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EXPERIMENTAL RESEARCH

Synthesis of copper (II) porphyrin complexes and their interaction with *c-myc* G-quadruplex DNA

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[Abstract] **Objective** To synthesize copper (II) porphyrin complexes and study their interactions with *c-myc* G-quadruplex DNA. **Methods** The 5-*p*-hydroxyphenyl-10, 15, 20-*tris* (*p*-methoxyphenyl) copper (II) porphyrin complex [*p*-HTMOPPCu (II)] was synthesized by the conventional heating method. Ultraviolet (UV) titration, fluorescence titration, fluorescence resonance energy transfer (FRET) melting point and competitive assays were used to study the interactions between *p*-HTMOPPCu (II) and *c-myc* G-quadruplex DNA. **Results** The UV absorption spectrum and fluorescence spectroscopy results indicated that the complex of *p*-HTMOPPCu (II) bound better with *c-myc* G-quadruplex DNA; the FRET melting point assay, and competitive melting point assay demonstrated that *p*-HTMOPPCu (II) could selectively bind and stabilize *c-myc* G-quadruplex DNA. **Conclusion** *p*-HTMOPPCu (II) can bind and stabilize *c-myc* G-quadruplex DNA and will potentially be developed into a class of small molecule inhibitors targeting *c-myc* G-quadruplex DNA for clinical applications in the treatment of tumors.

[Key words] Copper (II) porphyrin complexes; Synthesis; *c-myc* G-quadruplex DNA

1 Introduction

The G-quadruplexes (G4s)[1] are a nucleic acid secondary structure rich in guanine and widely present in the human genome, especially in the promoter region of oncogenes[2]. The proto-oncogene *c-myc* is an important transcription factor overexpressed in 70% of human cancers[3]. The nuclease hypersensitive element III1 (NHE III1) in the *c-myc* P1 promoter region, which controls 85%-90% of *c-myc* transcription, is guanine-rich and can fold to form G4s, thereby inhibiting *c-myc* transcription and expression[4]. Therefore, targeting *c-myc* has an important research value for developing novel antitumor drugs. Meanwhile, metal complexes achieve a better binding effect with G4s, attracting widespread attention as anti-cancer drugs[5].

Porphyrins and metalloporphyrins, widely found in nature and living organisms, play an important role in life processes; therefore, greater emphasis has been placed on the study

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of porphyrins[6-7]. To date, porphyrins and metalloporphyrins have many applications in medicine, biochemistry, synthetic chemistry, materials chemistry, and energy[8-9]. In 1979, Feil et al[10]. demonstrated that 5, 10, 15, 20-tetrakis (1-methyl-4-pyridinio) porphyrin tetra (*p*-toluenesulfonate) (TMPyP) could be embedded in DNA bases of calf thymus. Study on the interaction of TMPyP with various DNA and other biomolecules has since become a topic of profound interest. Research has shown that TMPyP4 binds and stabilizes *c-myc* Pu27 G4 DNA and inhibits the transcription and expression of the *c-myc* gene[11]. In addition, metalloporphyrin[12] can bind to nitrogenous bases on DNA and interact with polar groups through hydrogen bonding, electrostatic interactions, and hydrophobic interactions, which have good prospects for recognizing DNA. Therefore, it is valuable to develop further and design a metalloporphyrin that can bind and stabilize *c-myc* G4.

Previous studies[13-14] found that copper 5, 10, 15, 20-tetra (4-merhoxyphenyl) porphyrin (CuTMOPP), a copper (II) porphyrin complex, could bind and stabilize *c-myc* G4 DNA and inhibit its replication, but its poor water solubility limited related further study. Therefore, a hydroxyl group was introduced into the complex CuTMOPP to improve its chemical properties in this assay. According to the Alder-Longo method[15], the 5-*p*-hydroxyphenyl-10, 15, 20-tris (*p*-methoxyphenyl) porphyrin was synthesized by the reaction of 2, 5-unsubstituted pyrroles with aldehydes that provide endo-methylene, and further refluxed with copper sulfate pentahydrate to obtain 5-*p*-hydroxyphenyl-10, 15, 20-tris (*p*-methoxyphenyl) porphyrin copper (II) complexes [*p*-HTMOPPCu (II)]. Moreover, the interaction between this copper (II) porphyrin complex and *c-myc* G4 DNA was investigated by ultraviolet (UV) titration, fluorescence titration, fluorescence resonance energy transfer (FRET) melting point, and competitive assay.

2 Instruments and Reagents

2.1 Instruments

The RE-52A rotary evaporator (Shanghai Yarong Biochemical Instrument Co., Ltd.); TE214S one-millionth electronic analytical scale (Sartorius Scientific Instruments [Beijing] Co., Ltd.); DF-101S thermostatic magnetic stirrer (Yuhua Instrument [Gongyi] Co., Ltd.); SHZ-D (III) circulating water multi-purpose vacuum pump (Henan Province Yuhua Instrument Co., Ltd.); UV-2550 UV-visible spectrophotometer (Shimadzu [Japan] Corporation); RF-5301PC fluorescence spectrophotometer (Shimadzu [Japan] Corporation); UPLC-Q-Tof mass spectrometer (Micromass UK Limited); CFX86 real-time fluorescence quantitative PCR instrument (Bio-Rad [U.S.] Laboratory); PB-10 pH meter (Sartorius Scientific Instruments [Germany] Co., Ltd.).

2.2 Reagents

Propionic acid (Macklin Biochemical Reagent [Shanghai] Co., Ltd., GC); methanol (Tianjin Zhiyuan Chemical Reagent Co., Ltd., AR); trichloromethane (Guangzhou Chemical Reagent Factory, AR); pyrrole (Shanghai Aladdin Biochemical Technology Co., Ltd., CP); copper sulfate pentahydrate (Tianjin Damao Chemical Reagent Ltd., AR); *N*,*N*-dimethylformamide (DMF) (Guangdong Guanghua Technology Ltd., AR); absolute ethyl alcohol (Tianjin Zhiyuan Chemical Reagent Co., Ltd., AR); 4-methoxybenzaldehyde (Macklin Biochemical Reagent [Shanghai] Co., Ltd., AR); 4-hydroxybenzaldehyde (Sinopharm Chemical Reagent Co., Ltd., AR); tris(hydroxymethyl)aminomethane (Tris) (BioFRoxx, 99.9%); dimethyl sulfoxide (Tianjin Damao Chemical Reagent Ltd., AR); potassium chloride (Tianjin Zhiyuan Chemical Reagent Co., Ltd., AR); hydrochloric acid (Guangzhou Chemical Reagent Co., Ltd., AR); double-stranded DNA ds26 (General Biological Co., Ltd., biological grade);

c-myc G4 DNA (General Biological Co., Ltd., biological grade). Pyrrole was distilled before use.

Tris-HCl buffer solution (100 mmol/L KCl, pH 7.2): Precisely weigh 0.30 g Tris, 1.87 g KCl, add 200 mL double-distilled water to dissolve, adjust the pH to 7.2 with 1 mol/L hydrochloric acid solution, transfer to a 250 mL volumetric flask, fix the volume with double-distilled water to the scale line, and mix well to obtain the solution.

Tween buffer solution: Take 88 mL of Tris-HCl buffer solution (100 mmol/L KCl, pH 7.2), 10 mL of DMF, and 202 mL of Tween, and mix well to obtain the solution.

Preparation of *c-myc* G4 DNA: Dissolve 5OD1 in 219 μL of 10 mmol/L Tris-HCl, 100 mmol/L KCl buffer solution (pH 7.2), heat to 95℃ for about 5 minutes, cool to room temperature, and store at 4℃ for 24 hours to obtain the DNA.

Preparation of Fpu22T: Dissolve 1OD of double fluorescence-labeled pu22 (Fpu22) in 36 μL of 10 mmol/L Tris-HCl, 10 mmol/L KCl, 10 mmol/L Na_3AsO_4 buffer solution (pH 7.2), heat at 90℃ for 5 minutes, cool to room temperature, and store at 4℃ for 24 hours to obtain Fpu22T.

3 Methods

3.1 Synthetic Route

According to previous reports[16-17], the synthetic route of *p*-HTMOPPCu(II) was designed as follows (Fig. 1).

3.2 Synthesis Steps

3.2.1 Synthesis of 5-p-hydroxyphenyl-10, 15, 20-tris (p-methoxyphenyl) porphyrin (p-HTMOPP)

Add *p*-hydroxybenzaldehyde (1.82 g, 0.01 mol), *p*-methoxy benzaldehyde (8.75 g, 0.04 mol), and propionic acid (120 mL) to a round bottom flask, heat the solution at 140℃ for reaction, slowly adjust the speed to 40 r/min, stir and reflux, slowly add 80 mL of a mixture of propionic acid and freshly distilled pyrrole (3.99 g, 0.06 mol) dropwise, and continue stirring for 40 min. After the reaction, the solution was cooled to room temperature and left to stand overnight after 200 mL of absolute ethyl alcohol was added. After filtration, the filter cake was dried under a vacuum to obtain the crude purple product. The product was purified by silica-gel column chromatography and eluted with trichloromethane. The second purple band was collected and spun dry under reduced pressure to obtain 0.45 g of 5-*p*-hydroxyphenyl-10, 15, 20-tris (*p*-methoxyphenyl) porphyrin (*p*-HTMOPP) in 4.2% yield (calculated based on *p*-hydroxybenzaldehyde).

3.2.2 Synthesis of 5-p-hydroxyphenyl-10, 15, 20-tris (p-methoxyphenyl) porphyrin copper (II)(p-HTMOPPCu (II))

The 5-*p*-hydroxyphenyl-10, 15, 20-tris (*p*-methoxyphenyl) porphyrin (100 mg, 139 mmol)

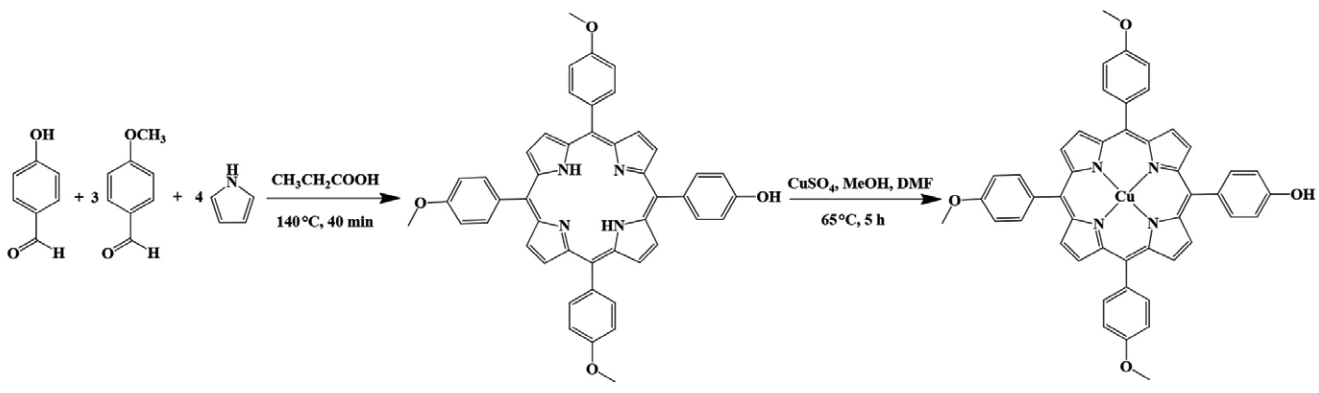

Fig. 1 The synthetic route of *p*-HTMOPPCu (II)

was accurately weighed and placed in a 50-mL three-necked flask, and 10 mL of DMF and 5 mL of methanol solution with anhydrous cupric sulfate (139 mg, 0.556 mmol) were added. The reaction was performed at 65℃ for 5 hours and then cooled to room temperature. The reaction solution was transferred to 200 mL of ice-cold deionized water, allowed to stand for a while, filtered, washed several times with distilled water, and dried in a vacuum drying oven to obtain the crude product. The crude product was dissolved with trichloromethane, undergone silica-gel column chromatography, and eluted with trichloromethane. The main target band was collected and spun dry under reduced pressure. The target product, 0.47 g of *p*-HTMOPPCu (II), was obtained in 43.38% yield (calculated based on porphyrin).

3.2.3 UV titration experiment

A 3 mL of Tween buffer was added to the cuvette, and the baseline was read with a UV-visible spectrophotometer. Subsequently, 18 μL of 1 mmol/L complex solution was added to the sample cell, making a 6 μmol/L complex solution; meanwhile, an equal volume of DMSO was added to the reference cell, and the UV absorption of the complex was measured after each was mixed respectively. The same volume of *c-myc* G4 DNA (100 μmol/L) solution was added to the reference cell, and the sample cell every 3 min to increase the concentration ratio of DNA to the complex in a certain ratio, and the changes of the absorption spectrum of the complexes were monitored in the range of 200-800 nm.

3.2.4 Fluorescent intercalator displacement (FID) assay

A 3 mL of Tween buffer, 1.43 μL of thiazole orange solution (TO) and 15 μL of *c-myc* G4 DNA (0.5 μM G4 DNA, 1.0 μM thiazole orange) were added to the fluorescent cuvette. They were mixed well and equilibrated for 0.5 hours. We set the excitation wavelength to 501 nm and recorded the fluorescence spectrum in the range of 500-660 nm. With 2 μL1 mmol/L copper(II) porphyrin complex solution added to the fluorescent cuvette each time, the solution was mixed well and equilibrated for 2 minutes. The fluorescence spectrum was recorded to observe the changes of TO-DNA fluorescence intensity with increasing concentration gradient of copper (II) porphyrin complex.

3.2.5 Fluorescence resonance energy transfer (FRET) melting point assay and competitive melting point assay

0.2 μmol/L Fpu22 (5'-FAM TGGGGAGGGTGGGGAGGGTGGGGAAGG-TAMRA-3', FAM: carboxyfluorescein, TAMRA: 6-carboxytetra methylrhodamine) were mixed with different concentrations of complexes (0, 3 and 6 μmol/L), respectively, and sodium arsenate buffer (pH 7.2) was used to top up to 25 μL. Three tubes of each concentration were incubated in parallel at 30℃ for 30 minutes, and the programmed temperature variation range was set from 30℃ to 95℃ with a temperature increase interval of 1℃, and the fluorescence values of FAM were recorded after equilibration for 30 seconds using the real-time fluorescence quantitative PCR instrument. The competitive melting point assay was similar to the FRET melting point assay, except that it was mixed with double-stranded DNA (ds26) (0, 1, and 2 μmol/L) into the system of 0.2 μmol/L Fpu22T solution and 3 μmol/L complex solutions, and detected by real-time fluorescence quantitative PCR instrument. Their data were processed by OriginPro 9.0.

4 Results and Discussion

4.1 Synthesis and characterization of compounds

The *p*-HTMOPP synthesized by the conventional method yielded 4.2% (calculated based on *p*-hydroxybenzaldehyde) and was characterized

using silica-gel column chromatography after purification was characterized using silica-gel column chromatography after purification was characterized by mass-spectrometry after being purified by silica-gel chromatography (Fig. 2). The *p*-HTMOPP showed a strong molecular ion peak (100%) at a mass-to-charge ratio (*m/z*) of 721.280 2, which can be attributed to $[M + H]^+$ of the compound *p*-HTMOPP, which is consistent with the calculated value of 721.277 0. In contrast, the mass-to-charge ratio (*m/z*) of 743.262 5 can be attributed to $[M + Na]^+$ of *p*-HTMOPP.

The *p*-HTMOPPCu (II) synthesized by conventional heating reflux synthesis had a yield of 43.38% (calculated based on porphyrins). The *p*-HTMOPPCu (II) was purified by 300-400 mesh silica-gel column chromatography and eluted with trichloromethane, where the main color band was collected as the target product (Fig. 3). The *p*-HTMOPPCu (II) showed a strong molecular ion peak at a mass-to-charge ratio (*m/z*) of 781.192 1 (100%), which can be attributed to $[M + H]^+$ of compound *p*-HTMOPPCu (II). This is generally consistent with the calculated value of 781.187 6. The results indicated that both obtained compounds are the target compounds.

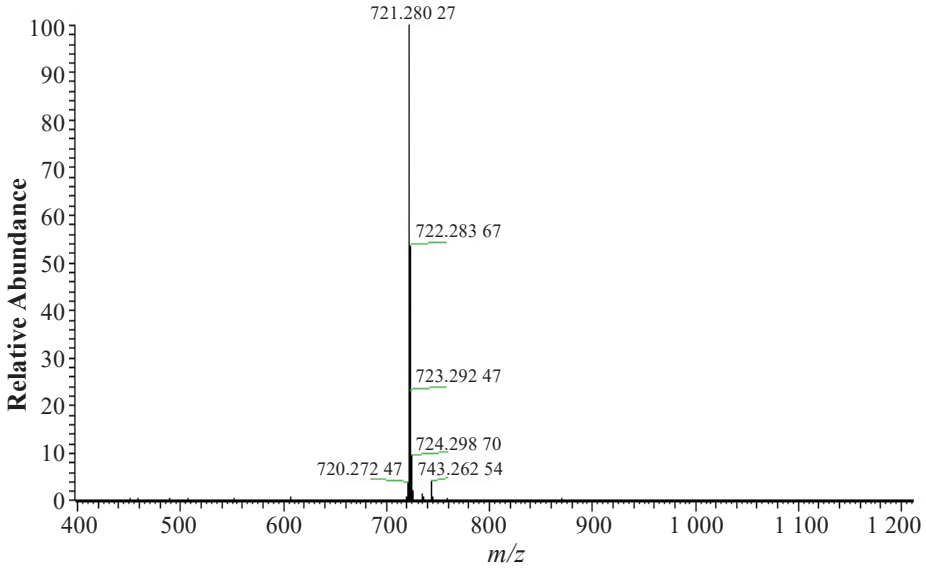

Fig. 2 ESI-MS spectrum of *p*-HTMOPP

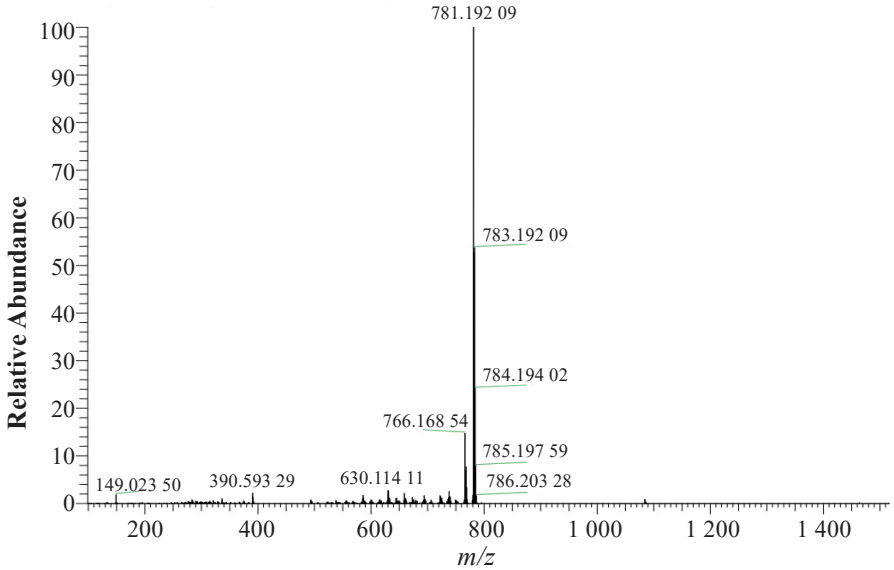

Fig. 3 ESI-MS spectrum of *p*-HTMOPPCu (II)

4.2 UV-visible absorption spectrum

The porphyrins and their metal complexes have strong electronic absorption peaks in the UV and visible regions, and the results are shown in Fig. 4. The porphyrin *p*-HTMOPP in trichloromethane solution showed a characteristic Soret band (i.e., B-band) absorption at 422 nm and a characteristic Q-band absorption of porphyrins at 650, 594, 554 and 518 nm; the *p*-HTMOPPCu (II) had a characteristic Soret absorption at 419 nm attributed to the porphyrin ring, and Q-band absorptions at 577 and 541 nm attributed to copper porphyrins, which are generally consistent with those reported in the literature[18-19].

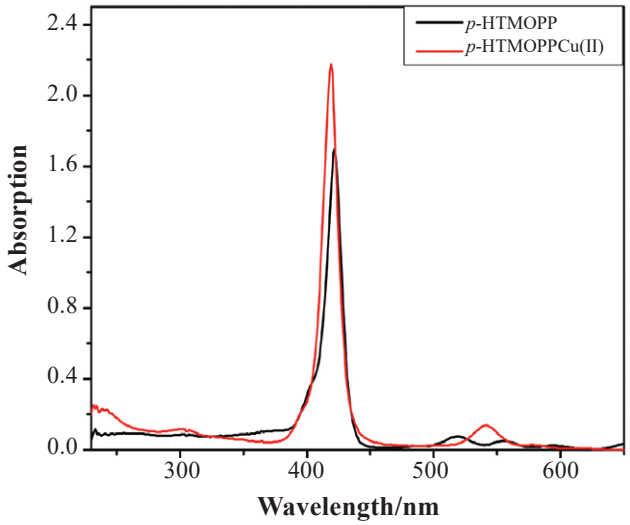

Fig. 4 Electronic absorption spectra of *p*-HTMOPPand *p*-CuHTMOPP (II)complexes. (The concentration is 6 μM, and the solvent is chloroform)

4.3 Experiment of DNA binding ability

4.3.1 UV titration assay

UV-visible absorption spectroscopy is a popular method used to study the interaction of complexes with DNA[20]. In general, the complexes show light absorption at certain wavelengths in the UV-visible spectrum. However, in the presence of DNA, the absorption peaks have signs of decolorization and bathochromic shift, and the intensity of the change depends on the binding strength of the complexes to DNA. Fig. 5 shows the UV-visible absorption spectrum of copper (II) porphyrin complex in Tween buffer with increasing concentration of *c-myc* G4 DNA. The copper (II) porphyrin complex had a characteristic Soret absorption (i.e., B-band) near 420 nm attributed to the porphyrin ring and a Q-band absorption at 500-750 nm attributed to the porphyrin ring. With the increase of *c-myc* G4 DNA concentration, the electronic absorption spectrum of *p*-CuTMOPP showed a significant color enhancement effect at 420 nm with 33.7% enhancement rate, and a color reduction effect at 450 nm with 14.2% reduction rate, indicating that *p*-HTMOPPCu (II) can interact with *c-myc* G4 DNA[21].

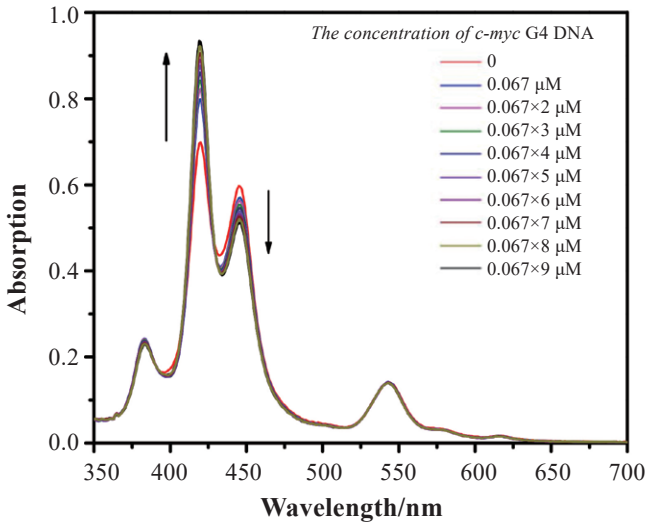

Fig. 5 UV-visible spectrum of copper (II)porphyrin complex *p*-HTMOPPCu (II)in buffer solution with increasing concentration of *c-myc* G4 DNA

[*p*-HTMOPPCu(II)]=6 μmol/L, [*c-myc*]=0.067 nμM, *n*=0, 1, 2, 3, 4, 5, 6, 7, 8, 9.

4.3.2 FID assay

Fluorescence spectroscopy[22] is one of the main tools to study the interaction of compounds with DNA. The copper (II) porphyrin complex is nonfluorescent in an aqueous solution due to a single electron in the 3D orbital of Cu^{2+}, which is paramagnetic; thus, the interaction of the copper (II) porphyrin complex with *c-myc* G4 DNA was investigated using the FID assay. TO is a DNA

organic small molecule fluorescent probe that is nonfluorescent in an aqueous solution and has a high fluorescence intensity when bound to G4 DNA. Therefore, the ability of *p*-HTMOPPCu (II) binding to *c-myc* G4 could be further investigated by observing the reduction rate (λ_{max}=533 nm) of the fluorescent substance of TO-DNA (thiazole orange [TO], a DNA organic small molecule fluorescent probe) upon binding to G4 DNA. Fig. 6 showed the fluorescence spectrum of TO-DNA in buffer solution with *p*-HTMOPPCu (II). As the concentration of *p*-HTMOPPCu (II) increased, the fluorescence intensity of its TO-DNA gradually decreased, which was due to the existence of DNA competition between *p*-HTMOPPCu (II) and TO, which indirectly reflected the binding ability of *p*-HTMOPPCu (II) to *c-myc* G4 DNA. The results showed that the copper (II) porphyrin complex *p*-HTMOPPCu (II) and *c-myc* G4 DNA had some binding ability.

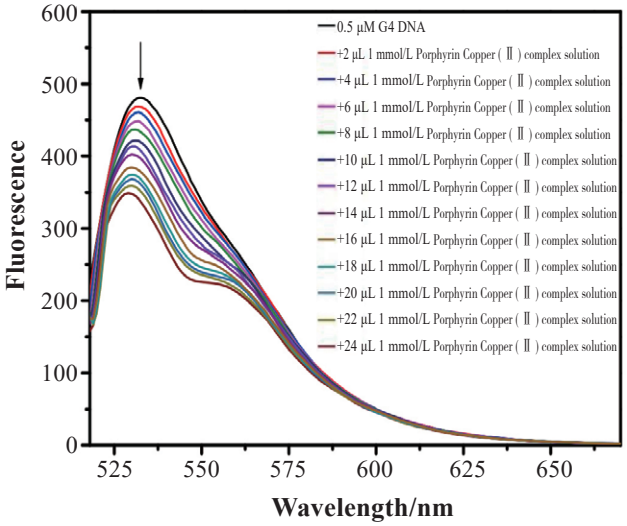

Fig. 6 The fluorescence spectrum of TO-*c-myc* with the increase of the concentration of *p*-HTMOPPCu (II)

4.3.3 FRET melting point and competitive melting point assays

The FRET melting point assay was further employed to investigate the stabilization ability of the copper (II) porphyrin complex *p*-HTMOPPCu (II) on *c-myc* G4 DNA. The melting point variation curves of 0.2 μmol/L *c-myc* at different concentrations of *p*-HTMOPPCu (II) were shown in Fig. 7A. As could be seen from the figure, the melting point temperature (T_m) of Fpu22T in buffer solution was 59 ℃ when no *p*-HTMOPPCu (II) complex was present. As *p*-HTMOPPCu (II)concentration increased, the *c-myc* melting point increased. When the *p*-HTMOPPCu (II) concentrations were 3 and 6 μmol/L, respectively, the melting points of *c-myc* increased, where the ΔTm was 5.1 and 8.0℃, respectively. The results indicated that *p*-HTMOPPCu (II) could bind and stabilize *c-myc* G4 DNA. In addition, FRET competitive melting point assay was used to investigate the selectivity of *p*-HTMOPPCu (II) for *c-myc* G4 DNA and ds26, as shown in Fig. 7B, where a higher than 5-fold and 10-fold of ds26

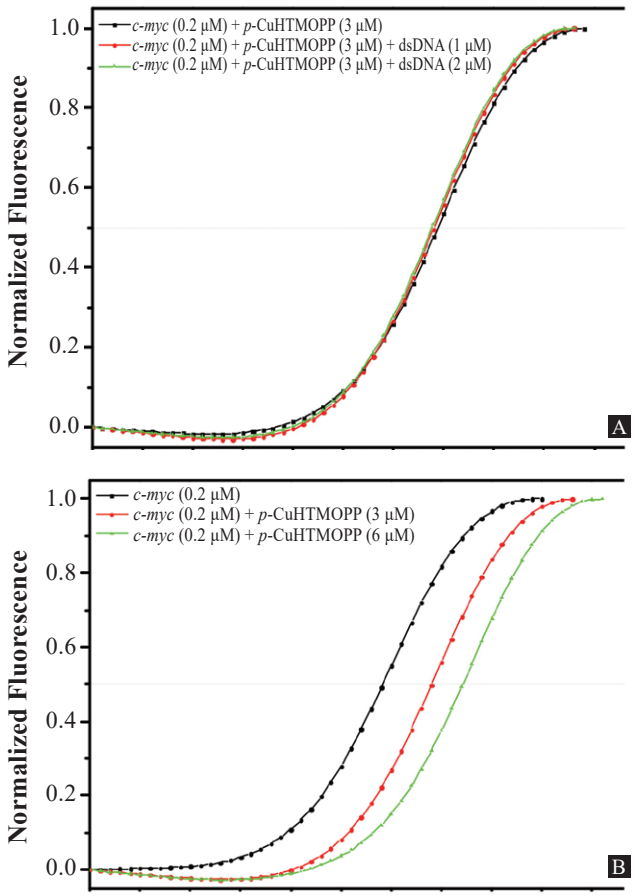

Fig. 7 Melting point curve of *c-myc* G4 DNA (0.2 μM) in K + buffer solution with increased *p*-HTMOPPCu (II) concentration

A. Competitive melting point plot of c-myc G4 DNA, *p*-HTMOPPCu (II) under the action of ds26; B. plot of *c-myc* G4 DNA, *p*-HTMOPPCu (II) under the action of ds26.

was added respectively to the *p*-HTMOPPCu (II) system of 0.2 μmol/L *c-myc* and 3 μmol/L *p*-HTMOPPCu (II) as the competitor. The results showed that the melting point temperature of *c-myc* G4 DNA remained almost unchanged, which indicated that *p*-HTMOPPCu (II) could selectively and preferentially bind and stabilize *c-myc* G4 DNA in the presence of both ds26 and *c-myc* G4 DNA[23-24] .

5 Conclusion

Porphyrins and metalloporphyrins have many applications in medicine, biochemistry, synthetic chemistry, materials chemistry, and energy. The study of their interactions with various DNAs and other biomolecules is a topic of great research interest today. Although CuTMOPP, a copper (II) porphyrin complex, can bind and stabilize *c-myc* G4 DNA and inhibit its replication, its poor water solubility has limited its further investigation. This experiment introduced hydroxyl groups into CuTMOPP to improve its chemical properties. Specifically, 5-*p*-hydroxyphenyl-10, 15, 20-tris (*p*-methoxyphenyl) porphyrin and its copper (II) complex were synthesized, their electronic absorption spectra were measured, and *p*-HTMOPPCu (II) was demonstrated to selectively bind and stabilize *c-myc* G-quadruplex DNA using UV titration, FID, and FRET melting point and competitive melting point assays. This compound will potentially be developed as a class of small molecule inhibitors targeting G4 DNA for clinical applications in tumor therapy.

6 Conflicts of Interest

These authors have no conflict of interest to declare.

7 Acknowledgements

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