$), $CuL(NO_3)_2$ H_2O (CuL, 1.54×10^{-5}mol Ł$^{-1}$) and $ZnL(NO_3)_2$ H_2O (ZnL, 1.54×10^{-5}mol Ł$^{-1}$) in pH=8.01 buffer (5 mM Tris-HCl/10 mM NaCl) at 37^{o}C for 9h; Lanes 1-6: DNA control, L, $Zn(NO_3)_2$ $6H_2O$, $Cu(NO_3)_2$ $3H_2O$, ZnL, CuL, respectively

Studies on mechanism of DNA cleavage

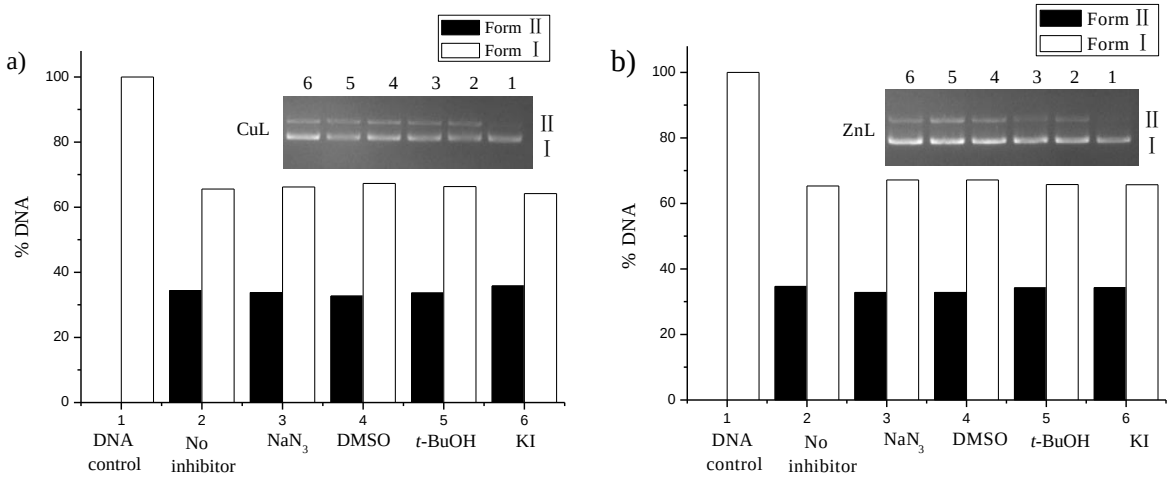
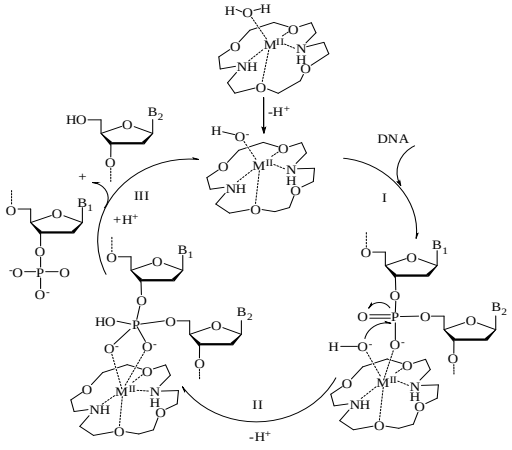


Fig. 7 Histogram representing of pUC19 plasmid DNA (0.025μg·μL^{-1}) cleaved by $CuL(NO_3)_2$ H_2O (CuL, 1.54×10^{-5} mol Ł$^{-1}$) and $ZnL(NO_3)_2$ H_2O (ZnL, 1.54×10^{-5}mol Ł$^{-1}$) in the presence of standard radical scavengers for singlet oxygen (NaN_3, 10 mM), for superoxide (KI, 10 mM) and for hydroxyl radicals (1 mM DMSO and 1 mM *t*-BuOH), incubated for 9 h at 37^{o}C in pH 8.01 buffer (5 mM Tris-HCl/10 mM NaCl). Lanes 1-6: DNA control, No inhibitor, NaN_3, DMSO, *t*-BuOH, KI, respectively.

Transition metal complexes can cleave nucleic acids or model phosphate esters by both hydrolytic pathway[2, 4, 13] and oxidative pathway.[14, 18, 19] In order to explore the mechanism of plasmid DNA cleavage by these complexes, the typical radical scavengers for singlet oxygen (NaN_3), for superoxide (KI), and for hydroxyl radical (DMSO and *t*-BuOH) were introduced to DNA cleavage assays (Figure 7).[2, 26] From figure 7, no significant inhibition effect on the DNA cleavage was observed in the presence of typical radical scavengers (NaN_3, DMSO, *t*-BuOH, KI), which indicate that the reactive oxygen species might not exist in the reaction.[26] Therefore, the reactive oxygen species is not necessary for the DNA cleavage catalyzed by the complexes CuL and ZnL, and DNA cleavage promoted by the two complexes might not take place *via* hydroxyl radical oxidative cleavage, but occur with a hydrolytic pathway.

It has been widely accepted that metal-bound hydroxyl species are the active species in the hydrolytic cleavage of DNA or model phosphate esters.[10, 20, 37, 38] Therefore, based on the pH-dependence of the plasmid DNA cleavage assay, lead us to put forward a hypothesis of that the deprotonation of a metal-coordinated water molecule to form the active nucleophile of a metal-bound hydroxide (CuL(OH) or ZnL(OH)). And such metal-coordinated hydroxide is an effective nucleophile in the DNA cleavage process. Hence, a tentative mechanism of DNA cleavage catalyzed by the metal complexes is proposed as Scheme 2. In Scheme 2, 5′-P-O scission of DNA is presented here, but 3′-P-O scission can also occur. Further studies should be conducted to satisfy the proposed mechanism in the future.



Scheme 2 Proposed hydrolytic mechanism of supercoiled DNA

Conclusions

In summary, both Cu(II) and Zn(II) complexes based on diaza-crown ether designed and synthesized as artificial nucleases exhibited high nuclease activities towards cleavage of supercoiled plasmid DNA. The DNA cleavage promoted by the two complexes takes place via the hydrolytic cleavage pathway and the hydrolytic mechanism is proposed. These results show that the transition-metal

complexes based on diaza-crown ether possessing a specific structure can be feasible artificial hydrolytic nuclease models. This study proved that the acidity of the catalytic system is one of the important factors in cleaving DNA, and the catalytic efficiency of the complexes is associated with the ionic radius of the transition metal ions. It is to be noted that the catalytic efficiency of Zn(II) complex is higher than that of Cu(II) complex at low concentration. And as for DNA cleavage catalyzed by the metal complexes based on diaza-crown ether, it is necessary to further study some other factors affecting the catalytic activity, such as the size of aza-crown ring, the nitrogen-oxygen atom number in aza-crown ring, the metal ionic radius and charge number and so on.

Acknowledgements

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