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Interaction of a New Fluorescent Probe with DNA and its Use in Determination of DNA

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Abstract In this paper we reported a metal complex **1-Zn** (2,5-di-[2-(3,5-bis(2-pyridylmethyl)amine-4-hydroxy-phenyl)-ethylene]-pyrazine-Zn) as a fluorescent probe sensing DNA. The result of the competitive experiment of the probe with ethidium bromide (EB) to bind DNA, absorption spectral change and polarization change in the presence and absence of DNA revealed that interaction between the probe and DNA was via intercalation. Ionic strength experiment showed the existence of electrostatic interaction as well. Scatchard plots also confirmed the combined binding modes. The fluorescence enhancement of the probe was ascribed to highly hydrophobic environment when it bound the macromolecules such as DNA, RNA or denatured DNA. The binding constant between the probe and DNA was estimated as 3.13×10^{7} mol^{-1} L. The emission intensity increase was proportional to the concentration of DNA. Based on this, the probe was used to determine the concentration of calf thymus DNA (*ct*-DNA). The corresponding linear response ranged from 2.50×10^{-7} to 4.75×10^{-6} mol L^{-1}, and detection limit was 1.93×10^{-8} mol L^{-1} for *ct*-DNA.

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Introduction

Binding of small molecules with deoxyribonucleic acid (DNA) has been studied extensively since DNA is the material of inheritance and controls the structure and function of cells [1]. Small molecules include metal complexes, porphyrins, natural antibiotics, simple aromatic hydrocarbons and some heterocyclic cations [2–7]. The studies contribute greatly to the design of new and promising anticancer agents for clinical use. Fluorescence assay is a powerful tool for sensing biomolecules because of its high sensitivity and easy operation. Due to the low fluorescence quantum yield of native DNA [8, 9], the search for extrinsic fluorescent probes presents great necessity [10, 11]. However, most sensing systems used fluorescence quenching mechanism [12]. Fluorescence enhancement assays possess some merits such as lower background and easy detection. Long excitation wavelength can avoid tissue burning and light bleaching while long emission wavelength can easily penetrate tissue and be detected. Therefore the design of fluorophore with both long excitation and emission wavelengths is very important [13–17]. Herein, a zinc complex (2,5-di-[2-(3,5-bis(2-pyridylmethyl)amine-4-hydroxy-phenyl)-ethylene]-pyrazine-Zn) (**1-Zn**) was presented as a new fluorophore to sense DNA. Zinc ion in metal complex enhanced the emission by inhibiting photon-induced electron transfer (PET) process [18]. The fluorescence increased greatly when **1-Zn** bound DNA because the microenvironment became more hydrophobic. The binding modes between

1-Zn and DNA were investigated to be intercalation as well as electrostatic interaction. Zinc ion attributed to the binding for a favorable electrostatic interaction with DNA phosphates.

Experimental

Apparatus

All fluorescence measurements were carried out on a F-4500 spectrofluorimeter (Hitachi, Japan) equipped with a xenon lamp source and a 1.0 cm quartz cell, and the scan speed was 240 nm min^{-1}. Absorption spectra were recorded on a Shimadzu-2501 UV-VIS spectrophotometer (Japan) using a 1.0 cm quartz cell. All pH measurements were made with a pHS-3 digital pH-meter (Shanghai REX instrument Corp., Shanghai, China) with a combined glass-calomel electrode.

Reagents

The stock standard solution of *ct*-DNA (Sigma Chemical Co.) was prepared by dissolving *ct*-DNA in 0.05 mol L^{-1}NaCl to reach a final concentration of 3.6×10^{-4} mol L^{-1}. DNA concentrations were determined by absorption spectroscopy using the molar extinction coefficient (mol^{-1} L cm^{-1}) of 6600 at the wavelength of 260 nm. They were stored at 4°C. The working solution was prepared by diluting the stock solution to reach a concentration of 1.0×10^{-4} mol L^{-1}. 5.0×10^{-2} mol^{-1} L Tris (Sigma Chemical Co.) buffer has a pH of 7.4 containing 5.0×10^{-2} mol^{-1} L NaCl. The stock solution of EB (Beijing Xiaxin Biology Corp.) was prepared into a concentration of 1.0×10^{-4} mol L^{-1}. Compound **1** was synthesized in our lab [18]. **1** was prepared into a concentration of 1.0×10^{-4} mol L^{-1} in DMSO. Zinc ion solution of 1.0×10^{-4} mol L^{-1} was prepared from $Zn(ClO_4)_2.6H_2O$ using DMSO. Metal complex was prepared by mixing 1:4 (v/v) of **1** and Zinc ion solution. DMSO was of chromatographic grade. Other chemicals were of analytical grade. Doubly distilled water was used throughout.

Procedures

20 μL **1-Zn** and different amounts of *ct*-DNA were added to a quartz cell and the solutions were diluted to 2.0 mL with Tris–HCl buffer and mixed. The fluorescence spectra from 445 to 750 nm of the above solutions were collected with excitation at 435 nm. Same experiments were performed on denatured *ct*-DNA and RNA as well.

30 μL **1-Zn** was transferred to a colorimetric cell and diluted to 3.0 mL with Tris–HCl buffer. A known volume of the standard solution of *ct*-DNA was added and the solution was mixed. Absorption spectra were collected.

20 μL EB solution and 100 μL *ct*-DNA was transferred to a quartz cell. Different amounts of Tris–HCl buffer and **1-Zn** were added to a total volume of 2.0 mL and mixed. The fluorescence spectra from 540 to 750 nm of the mixed solution were collected with excitation at 528 nm.

For fluorescence polarization measurements, various concentration of *ct*-DNA ranging from 1.0×10^{-6} to 2.2×10^{-5} mol L^{-1} was added to **1-Zn** with a concentration of 3.0×10^{-6} mol L^{-1}. The solutions were excited at 435 nm and the fluorescence was monitored at 530 nm through a pair of polarizers.

20 μL **1-Zn** and 40 μL *ct*-DNA and a certain amount of 4.0 mol L^{-1} NaCl were added to a quartz cell respectively. The solution was diluted to 2.0 mL with Tris–HCl buffer and mixed. Sodium ion concentration increased from 0.05 to 0.15 mol L^{-1}.The fluorescence spectra from 445 to 750 nm of the above solution were collected with excitation at 435 nm.

In order to prepare heat-denatured *ct*-DNA, 10 mL *ct*-DNA was heated in boiling water bath for 30 min. It was immediately cooled in ice water bath for 10 min. Fluorescent spectra were collected in the same way like *ct*-DNA.

Results and discussion

Fluorescent titration of **1-Zn** with *ct*-DNA

The fluorescent titration of **1-Zn** with *ct*-DNA was carried out in Tris–HCl aqueous solution of pH 7.4 and the results were presented in Fig. 1. **1-Zn** emitted weak fluorescence when excitation wavelength was set at 435 nm. However,

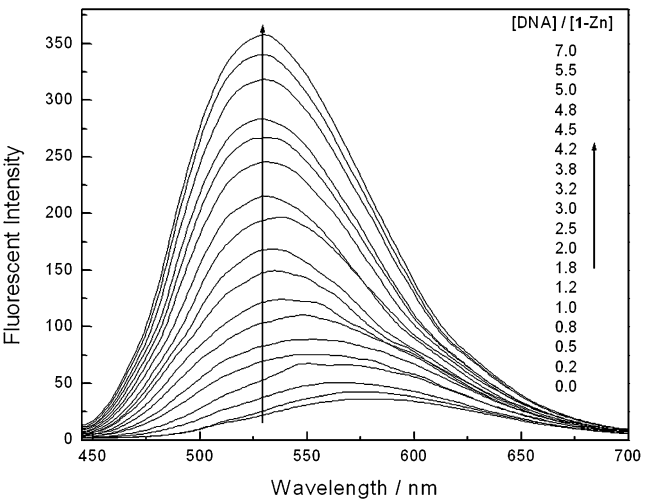

Fig. 1 Fluorescence titration of **1-Zn** (1.0×10^{-6} mol L^{-1}) with *ct*-DNA in pH 7.4 Tris–HCl aqueous solution ($DMSO/H_2O = 2/98$). Excitation wavelength set at 435 nm

the presence of *ct*-DNA resulted in a dramatic fluorescence enhancement along with a large spectral shift from 580 to 530 nm. Meanwhile, a strong green fluorescence was observed only via naked eyes. Such spectral change could be explained that **1-Zn** entered the double helices of DNA, which made its microenvironment more hydrophobic.

Absorption spectra investigation

The absorption spectral change of **1-Zn** was also investigated in the presence of *ct*-DNA. Figure 2 revealed the absorption spectral change of **1-Zn** upon addition of *ct*-DNA in pH 7.4 Tris–HCl buffer. The maximum wavelength of **1-Zn** centered at 435 nm. In the presence of increasing amounts of *ct*-DNA the spectrum of **1-Zn** red shifted along with decreasing absorbance at 435 nm. The phenomenon of hypochromism was suggested to be due to a strong interaction between the electronic states of the intercalating chromophore and those of the DNA bases [1, 19]. Thus it could be concluded that **1-Zn** intercalated into the base pairs of DNA's double helices.

Competitive binding of **1** and EB with *ct*-DNA

EB, a well known intercalator, binds DNA by intercalation [10–12]. EB emits weak fluorescence in aqueous solution, however the fluorescence of EB remarkably increases while binding DNA. It is supposed that EB intercalates into base pairs of DNA's double-helix structure which lead fluorescence intensity increase. While fluorescence intensity of EB-DNA decrease 50% in the presence of a fluorescent probe, the ratio of concentration between the probe and DNA is still less than 100 which is supposed that the probe possesses the same interaction model like EB does. It

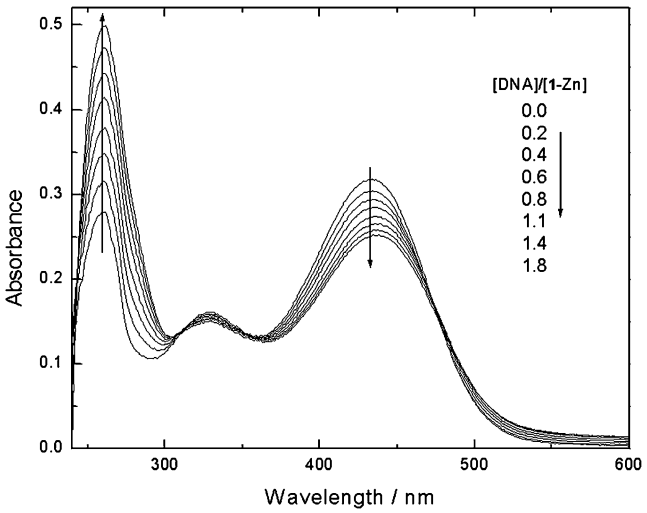

Fig. 2 Absorption spectral changes of **1-Zn** (1.0×10^{-5} mol L^{-1}) in the presence and absence of *ct*-DNA in pH 7.4 Tris–HCl buffer

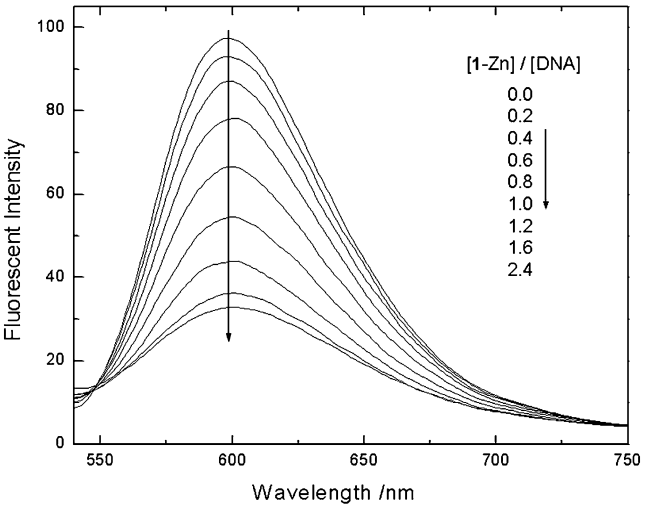

Fig. 3 Fluorescence spectral change of EB (1.0×10^{-6} mol L^{-1})–DNA (5.0×10^{-6} mol L^{-1}) upon addition of **1-Zn**. Excitation wavelength was 528 nm

means the existence of intercalation between the probe and *ct*-DNA. Figure 3 displayed the fluorescence spectral changes of EB-DNA in the presence of **1-Zn**. While the ratio of concentration between **1-Zn** and DNA is 2.4, $F/F_0 = 33.67\%$ which is less than 50%. Obviously, **1-Zn** competed with EB to bind DNA with certain amount of EB displaced as a result. Thus we can draw the conclusion that the binding ability of **1-Zn** with *ct*-DNA was strong. **1-Zn** is a good fluorescent probe for DNA.

Fluorescence polarization measurement

The fluorescence polarization change provided strong evidence to explain the binding mode between fluorophores and DNA. In the absence of DNA, the fluorescence of the small molecule was weakly polarized due to the molecule's rapid tumbling motion in aqueous media. However, if the molecule intercalates into the helix of DNA, its rotational motion should be restricted, thus, the fluorescence polarization of bound fluorophore should increase. Merely binding to the phosphate backbone or to the DNA grooves does not result in the enhancement of fluorescence polarization. Figure 4 reveals the polarization change of **1-Zn** upon the addition of *ct*-DNA with different concentrations. The large increase of fluorescence polarization suggested the intercalation of **1-Zn** into the double helices upon binding *ct*-DNA

Effect of ionic strength on the spectrum of **1-Zn**-DNA

Monitoring the change of ionic strength is an efficient method for distinguishing the binding modes between molecules and DNA. Increasing the cation's concentration will increase the complexation probability between the

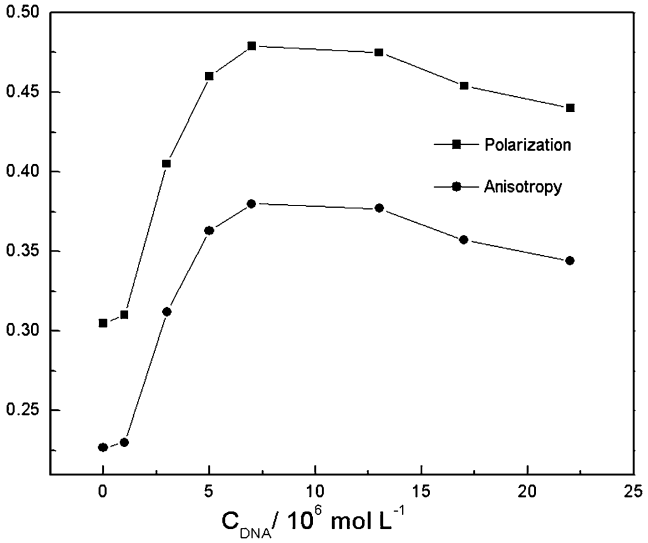

Fig. 4 Polarization and anisotropy changes of **1-Zn** (3.0×10^{-6} mol L^{-1}) upon addition of *ct*-DNA, G=1.281

cation and DNA phosphate backbone. Due to a competition for phosphate anion, the addition of the cation will weaken the surface-binding interactions which include electrostatic interaction and hydrogen binding between DNA and molecules [20]. The ionic strength effect on the system of **1-Zn**–DNA was studied and spectral changes were presented in Fig. 5 which displayed a strong dependence of fluorescence intensity on ionic strength. The fluorescence of **1-Zn**–DNA distinctly quenched with the increasing concentration of NaCl from 0.05 to 0.15 mol L^{-1}. The result indicated that the interaction between **1-Zn** and *ct*-DNA was electrostatic interaction besides intercalation.

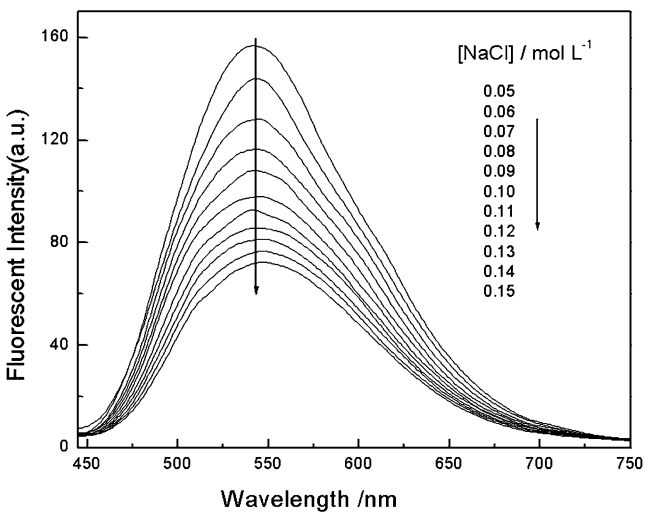

Fig. 5 Fluorescence spectra of **1-Zn** (1.0×10^{-6} mol L^{-1})–DNA (2.0×10^{-6} mol L^{-1}) in different concentration of NaCl. Excitation wavelength was 435 nm

Effect of metal complex on the binding of EB to DNA

Scatchard equation is used to describe the interaction between DNA and small molecules as Eq. 1 [21–23]. Fluorescence Scatchard plots for the binding of EB to *ct*-DNA in the presence of metal complex of varying concentrations were obtained according to r_E/c_E vs r_E.

$$\frac{r_E}{c_E} = (n - r_E)\cdot\frac{k_E}{1 + k_M c_M} \quad (1)$$

where r_E is the ratio of bound EB to total nucleotide concentration, c_E is the concentration of free EB, n is the maximum of r_E which is the size of binding site, k_E or k_M is the binding constant between EB or metal complex (**1-Zn**) with *ct*-DNA respectively, c_M is the concentration of free metal complex.

Using the fluorescence intensity change to determine r_E and corresponding Scatchard plots were obtained and shown in Fig. 6. It was clear that both the slop and intercept changed which implied that the binding action between **1-Zn** and *ct*-DNA was combined modes including intercalation and electrostatic interaction.

When **1-Zn** competes with EB to bind DNA, the binding can be characterized by the following equation:

$$\frac{r_M}{c_M} = (n - r_M)\cdot\frac{k_M}{1 + k_E c_E} \quad (2)$$

where r_M is the number of **1-Zn** bound to each nucleotide acid, c_M is the concentration of free **1-Zn**. n is the maximum of r_M which is size of the binding site. k_M is the binding constant between **1-Zn** with DNA. Since n=

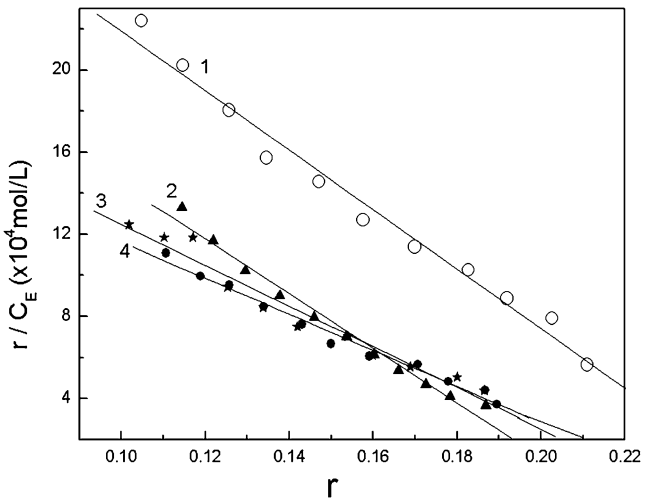

Fig. 6 Fluorescence Scatchard plots of the binding of EB to *ct*-DNA in the absence (line 1) and in the presence of various concentrations of metal complex (line 2–4). r_f, formal ratio of metal complex to nucleotide acid concentration, r_f increases in the order of 0, 0.1, 0.25, 0.5 for line 1–4 respectively. The nucleotide concentration was 1.5×10^{-5} mol L^{-1}, and the EB concentration varied from 2.0×10^{-6} mol L^{-1} to 2.0×10^{-5} mol L^{-1}

Table 1 Binding constant between complex 1-Zn and DNA*

$[E]_t$ (μmol L^{-1})	c_E (μmol L^{-1})	r_E	r/c_E (mol L^{-1})	c_M (μmol L^{-1})	k_M (mol L^{-1})	$\overline{k_M}$ (mol L^{-1})
1.0	0.91	0.036	3.9×10^4	8.56	2.71×10^7	
2.0	1.86	0.056	3.1×10^4	8.60	3.16×10^7	
4.0	3.79	0.084	2.2×10^4	8.66	3.53×10^7	$3.13\pm0.28\times10^7$

*The total concentrations of DNA and 1-Zn were 2.5 and 9.0 μmol L^{-1} respectively

0.22 and $k_E = 5.0 \times 10^7 mol^{-1}L$ for linear double stranded DNA [24],

$$c_{M=}[M]_t - r_M[DNA]_t \quad (3)$$

$$c_E = [E]_t - r_E[DNA]_t \quad (4)$$

where $[M]_t$, $[E]_t$ and $[DNA]_t$ are the total concentration of **1-Zn**, EB and DNA respectively. From Eqs. 1 and 2 we can get:

$$r_M = n - r_E - \frac{r_E}{k_E \cdot c_E} \quad (5)$$

$$k_M = \frac{(n - r_E) \cdot k_E \cdot \frac{c_E}{r_E} - 1}{c_M} \quad (6)$$

When the fluorescence intensity of EB/DNA system decreased to the lowest F′ because **1-Zn** competes with EB to bind DNA, then

$$c_E = \frac{F_0 - F}{F_0 - F'} \cdot [E]_t \quad (7)$$

with the detected fluorescence, c_E and r_E were obtained using Eqs. 7 and 4 while k_M was obtained using Eqs. 3, 5 and 6. The calculated results were presented in Table 1 and the binding constant between **1-Zn** and DNA was estimated as 3.13×10^7 mol^{-1} L.

Interaction between **1-Zn** and RNA or denatured *ct*-DNA

In order to further investigate the mechanism between **1-Zn** and *ct*-DNA, we also studied the effect of RNA or denatured *ct*-DNA on the fluorescent spectrum of **1-Zn**. The trend of spectral change which is similar to that of *ct*-DNA was obtained. The maximum emission wavelength of **1-Zn** blue shifted from 580 to 548 nm for denatured *ct*-DNA and from 580 to 558 nm for RNA respectively, and fluorescence intensity increased as well. Obviously, the structure of the double helix wasn't the only factor influencing binding action between **1-Zn** and *ct*-DNA. Figure 7 showed the emission intensity against the concentration of *ct*-DNA, RNA or denatured *ct*-DNA. The spectral shift and change of emission intensity were presented in Table 2. It was clear that the binding ability between **1-Zn** and nucleic acids following the order of *ct*-DNA > RNA ~ denatured *ct*-DNA. Therefore, it was concluded that the affinity between **1-Zn** and *ct*-DNA was stronger than that between **1-Zn** and RNA or denatured *ct*-DNA respectively.

Effect of ATP or pyrophosphate on the fluorescence spectrum of **1-Zn**

ATP has the similar structure unit as nucleic acids. The effect of ATP on **1-Zn**'s fluorescence was also investigated. The results revealed that the presence of ATP led fluorescence quenching. Because phosphate bound with **1-Zn**, zinc ion's ability to prohibit the PET process was weakened which lead to the fluorescence quenching. The fluorescence titration of **1-Zn** with pyrophosphate ($P_2O_7^{4-}$, PPi) was performed as well. We obtained the similar spectral change trends as those of ATP. Electronic interaction between **1-Zn** and *ct*-DNA indeed occurred, however, it should have caused fluorescence intensity decrease like ATP rather than increase. Thus, the enhancement of fluorescence

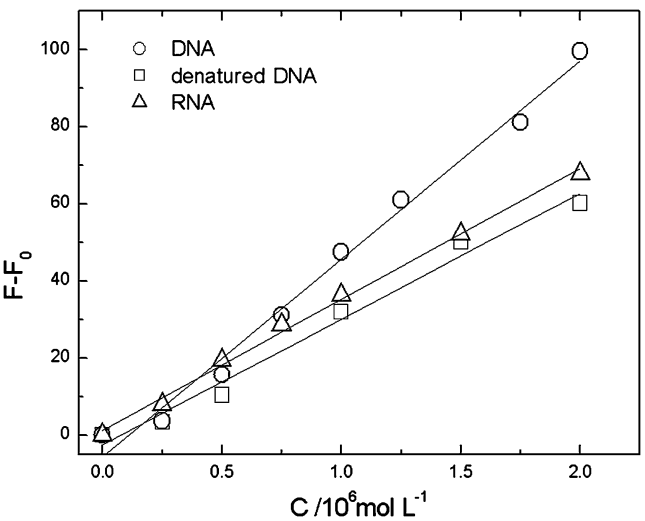

Fig. 7 Fluorescence intensity change of **1-Zn** in the presence of different concentrations of *ct*-DNA, denatured DNA and RNA. Excitation wavelength was 435 nm

Table 2 Spectral parameters of 1-Zn with nucleotide acid

	λ (nm)	$\Delta\lambda$ (nm)	Slop
1-Zn–DNA	530	50	5.14×10^{7}
1-Zn–denurated DNA	548	32	3.39×10^{7}
1-RNA	558	22	3.27×10^{7}

was ascribed to hydrophobic environment increase while **1-Zn** bound with DNA via intercalation.

Analytical application

Fluorescence titration result displayed that **1-Zn** showed a linear response to *ct*-DNA. A good linear relationship between fluorescence intensity and *ct*-DNA's concentration was obtained ranging from 2.5×10^{-7} to 4.75×10^{-6} mol L^{-1}. The equation followed as $\Delta F = 9.69 + 5.52\times10^{7}\ C_{DNA}$, ($n$= 15, r=0.998), and detection limit was 1.93×10^{-8} mol L^{-1}. **1-Zn** was highly sensitive to *ct*-DNA.

In order to explore the method's selectivity, the influence of various ions, amino acids, and glucose was tested according to the standard procedure. Under optimal experimental condition, in the mixture of 2.0×10^{-6} mol L^{-1} *ct*-DNA and 1.0×10^{-6} mol L^{-1} **1-Zn**, the presence of following amounts of foreign ions and compounds didn't interfere with the determination of *ct*-DNA while the error was less than ±5%: 1,000-fold Na^{+} and K^{+}, 120-fold Mg^{2+}, 70-fold urea, 30-fold Ba^{2+}, Fe^{3+}, Ag^{+}, cysteine and glucose, 25-fold Ca^{2+}, Al^{3+}, and cystine, 20-fold Glycine, methionine, 10-fold Pb^{2+}, Ni^{2+}, Cd^{2+}, Hg^{2+}, tryptophan, tyrosine, and 0.25-fold Cu^{2+}.

Conclusion

The present work reports the study of the interaction between a fluorescent probe **1-Zn** and *ct*-DNA or RNA. The interaction mechanism of **1-Zn**'s binding *ct*-DNA was investigated in detail and results suggested that the interaction modes were mainly intercalation along with electrostatic interaction. The fluorescence enhancement of **1-Zn** in the presence of nucleic acid was ascribed to the intercalation interaction. **1-Zn** could be used for the determination of *ct*-DNA and the detection limit was 1.93×10^{-8} mol L^{-1}.

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