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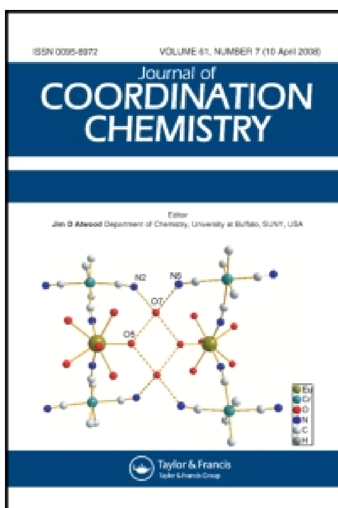

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Synthesis, interaction with DNA and nuclease activity of zinc complexes of 2,2′-bipyridine derivatives with tetraalkylammonium groups

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Two zinc complexes of disubstituted 2,2′-bipyridine with tetraalkylammonium groups, $[Zn(L^1)_3](ClO_4)_8 \cdot 12H_2O$ (**1**) and $[Zn(L^2)_3](ClO_4)_8 \cdot 2H_2O$ (**2**) ($L^1 = [4,4'-(Et_3NCH_2)_2\text{-bpy}]^{2+}$, $L^2 = [4,4'-((n\text{-Bu})_3NCH_2)_2\text{-bpy}]^{2+}$ and bpy = 2,2′-bipyridine), have been synthesized and characterized. Species distributions in aqueous solution of both complexes were determined by potentiometeric titration. The interaction of these complexes with calf thymus DNA was explored by UV spectroscopic titration and CD spectroscopy, suggesting outside electrostatic binding mode. Both complexes were applied to the cleavage of plasmid pBR322 DNA under hydrolytic conditions; **1** can effectively cleave DNA from supercoiled to nicked form, showing quite high nuclease activity, while **2** has limited ability to cleave DNA.

Keywords: Nuclease activity; Zinc complexes; Tetraalkylammonium groups; 2,2′-Bipyridine; DNA cleavage

1. Introduction

Phosphodiester bonds of DNA are extraordinarily resistant to hydrolysis under uncatalyzed physiological conditions. Natural nucleases markedly accelerate the hydrolysis of DNA. Activity of these enzymes mostly depends on their active sites containing transition metal ions, such as Zn(II), Mn(II), Fe(III) and Ca(II), etc. Although many of these enzymes are currently used in the laboratory [1], the development of artificial nucleases has attracted interest [2–8]. Artificial nucleases are usually based on Cu(II), Co(III), Zn(II), Fe(III) and lanthanide ions [9]. Zinc is the only metal frequently encountered in natural and artificial nucleases. Since the activity of nucleases based on Zn(II) is somewhat lower than other usually employed transition metal complexes, zinc-based artificial nucleases reported to date are rare. In spite of their relatively low activity, zinc-based nucleases offer some advantages over other metal-based nuclease [1, 8, 9]: Zn(II) is a strong Lewis acid, and its complexes exchange ligands rapidly in solution; it is less toxic than other transition metal ions; it has no

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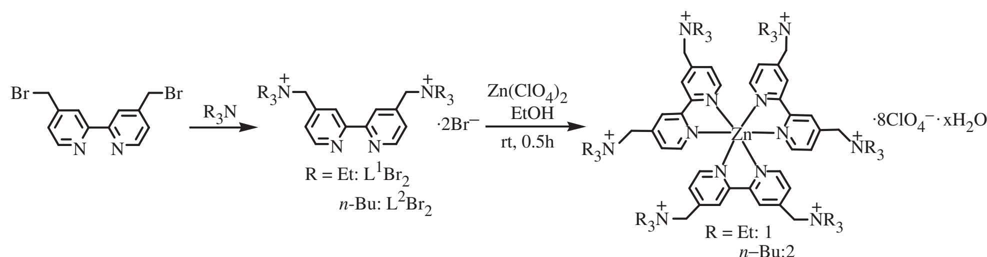

Scheme 1. Synthesis of zinc complexes **1** and **2**.

ligand field stabilization energy adopting suitable coordination geometry to fulfill the structural requirement of a reaction; the final and most important factor is that Zn(II) is not redox active, ruling out oxidative cleavage pathways which may induce radical damage to DNA. Therefore, the development of zinc-based artificial nucleases is significant.

Many reported zinc-based artificial hydrolases can be categorized into several groups: a family based on macrocyclic polyamine [1, 10–12], a family based on peptide derivatives [10, 13–16] and a family based on ligands with appended intercalating groups [1, 10, 13, 14]. The design of some of the above-mentioned artificial hydrolases focuses on increasing DNA affinity by introduction of intercalating groups. Another important strategy, the introduction of functional groups (for example, amino, guanidine, ammonium groups, etc.), has been applied to some artificial hydrolases [17–22] to increase their activity. However, most of these hydrolases were used to cleave model nucleic acids. The cleavage of DNA (one of the most challenging hydrolysis substrates) by zinc-based artificial nucleases with function groups has seldom been reported.

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In this work, two Zn(II) complexes of disubstituted 2,2′-bipyridine with tetraalkylammonium groups, $[Zn(L^1)_3](ClO_4)_8 \cdot 12H_2O$ (**1**) and $[Zn(L^2)_3](ClO_4)_8 \cdot 2H_2O$ (**2**) ($L^1 = [4,4'\text{-}(Et_3NCH_2)_2\text{-bpy}]^{2+}$, $L^2 = [4,4'\text{-}((n\text{-Bu})_3NCH_2)_2\text{-bpy}]^{2+}$ and bpy = 2,2′-bipyridine), have been synthesized (scheme 1). We describe here the preparation, characterization, interaction with DNA and DNA cleavage activities of these Zn(II) complexes.

2. Experimental

2.1. *Chemicals and reagents*

4,4′-Dimethyl-2,2′-bipyridine was purchased from Alfa Aesar Co., Ltd.; pBR322 DNA was purchased from MBI Co.; calf thymus DNA (CT-DNA) and ethidium bromide were purchased from Sino-American Biotechnology. Other reagents of analytical grade were obtained from commercial sources and used as received. Milli-Q water was used in all physical measurement experiments.

CAUTION! Perchlorate salts are potentially explosive and should always be handled with care and in small scale.

2.2. *Physical methods*

UV/VIS spectra were scanned on a Varian Cary 300 UV/VIS spectrophotometer. CD spectra were recorded on a Jasco J-810 spectropolarimeter. ^{1}H NMR spectra were collected on a Varian Mercury-Plus 300 NMR spectrometer. ESI-MS spectra were recorded on a LCMS-2010A Liquid Chromatograph Mass Spectrometer. Elemental analyses were performed on an Elementar Vario EL elemental analyzer. Potentiometric titrations were carried out on a Metrohm 702 SM Titrino titrimeter.

2.3. *Synthesis*

The synthesis of **1** and **2** is shown in scheme 1. ESI-MS spectra of ligands, ^{1}H NMR spectra of ligands and complexes are presented in supplementary materials (figures S1–S3).

2.3.1. 4,4′-*Bis*(triethylaminomethyl)-2,2′-bipyridine dibromide (L^1Br_2). To a stirred solution of 4,4′-dibromomethyl-2,2′-bipyridine [23–26] (0.200 g, 0.585 mmol) in chloroform (5 mL), triethylamine (1.0 mL, 7.2 mmol) was added and the solution was stirred at room temperature for 2 h. The precipitate was filtered, washed with chloroform and dried to offer L^1Br_2 as pale pink powder (0.350 g, 98.5%). ^{1}H NMR (D_2O, 300 M): δ 8.690 (d, $J=5.1$ Hz, 2H, bpy-H); 8.126 (s, 2H, bpy-H); 7.578 (d, 2H, $J=5.1$ Hz, bpy-H); 4.489 (s, 4H, bpy-CH2); 3.241 (q, $J=7.2$ Hz, 12H, Me-CH_2); 1.340 (t, $J=7.2$ Hz, 18H, CH_3). ESI-MS, m/z (%): 192.25 ($[L^1]^{2+}$, 100); 362.35 ($[L^1+Br–Et_3N]^+$, 18). Anal. Calcd for $C_{24}H_{40}N_4Br_2 \cdot 3.5H_2O$ (%): C, 47.45; H, 7.80; N, 9.22. Found: C, 47.65; H, 7.85; N, 9.19.

2.3.2. $[Zn(C_{24}H_{40}N_4)_3](ClO_4)_8 \cdot 12H_2O$ (1). To a stirred ethanol solution (5 mL) of L^1Br_2 (0.100 g, 0.165 mmol), was added 3 mL ethanol solution of $Zn(ClO_4)_2 \cdot 6H_2O$ (0.100 g, 0.269 mmol). Pink precipitate appeared immediately, was filtered, washed with ethanol several times, and dried to give **1** as pink powder (0.116 g, 94.5%). UV (H_2O): λ_{max} 238 nm ($\varepsilon = 4.55 \times 10^4$ M^{-1} cm^{-1}, $\pi \rightarrow \pi^*$); 289 ($\varepsilon = 5.78 \times 10^4$, $n \rightarrow \pi^*$). ^{1}H NMR (DMSO-d_6, 300 MHz): δ 8.855 (d, $J=4.2$ Hz, 6H, bpy-H); 8.574 (s, 6H, bpy-H); 7.623 (s, with a shoulder, 6H, bpy-H); 4.661 (s, 12H, bpy-CH_2); 3.266 (d, with two shoulders, $J=6.6$ Hz, 36H, Me–CH_2); 1.352 (t, $J=6.6$ Hz, 54H, CH_3). Anal. Calcd for $C_{72}H_{144}N_{12}Cl_4O_{44}Zn$ (%): C, 38.76; H, 6.51; N, 7.53. Found: C, 38.42; H, 5.95; N, 7.24.

2.3.3. 4,4′-*Bis*(tri-*n*-butylaminomethyl)-2,2′-bipyridine dibromide (L^2Br_2). To a stirred solution of 4,4′-dibromomethyl-2,2′-bipyridine (0.200 g, 0.585 mmol) in acetonitrile (15 mL), tri-*n*-butylamine (0.70 mL, 2.95 mmol) was added and the solution refluxed for 2 h; then the solvent was evaporated, and the residue was dissolved in minimum ethanol and precipitated by adding petroleum ether. The precipitate was filtered, washed with petroleum ether and dried to give L^2Br_2 as white powder (0.425 g, 98.2%). ^{1}H NMR (DMSO-d_6, 300 M): δ 8.841 (d, $J=4.8$, 2H, bpy-H); 8.557 (s, 2H, bpy-H); 7.601 (d, 2H, $J=4.8$, 2H, bpy-H); 4.719 (s, 4H, bpy-CH_2); 3.184 (t, $J=7.7$ Hz, 12H, (*n*-Pr)-CH_2); 1.780–1.757 (m, 12H, Et-CH_2); 1.372–1.299 (m, 12H, Me–CH_2); 0.961 (t, $J=7.2$ Hz, 18H, CH_3). ESI–MS, m/z (%): 276.95 ($[L^2]^{2+}$, 100); 367.55 ($[L^2–Bu_3N]^+$, 10).

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Anal. Calcd for $C_{36}H_{64}N_4Br_2 \cdot 1.5H_2O$ (%): C, 58.45; H, 9.13; N, 7.57. Found: C, 58.68; H, 9.20; N, 7.23.

2.3.4. $[Zn(C_{36}H_{64}N_4)_3](ClO_4)_8 \cdot 2H_2O$ (2). To a stirred acetonitrile solution (5 mL) of L^2Br_2 (0.150 g, 0.203 mmol), was added 3 mL ethanol solution of $Zn(ClO_4)_2 \cdot 6H_2O$ (0.150 g, 0.404 mmol). The solution was stirred for 15 min; then the solvent was evaporated. The resulting solid was washed with water several times, then filtered and dried to offer **2** as offwhite powder (0.167 g, 96.1%). UV (H_2O, trace CH_3CN): λ_{max} 239 nm ($\varepsilon = 3.76 \times 10^4\ M^{-1}\ cm^{-1}$, $\pi \rightarrow \pi^*$); 289.5 ($\varepsilon = 4.80 \times 10^4$, $n \rightarrow \pi^*$). ^1H NMR (DMSO-d_6, 300 MHz): δ 8.847 (d, $J = 4.8$ Hz, 6H, bpy-H); 8.563 (s, 6H, bpy-H); 7.588 (d, $J = 4.8$ Hz, 6H, bpy-H); 4.702 (s, 12H, bpy-CH_2); 3.181 (t, $J = 8.1$ Hz, 36H, (*n*-Pr)–CH_2); 1.786 (br, 36H, Et–CH_2); 1.400-1.281 (m, 36H, Me–CH_2); 0.967 (t, $J = 7.4$ Hz, 54H, CH_3). Anal. Calcd for $C_{108}H_{196}N_{12}Cl_4O_{34}Zn$ (%): C, 50.75; H, 7.73; N, 6.58. Found: C, 50.93; H, 7.85; N, 6.15.

2.4. *Potentiometric titration*

Species distribution in aqueous solution of these complexes was studied by potentiometric titration. The final 25.0 mL test solution contains 1.0 mM $Zn(ClO_4)_2$, 3.0 mM ligands, 6.0 mmol $HClO_4$ and 0.1 M NaBr which was used to control ionic strength. Titration was carried out under argon at $25 \pm 0.1^\circ C$, and the test solution was purged with argon for 10 min before titration. Titration was performed by titrating NaOH aqueous solution (0.1017 M) to the test solution. The titration volume step was 0.01 mL, and the equilibrium time for each titration was not less than 30 s. The titration points for metal-L^1 and metal-L^2 systems are 248 and 243, respectively. Titration data were processed with HYPERQUAD 2000 program. The pH-region used in calculations for metal-L^1 and metal-L^2 systems are 2.23–11.36 and 2.41–11.35, respectively.

2.5. *UV spectroscopic titration*

Stock solution (3.0 mM) of **1** in buffer solution (5 mM Tris-50 mM NaCl, pH 7.75) and **2** in 1 : 1 CH_3CN-buffer were used. CT-DNA was dissolved in buffer and stored at 4°C as stock solution. The concentration of DNA was determined according to the literature [27]. Stock solution of complexes was diluted into 20 μM with buffer solution. To the test solution of complexes (20 μM, initial volume 3.0 mL), 10 μL stock solution of CT-DNA (4.0 mM) was added, then the solution was scanned in the range of 400–200 nm. For each sample, the addition of CT-DNA and the scan were repeated five times. The change in solution volume was taken into account. Trace CH_3CN has no effect on the UV spectra.

2.6. *CD spectroscopy*

Buffer solution, stock solutions of complexes and CT-DNA were the same as UV spectroscopic titration. CD spectra of DNA (100 μM) were scanned in the absence and

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presence of complexes ($r = 0, 0.15, 0.30, r = [\text{complex}]/[\text{DNA}]$) at room temperature. Each sample was scanned for ten repetitions at a speed of $100\,\text{nm}\,\text{min}^{-1}$, and the average spectra were adopted. The buffer background was subtracted automatically.

2.7. *DNA cleavage*

The cleavage of plasmid DNA (pBR322, 38 µM base pair) was performed in 20 mM buffer (MES, HEPES, TAPS or CHES according to pH) by treating DNA with a certain concentration of complex. The total volume of 10 µL sample solution was incubated in a sealed plastic vessel and the cleavage was carried out at 37°C for a defined time, then 4 µL loading buffer (bromophenol blue, 50% glycerol and 2 mM EDTA) was added. The sample was stored at −20°C, then loaded onto a 0.9% agarose gel and electrophoresed at a constant voltage of 120 V for 100 min in TBE buffer (100 mM Tris-2 mM EDTA-HBO_3, pH 8.3). DNA bands were visualized in an electrophoresis documentation and analysis system 120. Densitometeric calculations were made using the analysis method in Image Tools 3.00. A correction factor of 1.47 was used for supercoiled DNA taking into account its weaker staining capacity by ethidium bromide compared to nicked and linear DNA [18]. The apparent rate constant k_{obs} was determined according to the literature [18].

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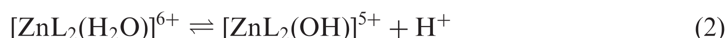
3. Results and discussion

3.1. *Identification of hydrolytically active species*

Scheme 1 shows the proposed structures of solid-state **1** and **2**. In general, bipyridine and its derivatives form stable 1 : 3 Zn(II) complexes (ZnL_3) in solid state [22, 28]. However, it has been widely accepted that hydroxyl-coordinated species are potential reactive nucleophilic agents in the hydrolysis of phosphate groups [1, 10, 16, 29-31]. Previous reports have pointed out that Zn(II)-coordinated ligands can exchange rapidly with solvent molecules in aqueous solution [1, 8, 9], that is to say, $[ZnL_3]^{8+}$ can dissociate at least one of its ligands to form $[ZnL_2(H_2O)]^{6+}$:

$$[ZnL_3]^{8+} + H_2O \rightleftharpoons [ZnL_2(H_2O)]^{6+} + L^{2+} \quad (1)$$

The equilibrium between $[ZnL_3]^{8+}$ and $[ZnL_2(H_2O)]^{6+}$ species is shown by the results of potentiometric titration (shown as ZnL_3 and ZnL_2, respectively, in the distribution diagrams, figure 1). In $[ZnL_2(H_2O)]^{6+}$, the Zn(II)-coordinated water molecule can readily be deprotonated to produce $[ZnL_2(OH)]^{5+}$, which is a good species for cleavage of DNA:



$$[ZnL_2(H_2O)]^{6+} \rightleftharpoons [ZnL_2(OH)]^{5+} + H^+ \quad (2)$$

As can be seen from figure 1, $[ZnL_2(OH)]^{5+}$ exists in basic aqueous solution of both complexes.

Calculated formation constants of some species are listed in table 1, and the titration and fitted curves of both complexes are presented in supplementary material (figure S4).

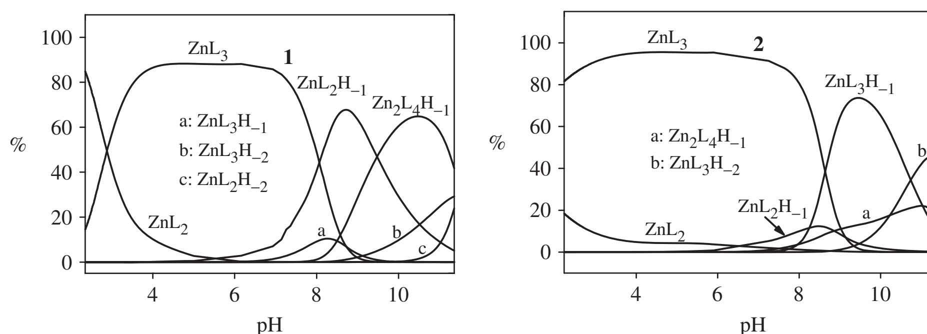


Figure 1. Species distribution diagrams in aqueous solution of **1** and **2**. Titration conditions: $[Zn(ClO_4)_2] = 1.0\,mM$, [ligands] $= 3.0\,mM$, $[HClO_4] = 6.0\,mM$, $I = 0.1\,M$ NaBr, $25 \pm 0.1°C$. ZnL_3: $[ZnL_3]^{8+}$; ZnL_2: $[ZnL_2(H_2O)]^{6+}$; ZnL_2H_{-1}: $[ZnL_2(OH)]^{5+}$; ZnL_2H_{-2}: $[ZnL_2(OH)_2]^{4+}$; ZnL_3H_{-1}: $[ZnL_3(OH)]^{7+}$; ZnL_3H_{-2}: $[ZnL_3(OH)_2]^{6+}$; $Zn_2L_4H_{-1}$: $[(ZnL_2)_2\text{-}\mu\text{-}OH]^{11+}$. "OH" represents hydroxyls bound to the metal ion or tetraalkylammonium groups.

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Table 1. Calculated formation constants of some species in aqueous solution of **1** and **2**.

Species/Reaction		1	2
$L + H^+ = [LH]^+$	log β	2.2	3.4
$L + 2H^+ = [LH_2]^{2+}$		–	5.8
$[Zn(H_2O)_6]^{2+} + 2L = [ZnL_2(H_2O)]^{6+} + 5H_2O$		6.4	6.9
$[Zn(H_2O)_6]^{2+} + 3L = [ZnL_3]^{8+} + 6H_2O$		8.9	9.3
$[ZnL_2(H_2O)]^{6+} = [ZnL_2(OH)]^{5+} + H^+$	pK_a	7.6	7.4

[a]Standard deviation of all data <0.1.

3.2. *Interaction with CT-DNA*

Interaction of **1** and **2** with CT-DNA was first studied by UV spectroscopic titration, and absorption spectra are shown in figure 2. With addition of CT-DNA ($0\text{–}6.54 \times 10^{-5}\,M$) to the solution of both complexes ($2.0 \times 10^{-5}\,M$), hyperchromicity was observed. The hyperchromicity in absorbance indicates the binding mode of these complexes towards CT-DNA is not intercalation, since intercalation would result in hypochromicity [32]. The change of absorbance at ~288 nm with increasing concentration of CT-DNA was followed and the intrinsic binding constant (K_b) of these complexes was determined according to equation 3:

$$[DNA]/(\varepsilon_A - \varepsilon_F) = [DNA]/(\varepsilon_B - \varepsilon_F) + 1/K_b(\varepsilon_B - \varepsilon_F) \tag{3}$$

In equation 3, ε_A, ε_F and ε_B represent apparent, DNA free and fully bound, extinction coefficients of complexes in solution [33]. The K_b values of **1** and **2** were calculated as $3.55 \times 10^3\,M^{-1}$ and $2.72 \times 10^3\,M^{-1}$, respectively, which are much smaller than those reported for typical intercalators (e.g. EB-DNA, $\sim 10^6\,M^{-1}$) [32, 34], and are comparable with some reported non-intercalators [35, 36]. The quite low K_b values do not favor intercalation binding either. Furthermore, the lower K_b value of **2** than that of **1** indicates steric hindrance has an effect on the interaction with DNA.

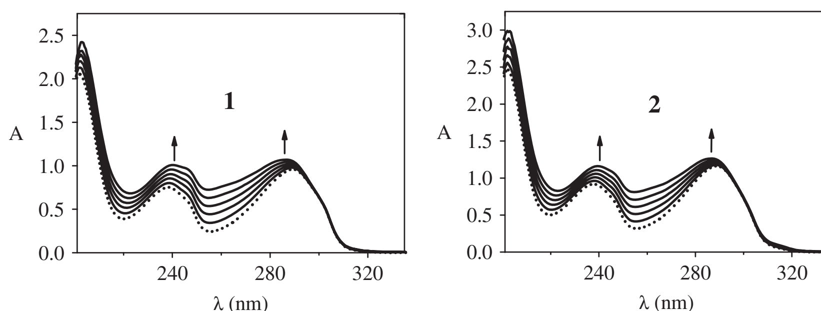

Figure 2. Absorption spectra of 2.0×10^{-5} M **1** and **2** in the absence (dotted line) and presence (solid line) of increasing amount of CT-DNA (0–6.54×10^{-5} M) in 5 mM Tris-50 mM NaCl buffer (pH 7.75) at 25°C.

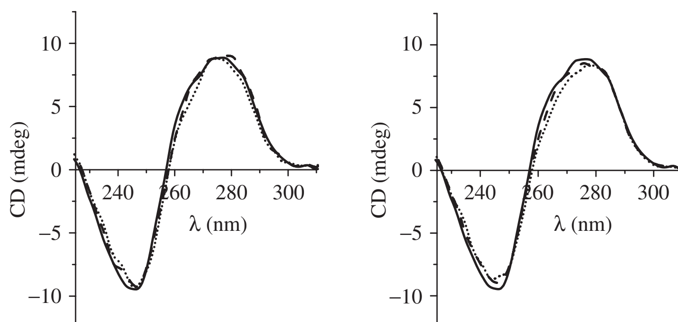

Figure 3. CD spectra of 100 μM CT-DNA in the absence and presence of **1** and **2**: $r = 0$ (—), $r = 0.15$ (– – –), $r = 0.30$ (••••••), $r =$ [complex]/[DNA]. Experimental conditions: 5 mM Tris-50 mM NaCl buffer, pH 7.75, room temperature; scan rate 100 nm min^{-1}.

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The interaction between these complexes and CT-DNA was also studied by CD spectra. As shown in figure 3, the addition of complexes to the solution of DNA did not result in change of conformation and remarkable decrease in the ellipticity of bands, implying that intercalation binding should be excluded again, because this binding mode would cause a characteristic decrease in both positive and negative bands [37]. Bearing so many positive and steric-hindrance groups, these complexes would interact with DNA *via* outside electrostatic binding mode.

3.3. *Nuclease activity*

Complexes **1** and **2** were applied to the cleavage of plasmid DNA (pBR322) to assess their nuclease activity. First, DNA cleavage was performed at different complex concentration. It can be seen from figure 4, the cleavage of DNA by **1** is concentration-dependent. When the concentration of **1** was 50 μM, only a little supercoiled DNA (from I) was converted into nicked DNA (form II). With increase of complex concentration, the percent of form II DNA increased and arrived at the plateau at 250 μM. Complex **2** can hardly cleave DNA even at 400 μM. The stronger steric hindrance caused by bigger functional groups (tri-*n*-butylaminomethyl groups) may be

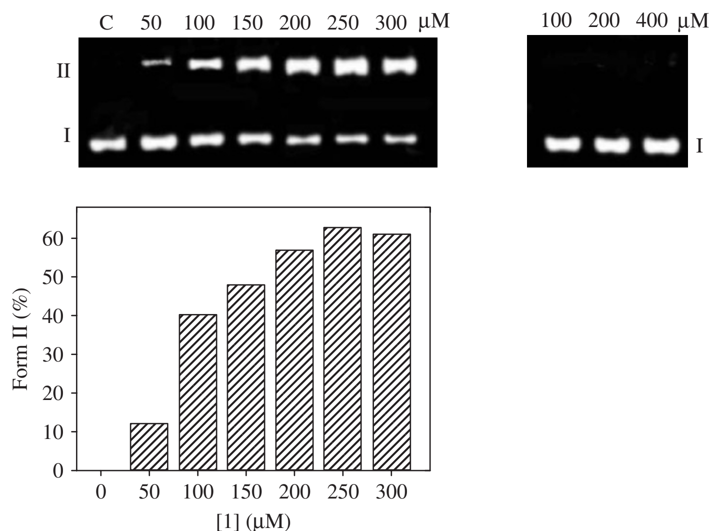

Figure 4. Cleavage of pBR322 DNA (38 μM bp) by **1** and **2** at the indicated concentrations in 20 mM HEPES buffer (pH 7.75) at 37°C after 16 h incubation. 'C' represents DNA control, the same hereinafter.

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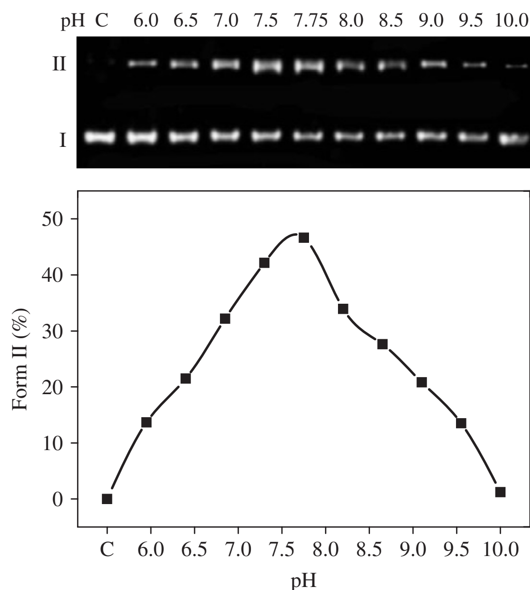

Figure 5. The pH-dependent cleavage of pBR322 DNA (38 μM bp) promoted by **1** (200 μM) in 20 mM buffer (MES, HEPES, TAPS or CHES according to pH) at 37°C after 16 h incubation.

responsible for this result, which make it very difficult for **2** to approach the phosphodiester groups of DNA.

In order to investigate the effect of buffer pH on the nuclease activity of **1**, DNA cleavage catalyzed by **1** was performed at different pHs (pH 6.0–10.0), as shown in figure 5. The pH-dependent cleavage of DNA was observed, and the percent of form II DNA displays a bell-like profile with the maximum value at pH 7.75 (HEPES buffer).

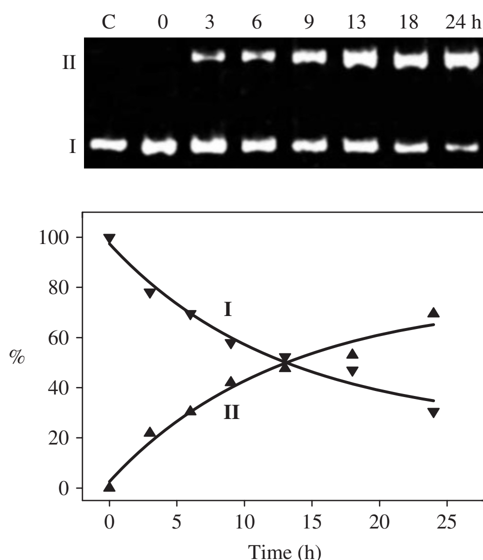

Figure 6. Electrophoresis pattern and time course plot showing the time-dependent cleavage of pBR322 DNA (38 µM bp) promoted by **1** (200 µM) in 20 mM HEPES buffer (pH 7.75) at 37°C.

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The hydrolysis kinetics of DNA cleavage were studied with 200 µM of **1** in 20 mM HEPES buffer (pH 7.75). The electrophoresis pattern and corresponding time course plot are shown in figure 6. The decrease of form I and increase of form II are well fit for single exponential decay. Apparent rate constants (k_{obs}) for the decrease of form I DNA was calculated as $2.18 \times 10^{-5}\,s^{-1}$. The k_{obs} values of reported zinc complex are usually in the range of 10^{-5}–$10^{-6}\,s^{-1}$ [1, 10, 16, 38–40], and some higher k_{obs} values were obtained at higher complex concentration (10^{-3} M) [38–40] or higher reaction temperature (50°C) [16]. The obtained k_{obs} values imply **1** possesses quite high DNA cleavage activity.

4. Conclusion

Two zinc complexes with tetraalkylammonium groups were synthesized and characterized. The results of UV spectroscopic titration and CD spectroscopy indicate that these complexes interact with DNA *via* outside electrostatic binding. Species distribution in aqueous solution of both complexes suggests that Zn(II)-coordinated hydroxyl species are the hydrolytically active species. Both complexes were applied to the hydrolytic cleavage of DNA to assess their nuclease activities, and the results show that **1** with smaller functional groups can cleave DNA, showing quite high nuclease activity, while **2** with bigger functional groups can hardly cleave DNA.

Supplementary materials

ESI-MS spectra of ligands, ^{1}H NMR spectra of ligands and complexes, titration and fitted curves of potentiometric titration.

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