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Synthesis, Crystal Structure and Interactions with Different G-quadruplex DNA and ctDNA of Zn-CAP

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Abstract: In this study, one mononuclear zinc(II) complex with 1,2-bis CAP ((5-chlorosalicylidene amino)-phenylene): $C_{22}Cl_3N_2O_{3.5}Zn_{1.5}H_{0.125}$ (Zn-CAP) was synthesized. The binding properties of Zn-CAP with G-quadruplex DNA and ctDNA (calf thymus DNA) were examined by fluorescence, CD (circular dichroism) spectroscopic and FRET (fluorescence resonance energy transfer) assay. In the fluorescence emission spectral analysis, the addition of three series of G-quadruplex DNA (G_4-HTG21, G_4-Pu27 and G_4-c-kit-1) into the Zn-CAP solution induced moderate or add hypochromicity with total quenching ratios of 10.73%, 15.07% and 8.59% in the presence of K^+ were achieved, respectively. While the addition of ctDNA under same condition only caused 7.08% quenching on the fluorescence emission of Zn-CAP. In the CD spectral analysis, the interaction with Zn-CAP could induce significant spectral changes on the CD absorption of G_4-HTG21, G_4-Pu27 and G_4-c-kit-1, with 106.00%, 93.06%, 113.47% increment at 232 nm absorption, along with a 81.11%, 92.80%, 83.72% decrement at 295 nm or 270 nm absorption, which demonstrated that the antiparallel structure of G-quadruplex DNA is more stable in the presence of Zn-CAP. Comparatively, the addition of Zn-CAP could induce significant spectral changes on the CD absorption of double helix ctDNA, with 64.17% decrement on the positive peak absorption, along with a 90.91% increment on the negative peak absorption. On the other hand, in the FRET-melting assay analysis, it was clear that Zn-CAP at 0.5 equivalences could raise the melting temperature of G-quadruplex (F21T or FPu18T) by 3.45 °C and 15.85 °C, indicating an obvious stabilization effect of Zn-CAP on G-quadruplex in Pu27. All the results indicated that Zn-CAP exhibited higher binding affinity and binding intensity to G-quadruplex DNA than ctDNA, especially G-quadruplex Pu27.

Key words: Zn-CAP, G-quadruplex DNA, ctDNA, spectroscopy, FRET assay.

1. Introduction

DNA is the molecular target for a large number of antitumor [1]. However, a main problem for these conventional anticancer agents is their significant toxicity because of their nonspecific interaction with the duplex DNA. In order to improve the selectivity and reduce the side effects of DNA-interactive drugs, many researchers have focused on designing molecules targeting to the specific DNA secondary structures, such as G-quadruplex DNA.

Telomeres are specialized class of functional DNA-protein structures [2]. One of the most abundant sources of DNA sequences capable of forming G-quadruplex structures is found in telomeres. Telomeric DNA plays an important biological role on protecting chromosomes from nuclease attacks and losing information in the process of cell divisions [3-5]. Telomeric DNA is composed of a repeated double-stranded $[TTAGGG/CCCTAA]_n$ sequence except in the 3'-terminal region, which consists of a single-stranded tandem [TTAGGG] repeated sequence over several hundred bases [6, 7]. The rich sequence (TTAGGG) formation of planar molecular G-tetrads by hydrogen-bonding interactions between the Watson-Crick edge and the Hoogsteen edge, the G-tetrad can be further stabilized by alkali metal cations and consequently form G-quadruplex DNA

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structures [8, 9]. Previous studies have shown that ligand-induced quadruplex formation or stabilization of G-quadruplexes by the telomeric G-rich strand can inhibit the activity of telomerase [10-13]. Therefore, G-quadruplex DNA has become an important molecular target for developing new anticancer drugs [14].

In the last decades, many researchers focused on metal complexes as effective stabilizers of quadruplex DNA [15]. Some of the metal complexes interact strongly with G-quadruplex DNA and exhibit high selectivity for G-quadruplex DNA [16]. Zn-CAP formed as a Zn(II) complex from 1,2-bis (5-chlorosalicylidene amino)-phenylene of large aromatic planarity is widely existed in biological systems (Fig. 1). It is apt to externally stack on the terminal G-tetrads of the G-quadruplexes [17, 18]. On the other hand, hydrogen bonds between the carboxyl groups of Zn-CAP with the quadruplex phosphate backbone would enhance the interaction between the Zn-CAP and the quadruplex DNA [19]. In this work, we report the interactions between Zn-CAP and different G-quadruplex DNA as well as ctDNA in the presence of K^+ by means of fluorescence, CD (circular dichroism) spectroscopy and the FRET (fluorescence resonance energy transfer) assay.

2. Experiments

2.1 Materials and Instruments

All chemical reagents were commercially available and received without further purification, unless noted specifically. ctDNA (calf thymus DNA) and Tris were purchased from Sigma. C-kit-1 (CGGGCGGGCACGAGGGAGGGT), HTG21 (GGGTTAGGGTTAGGGTTAGGG), Pu27 (TGGGGAGGGT GGGGAGGGTGGGGAAGG), F21T (5'-FAM-GGGCTAGGGCTAGGGCTAGGG-TAMRA-3') and FPu18T (5'-FAM-AGGGTGGGGAGGGTGGGG-TAMRA-3') were purchased from Sangon Biotech Co. Ltd. (Shanghai). Fluorescence spectra were obtained on Shimadzu RF-5301PC Spectrofluorophotometer. CD spectrum was recorded on Jasco J-810-150L Spectropolarimeter. The FRET-melting assay was made on 7500 Fast Real Time PCR (ABI Co. Ltd., USA).

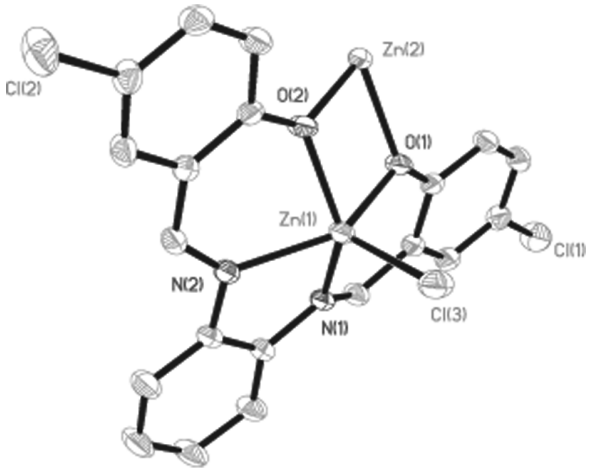


Fig. 1 Crystal structure of Zn-CAP.

In the spectral analyses, Zn-CAP was dissolved in DMSO to prepare the stock solution of 2.0 mM. Tris-KCl buffer solution (10 mM Tris, 100 mM KCl, adjusted to pH = 7.35 by hydrochloric acid) was prepared using double distilled water. The calf thymus DNA was dissolved in Tris-KCl buffer solution to prepare the stock solution of 2.0 mM. G-quadruplex DNA was dissolved in Tris-KCl buffer solution to prepare the stock solution of 100 μM. The calf thymus DNA and G-quadruplex DNA stock solution were stored at 4 ^oC for no more than 5 days before use.

2.2 Synthesis

2.2.1 Preparation of Ligand CAP

A mixture of 1,2-diaminobenzene (1.08 g, 0.01 mol), 5-chlorosalicylaldehyde (3.12 g, 0.02 mol) dissolved in distilled water (20 cm^3) was refluxed with stirring. The solution was continuously refluxed in water-bath at 75 ^oC for 6 h. After cooled to the room temperature, the yellow precipitate appeared, then filtration. And then recrystallized from methanol, and dried (yield, 71%).

2.2.2 Preparation of Zinc(II) Complex Zn-CAP

The mixture of $ZnCl_2$ (0.1 mmol, 0.014 g), CAP (0.12 mmol, 0.046 g), and ethanol (1 cm^3) was heated

to 80 ºC and was stirred for 12 h. After cooling down to the room temperature, the yellow-brown crystals were thus obtained. Yield: 0.035 g, 58%. Single crystals suitable for X-ray diffraction analysis were harvested by slow evaporation of the re-crystallized ethanol solution.

2.3 X-ray Crystallography

The data collection of single crystal of Zn-CAP was carried out on a Bruker Smart Apex II CCD diffractometer equipped with graphite monochromated Mo-Kα radiation (λ = 0.71073 Å) at room temperature. The structures were solved with direct methods and refined using SHELX-97 programs. The non-hydrogen atoms were located in successive difference Fourier synthesis. The final refinement was performed by full-matrix least-squares methods with anisotropic thermal parameters for no-hydrogen atoms on F2. The hydrogen atoms were added theoretically and riding on the concerned atoms. The paramerters used intensity collection and refinements are summarized in Table 1 together with the crystal data.

Table 1 Crystal data and structure refinement for [Zn-CAP].

Empirical formula	$C_{44}H_{38}Cl_6N_4O_7Zn_3$
Formula weight (*M*)	1,143.59
Crystal system	Monoclinic
Space group	C_2/c
a (Å)	24.8524(13) Å
b (Å)	8.4643(5) Å
c (Å)	23.0873(11) Å
α (°)	90.00°
β (°)	102.973(5)°
γ (°)	90.00°
V (Å^3)	4,732.7 (4) Å^3
Z	4
F (000)	2,312
θ range for data collection (°)	6.04-52.74°
Reflections collected/unique	23704/4845 [*R*(int) = 0.0420]
Goodness-of-fit on F^2	1.058
Final *R* indices [$I > 2\sigma(I)$]	R_1 = 0.0394 ω R_2 = 0.0946
R indices (all data)	R_1 = 0.0522 ω R_2 = 0.1027

2.4 Stability in Solution

Complex Zn-CAP was tested on its stability in physiological conditions (TBS buffer solution with pH value of 7.35, containing 1% DMSO, at 25 ^{o}C) by means of UV-Vis spectroscopy.

2.5 Spectroscopic Studies on DNA Interaction

The fluorescence emission spectra were recorded under slit width as 3 nm/3 nm with excitation wavelength (E_x) as 410 nm. The concentration of Zn-CAP was kept for 20 μM as working solutions. The G-quadruplex DNA was added with increasing [G_4-DNA]/[Zn-CAP] ratios ranging from 0.02 to 0.2 in every 0.02 interval, while the increasing [ctDNA]/[Zn-CAP] ratios ranged from 0.2 to 4.0 in every 0.4 interval. The CD absorption spectra of DNA were measured in Tris-KCl buffer solution at a 100 nm/min scan rate in the wavelength range from 200 nm to 400 nm. The concentration of Zn-CAP was kept for 25 μM as working solutions. The concentrations of G-quadruplex DNA and ctDNA were maintained at 5 μM and 100 μM, with [G_4-DNA]/[Zn-CAP] = 1:5 and [ctDNA]/[Zn-CAP] = 4:1, respectively. For FRET-melting assay analysis, Oligonucleotides were initially diluted in 10 mM K^+-buffer solution (pH 7.35) to the required concentration (10 μM) and then annealed by heating to 90 ^{o}C for 5 min, followed by cooling to room temperature in the heating block. The resulting 20 μL solutions were added into 96-well RT-PCR plates (Bio-Rad) (MJ Research, Waltham, MA). Measurements were made on 7500 Fast Real Time PCR (ABI Co. Ltd., USA) with excitation at 470 nm and detection at 530 nm. Fluorescence readings were taken at intervals of 0.87 ^{o}C over the range 37-95 ^{o}C, with a constant temperature being maintained for 30 s prior to each reading to ensure a stable value. All experimental values were determined in triplicate. Final analysis of the data was carried out using a script written in the program Origin 7.5 (OriginLab Corp.). All the spectroscopic experiments were performed at room temperature.

3. Results and Discussion

3.1 Chemistry

The tetradentate Schiff base as ligand CAP was synthesized by the condensation reaction of *o*-phenylenediamine and 5-chlorosalicylaldehyde (Scheme 1). Corresponding zinc(II) complex Zn-CAP was prepared by the reaction of $ZnCl_2$ and CAP in the presence of ethanol under solvothermal conditions (Scheme 1). The formula of compound Zn-CAP was confirmed by spectroscopic studies as well as single crystal X-ray diffraction analysis.

3.2 Mass Spectra

The mass spectra of the soluble compound Zn-CAP was recorded in DMSO solution. The monomer zinc(II) complex Zn-CAP shows peak at *m*/*z* 482.7 amu due to the parent ion fragment $[Zn\text{-}CAP+Cl]^-$ (Fig. 6). Each spectrum is associated with daughter peaks due to fragmentation.

3.3 Crystal Structures

The crystallographic data and refinemental details of the complex Zn-CAP was summarized in Table 1. The selected bond lengths (Å) and bond angles (°) for complex Zn-CAP was given in the caption of Fig. 1. The crystal structure shows that in complex Zn-CAP the Schiff base ligand (CAP) is four-fold coordinated at the zinc(II) via the imine nitrogens and the oxygen atom of the hydroxyl groups. The zinc atom resides in a square-planar geometry. The bond angles at the zinc atom (N1-Zn1-N2, N1-Zn1-O1, N2-Zn1-O2 and O1-Zn1-O2) lie in the range of 75.0-90.0°.

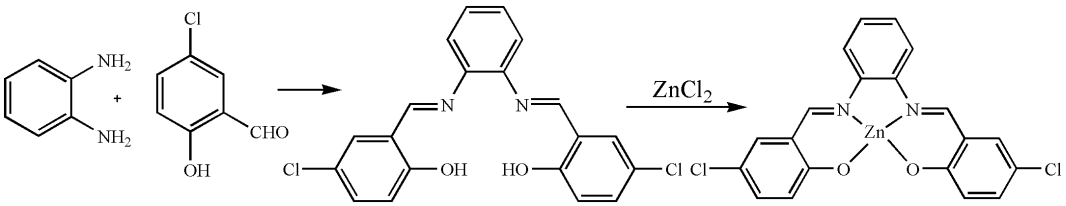
3.4 Stability in Solution

As shown in Fig. 2, complex Zn-CAP in TBS buffer solution was screened on their UV-Vis absorption spectrum at 0, 12 and 24 h, respectively. It could be found that no obvious changes on the absorption peaks and shapes for the complex Zn-CAP over the time, indicating that no structural alterations or decompositions on the complex Zn-CAP [20]. On the other hand, the absorption intensities for the complex Zn-CAP decreased slightly and regularly with time, which could be explained as the slow precipitation of the complex due to their poor water solubility [21].

3.5 Fluorescence Emission Spectral Analysis

In aqueous solution system, the fluorescence emission of small molecules can be quenched by polyanionic nucleotide [22], and a collisional quenching mode is usually considered. The collisional quenching mode can be caused by the electronic transitions from the excited state (π^* orbital) of the fluorescent molecule to the ground state (π orbital) of the quencher (such as nucleic acid). It is based on the π-π stacking between the aromatic groups of fluorescent molecules and base pairs of DNA, which is generally attributed to an intercalative binding mode of Zn-CAP with DNA [23].

The fluorescence emission spectra of Zn-CAP in the absence and presence of ctDNA or G-quadruplex DNA are shown in Fig. 3. Zn-CAP solution gave the characteristic fluorescence emission at around 530 nm under exciting wavelength of 420 nm. The addition of ctDNA or G-duadruplex DNA all caused obvious quenching on the fluorescence emission of Zn-CAP, but with different quenching extents. Under the addition of G-duadruplex with increasing $[G_4\text{-DNA}]/[Zn\text{-CAP}]$ ratios in the range from 0.02:1 to 0.20:1 (every 0.02 interval), quenching ratios of 10.73%, 15.07% and 8.59% were achieved, respectively.



Scheme 1 Preparation of Zn-CAP from 1,2-diaminobenzene,5-chlorosalicylaldehyde and $ZnCl_2$.

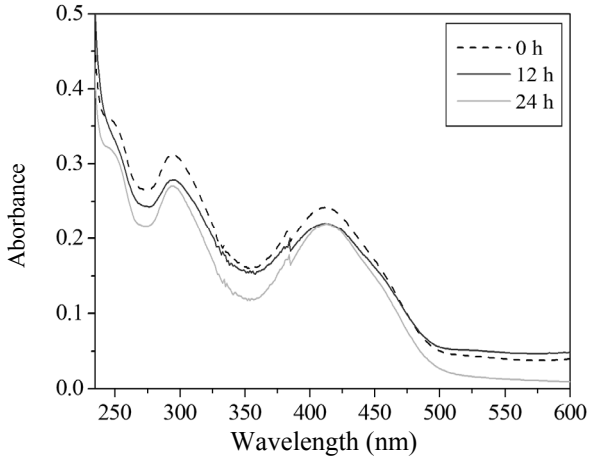


Fig. 2 Time-dependent stability studies on complex Zn-CAP in TBS buffer solution monitored by UV-vis absorption spectra.

In contrary, under the increasing [ctDNA]/[Zn-CAP] ratios in the range from 0.2:1 to 4.0:1, only 7.08% as that Zn-CAP bound with G-quadruplex DNA more intensively than with the ctDNA, especially G-quadruplex Pu27.

3.6 Circular Dichroism Spectra

The CD is a useful technique to assess whether the nucleic acids undergo conformational changes as a result of complex formation or changes in environmental conditions [24], including monitoring the folding of G-quadruplex and the influence of a ligand when binding to a quadruplex structure [25].

As shown in Fig. 4, the CD spectra of ct-DNA showed a positive absorption peak at 275 nm and a negative absorption peak at 242 nm due to π-π base

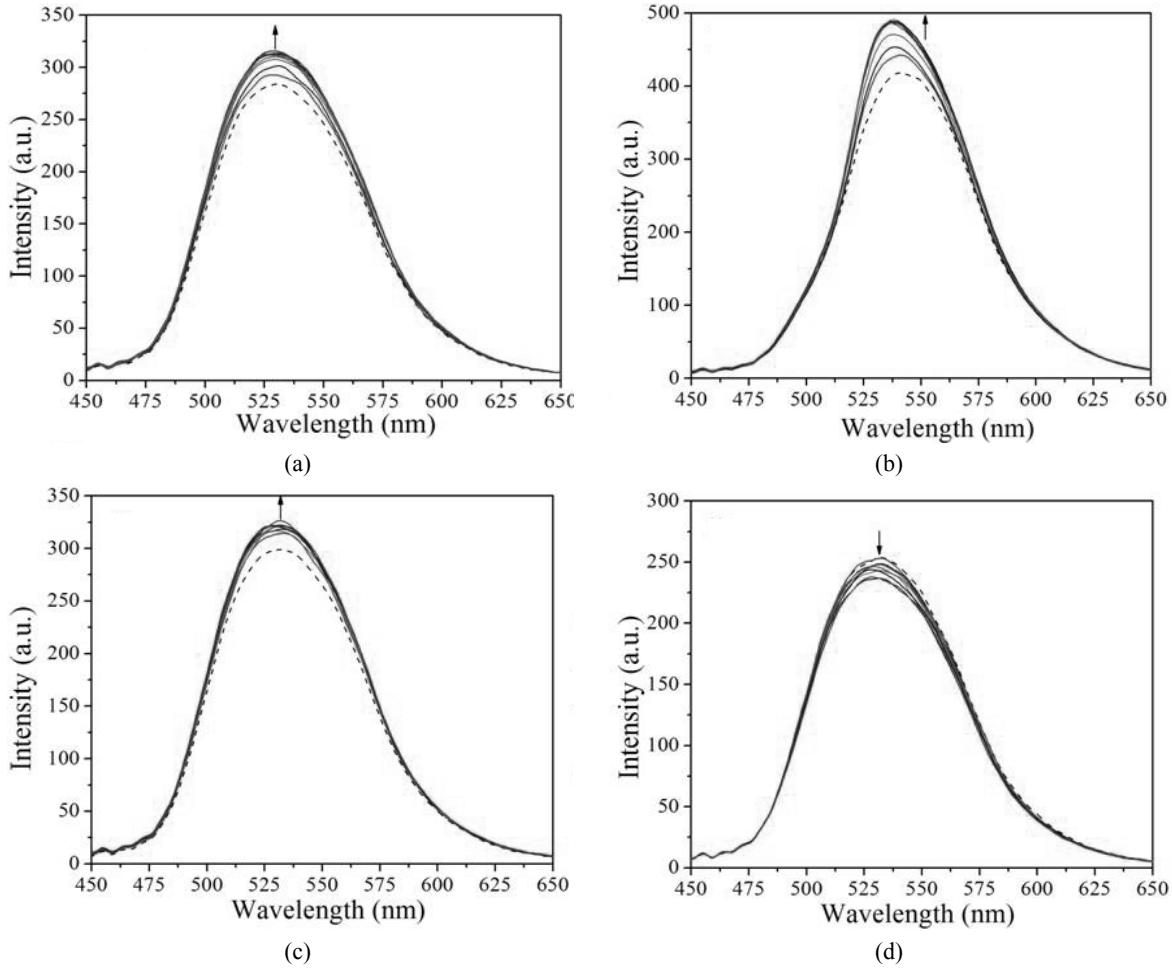


Fig. 3 Fluorescence emission spectra of Zn-CAP (20 μM) in 100 mM K^+ solution in the absence and presence of ctDNA or different G-quadruplex DNA: (a) G_4-HTG21; (b) G_4-Pu27; (c) G_4-c-kit-1 and (d) ct-DNA.

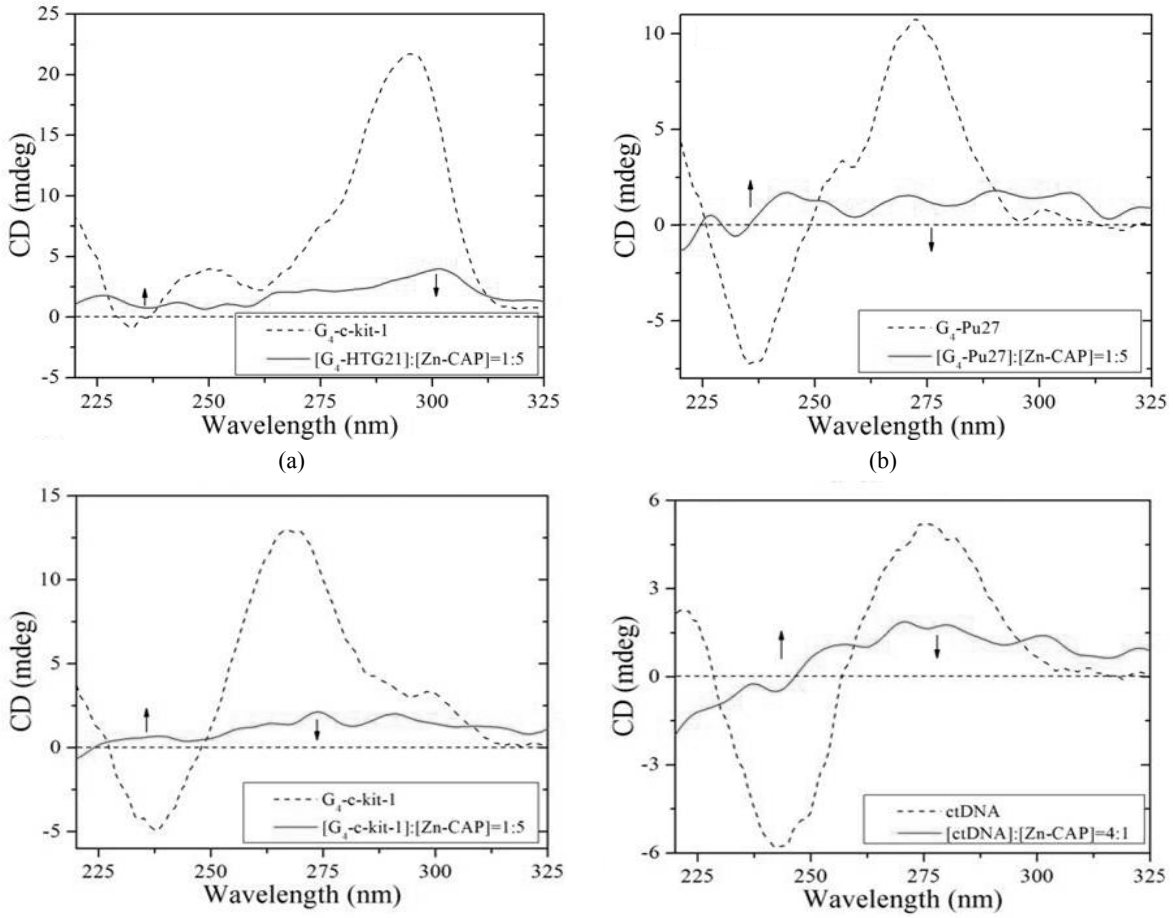


Fig. 4 CD spectra of between ct-DNA with G-quadruplex DNA in the presence of K^+ upon addition of Zn-CAP: (a) [G_4-HTG21] = 5 μM, [Zn-CAP] = 25 μM; (b) [G_4-Pu27] = 5 μM, [Zn-CAP] = 25 μM; (c) [G_4-c-kit-1] = 5 μM, [Zn-CAP] = 25 μM; (d) [ctDNA] = 100 μM, [Zn-CAP] = 25 μM.

stacking and right-hand helicity, respectively, being consistent with the characteristic B conformation of DNA [26]. The CD spectra of G_4-HTG21, G_4-Pu27 and G_4-c-kit-1 show a negative peak at 235 nm and a positive peak at about 270 nm or 295 nm, which is indicative of an antiparallel or parallel G-quadruplex conformation [27].

The addition of 5 equiv. of Zn-CAP to G-quadruplex DNA solution induced different CD spectral changes from that of to ctDNA. As showed in Fig. 4, the interaction with Zn-CAP could induce significant spectral changes on the CD absorption of G_4-HTG21, G_4-Pu27 and G_4-c-kit-1, with 106.00%, 93.06%, 113.47% increment at 232 nm absorption, along with a 81.11%, 92.80%, 83.72% decrement at 295 nm or 270 nm absorption, which demonstrated that the antiparallel or parallel structure of different G-quadruplex DNA are more stable in the presence of Zn-CAP. Comparatively, the addition of Zn-CAP could induce significant spectral changes on the CD absorption of double helix ctDNA, with 90.91% increment on the negative peak absorption, along with a 64.17% decrement on the negative peak absorption. The results clearly indicated that the competitive binding of Zn-CAP induce the formation of quadruplex DNA more obvious change than that of ctDNA.

3.7 FRET Assay on Quadruplex

The stability of F21T or FPu18T treated with Zn-CAP was investigated by FRET melting assay. The oligomer F21T or FPu18T containing a fluorophore at the 5'-end and a fluorescence quencher at the 3'-end

were used in this assay. Intramolecular folding should bring the two chromophores in close enough proximity for energy transfer to be observed. In the unfolded form, little transfer is expected, as the average distance of the two chromophores should be larger than the 5.0 nm distance [28, 29]. The stabilising ability of Zn-CAP appears to be concentration-dependent with much higher activity observed at 0.5 μM compared to F21T or FPu18T alone (Fig. 5). The ΔT_m values were 3.45 ^{o}C and 15.85 ^{o}C, respectively, from which it is clear that Zn-CAP is a potent and selective stable of the G-quadruplex FPu18T compared to F21T. The results further suggested that Zn-CAP bound with G-quadruplex Pu27 more intensively than with the human telomeric G-quadruplex HTG21, which is consistent with what was suggested by fluorescence emission spectral and circular dichroism spectra analysis.

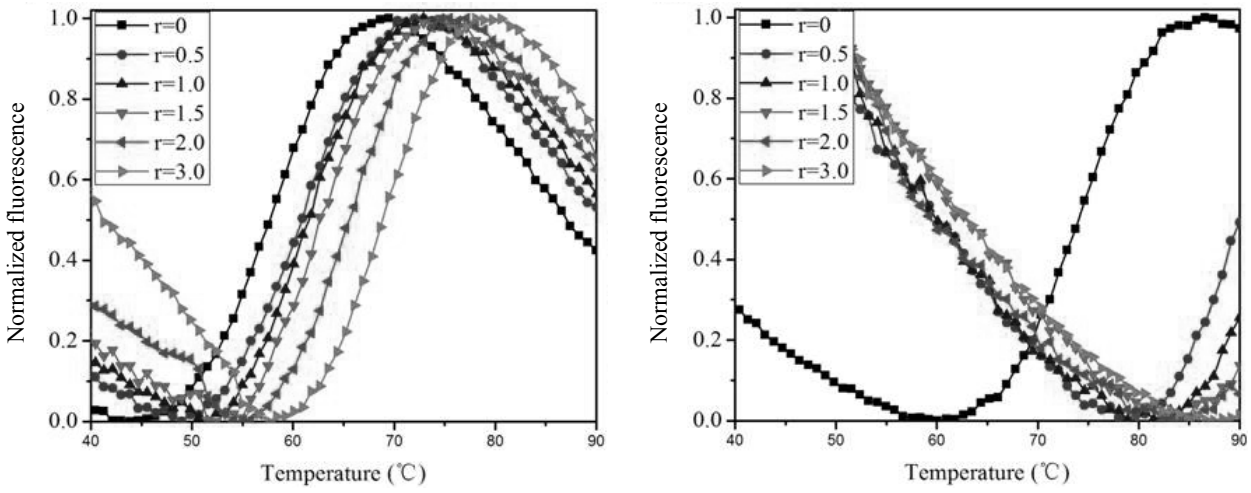


Fig. 5 FRET melting curves for experiments carried out with (a) F21T or (b) FPu18T (1 μM in 10 mM Tris-KCl-HCl buffer pH 7.35) with Zn-CAP: *r* = [Zn-CAP]/[F21T or FPu18T].

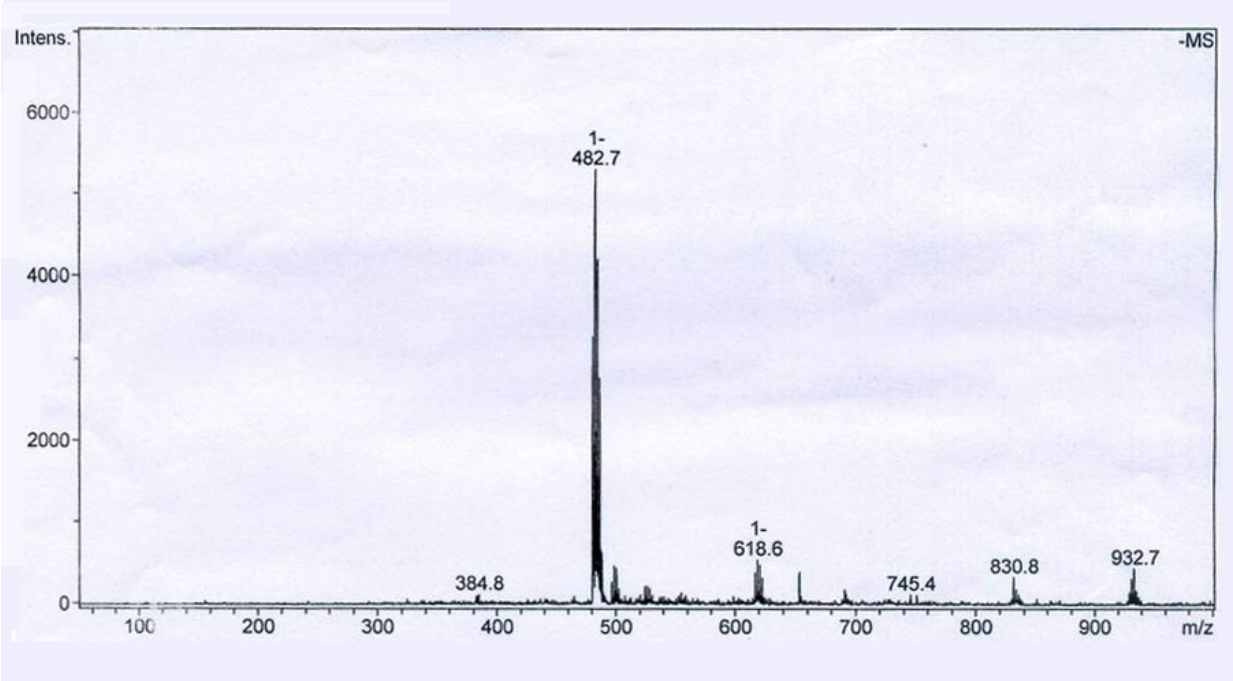


Fig. 6 ESI-MS spectrum of Zn-CAP.

4. Conclusions

In summary, the interactional mechanism of Zn-CAP with three types of G-quadruplex DNA as well as ctDNA was studied by fluorescence, CD spectroscopic and FRET assay. The results indicated that Zn-CAP exhibited higher binding affinity with G-quadruplex DNA than to ctDNA, especially G-quadruplex Pu27. This may be explained as the more expanded planar aromatic structure of G-quadruplex DNA facilitates its π-π stacking with Zn-CAP than does the ctDNA. And the antiparallel or parallel G-quadruplex form DNA is more stable than double helix form DNA in the presence of Zn-CAP.

Acknowledgments

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