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Experimental and density functional theory (DFT) methods studies on the interactions of Ru(II) polypyridyl complexes with the RAN triplex poly(U)•poly(A)*poly(U)

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There is renewed interest in investigating triple helices because these novel structures have been implicated as a possible means of controlling cellular processes by endogenous or exogenous mechanisms. Due to the Hoogsteen base pairing, triple helices are, however, thermodynamically less stable than the corresponding duplexes. The poor stability of triple helices limits their practical applications under physiological conditions. In contrast to DNA triple helices, small molecules stabilizing RNA triple helices at present are less well established. Furthermore, most of these studies are limited to organic compounds and, to a far lesser extent, on metal complexes. In this work, two Ru(II) complexes, $[Ru(bpy)_2(btip)]^{2+}$ (Ru**1**) and $[Ru(phen)_2(btip)]^{2+}$ (Ru**2**), have been synthesized and characterized. The binding properties of the two metal complexes with the triple RNA poly(U)•poly(A)*poly(U) were studied by various biophysical and density functional theory methods. The main results obtained here suggest that the slight binding difference in Ru**1** and Ru**2** may be attributed to

the planarity of the intercalative ligand and the LUMO level of Ru(II) complexes. This study

further advances our knowledge on the triplex RNA-binding by metal complexes, particularly

Ru(II) complexes.

1. Introduction

Nucleic acid triple helices (also called triplexes), containing triple-strand DNA-type and

RNA-type structures, have attracted considerable attention in recent years.[1–5] The reason for

this is that these novel structures have been implicated as a possible means of controlling

cellular processes by endogenous or exogenous mechanisms. Stabilization of triplexes is

extremely important for carrying out their biological functions,[6] such as antigene therapy and

gene regulation. Due to the Hoogsteen base pairing, triplexes are, however,

thermodynamically less stable than the corresponding duplexes. The poor stability of these

structures limits their use under physiological conditions.[6,7] In this regarding, small molecules

able to stabilize the specific sequences of triplexes are of very importance.[8]

In recent years, many efforts are under way to use natural and synthetic small molecules to

modulate the properties of triplex structures under physiological conditions.[9–11] In contrast to

DNA triple helices, however, investigations of small molecules stabilizing RNA triple helices

at present are less well established. Furthermore, most of these studies are limited to organic

compounds[12–14] and, to a far lesser extent, on metal complexes.[15–17] In general, the

stabilization of RNA triple helices can be achieved by the action of intercalators,[18,19] in

particular when covalently linked to the third strand.[20] However, intercalators not covalently

linked can either stabilize or destabilize RNA triplexes.[21,22] For example, the melting

experiments reveal that proflavine (PR) and its platinum(II)-proflavine complex (PtPR)[15] and

ethidium[23] tend to destabilize the triplex, whereas berberine analogs[13] are able to strongly

stabilize the Hoogsteen base paired third strand of RNA triplexes by intercalation. In addition,

some alkaloids stabilize the Hoogsteen base-paired third strand of RNA triplexes almost

without affecting the stability of the duplex, such as berberine, palmatine and coralyne.[18]

Interestingly, studies of the interaction mechanisms of coralyne with

poly(U)•poly(A)*poly(U) (Fig. 1, where •denotes the Watson-Crick base pairing and *

denotes the Hoogsteen base pairing) reflect that coralyne tends to stabilize the triplex structure

because of this small molecule being able to induce the triplex−to−duplex conversion and also

the duplex−to−triplex conversion. These researches reveal that the intercalative process and

modes effect on the stability of RNA triple helices are more complicated than previously

thought and very sensitive to the structure of the bound compound.[14b] In our quest for small

molecules to improve the stabilization of RNA triplexes, we recently reported a Ru(II)

polypyridyl complex, $[Ru(phen)_2(mdpz)]^{2+}$ {phen = 1,10-phenanthroline, mdpz

=7,7′-methylenedioxyphenyl-dipyrido-[3,2-a:2′,3′-c]-phenazine}, could serve as a prominent

molecular "light switch" for poly(U)•poly(A)*poly(U) under physiological conditions and

enhance the triplex stabilization through intercalation.[16] This complex is also a first emission

'light switch' for RNA triplexes.

   To further evaluate and understand the factors effect on the stability of RNA triplexes, it is

quite necessary and really significant to study the interactions between RNA triplexes and

small molecules with different shapes and electronic properties. It's well established that Ru(II)

polypyridyl complexes, due to a combination of easily constructed rigid chiral structures

spanning all three spatial dimensions and a rich photophysical repertoire, prominent DNA

binding properties and promising biological activity, have attracted considerable attentions in

recent years.[24–26] Unfortunately, the study of Ru(II) polypyridyl complex−RNA triplex interactions is very scarce at present.[15,16]

To explore the factors effect on the stabilization of RNA triplexes by octahedral Ru(II) complexes, $[Ru(bpy)_2(btip)]^{2+}$ {Ru**1**; btip = 2-benzo[*b*]thien-2-yl-1*H*-imidazo[4,5-*f*]-[1,10]phenanthroline} and $[Ru(phen)_2(btip)]^{2+}$ (Ru**2**; phen = 1,10-phenanthroline) (Fig. 1) have been synthesized and characterized In this study. The binding properties of Ru**1** and Ru**2** with the triplex RNA poly(U)•poly(A)*poly(U) have been explored by using biophysical techniques and density functional theory (DFT) methods. The main results obtained here further advance our knowledge on the binding of RNA triplexes with metal complexes, particularly Ru(II) complexes.

2. Experimental Sections

2.1. Materials

1,10-phenanthroline-5,6-dione,[27] *cis*-[Ru(phen)$_2$Cl$_2$]·2H$_2$O and *cis*-[Ru(bpy)$_2$Cl$_2$]·2H$_2$O[28] were prepared according to literature procedures. Polynucleotide samples of double stranded poly(A)•poly(U) and single stranded poly(U) were obtained from Sigma-Aldrich Corporation (St. Louis, MO, USA) and were used as received. The RNA triplex poly(U)·poly(A)*poly(U) was prepared as reported earlier.[16] The concentration of poly(U)·poly(A)*poly(U) was determined optically using molar extinction coefficients, ε (M^{-1} cm^{-1}) reported in the literature.[29–31] All titration experiments were conducted at 20 °C in pH 7.0 phosphate buffer (6 mmol/L Na$_2$HPO$_4$, 2 mmol/L NaH$_2$PO$_4$, 1 mmol/L Na$_2$EDTA, 19 mmol/L NaC1).

2.2. Physical measurement

Microanalyses (C, H and N) were carried out on a Perkin–Elmer 240Q elemental analyzer. ^{1}H

NMR spectra were recorded on an Avance-400 spectrometer with d_6-DMSO as solvent at room temperature and TMS (tetramethylsilane) as the internal standard. Mass Spectrometer was performed on an Autoflex III™ Maldi-Tof mass spectrometer (Bruker) using DMSO as the mobile phase. UV–visible spectra were recorded on a Perkin–Elmer Lambda-25 spectrophotometer, and emission spectra were recorded on a Perkin–Elmer LS-55 luminescence spectrometer at room temperature. Circular dichroism (CD) spectra were measured on a JASCO-810 spectropolarimeter.

2.3. Synthesis of 2-benzo[*b*]thien-2-yl-1*H*-imidazo[4,5-*f*][1,10]phenanthroline (btip)[32]

A mixture of 1,10-phenanthroline-5,6-dione (0.16 g, 0.5 mM), benzo[*b*]thiophene-2-carbaldehyde (0.07 g, 1.0 mM), ammonium acetate (1.54 g, 20 mM), and glacial AcOH (10 ml) was heated at reflux with stirring for 1.5 h. The cooled solution was filtered and diluted with water, then neutralized with concentrated aqueous ammonia. The yellow precipitate was collected and purified by column chromatography (Alox; EtOH/toluene 5:1) to afford btip (0.17 g, 96%). Maldi-Tof-MS (m/z): 492.2 [M+1]. Anal. calc. for $C_{21}H_{12}N_4S$: C 71.57, H 3.43, N 15.90; found: C 71.35, H 3.57, N 15.74.

2.4. Synthesis of [Ru(bpy)₂(btip)](ClO₄)₂·H₂O (Ru1)[32]

A mixture of *cis*-[Ru(bpy)₂Cl₂]·2H₂O (104 mg, 0.20 mM), btip (71 mg, 0.20 mM), and ethylene glycol (20 ml) was thoroughly deoxygenated. The purple mixture was heated for 8 h at 150 ^{o}C under argon atmosphere. When the solution finally turned red, it was cooled to room temperature and equal volume of saturated aqueous sodium perchlorate solution was added under vigorous stirring. The red solid was collected and washed with small amounts of H_2O, EtOH, and Et₂O, respectively. After that, the red solid was dried under vacuum and purified on

a neutral alumina column with MeCN/toluene (2:1, v/v) to afford the title complex (143 mg, 73%). Maldi-Tof-MS (m/z): 765.1 ([M-2ClO$_4$-H]$^+$). Anal. calc. for C$_{41}$H$_{30}$Cl$_2$N$_8$O$_9$RuS: C 50.11, H 3.08, N 11.40; found: C 50.02, H 3.10, N 11.39. ^{1}H NMR (400 MHz, ppm, D$_6$-DMSO; d, doublet; s, singlet; t, triplet; m, multiplet): 8.95（d, J = 7.6, 2H）, 8.85 (dd, J$_1$ = 8.8, J$_2$ = 8.8, 4H), 8.20 (t, 2H), 8.09 (t, 2H), 7.96 (d, J = 8.0, 1H), 7.91 (d, J = 6.4, 1H), 7.86 (d, J = 4.8, 2H), 7.78 (d, J = 7.2, 2H), 7.57 (t, 6H), 7.34–7.40 (m, 5H).

2.5. Synthesis of [Ru(bpy)$_2$(pnip)](ClO$_4$)$_2$·H$_2$O (Ru2)

The dark red complex [Ru(phen)$_2$(btip)](ClO$_4$)$_2$ was obtained by a similar procedure to that described above. Yield: 132 mg, 64%. Anal.Calcd for C$_{45}$H$_{30}$Cl$_2$N$_8$O$_9$RuS: C, 52.43; H, 2.93; N, 10.87. Found: C, 52.35; H, 2.98; N, 10.82. Maldi-Tof-MS (m/z): 813.0 ([M-2ClO$_4$-H]$^+$). ^{1}H NMR (ppm, DMSO-d^6, 400 MHz): 8.90 (d, J = 8.4, 2H); 8.75 (d, J = 8.0, 4H); 8.39 (d, J = 14.4, 4H); 8.09 (d, J = 4.8, 4H); 8.02 (s,1H); 7.93 (d, J = 7.2, 2H); 7.88 (d, J = 8.0, 1H); 7.74–7.81 (m, 6H), 7.62 (t, 1H),7.35 (dd, J$_1$ = 7.6, J$_2$ = 8.4 , 2H).

2.6. RNA-binding experiments

The methods for spectroscopic titrations and viscosity measurements of the two Ru(II) complexes binding with poly(U).poly(A)*poly(U) are the same as before.[16] The intrinsic binding constants (K_b) and the binding site size (s) are determined the changes of absorbance at 463 nm for Ru**1** and 464 nm for Ru**2** by using the following equation.[33]

$$\frac{\varepsilon_a - \varepsilon_f}{\varepsilon_b - \varepsilon_f} = \frac{\sqrt{b - (b^2 - 2K_b^2 C_t [RNA]/s)}}{2K_b C_t} \quad (1a)$$

$$b = \frac{1 + K_b C_t + K_b [RNA]}{2s} \quad (1b)$$

where [RNA] is the concentration of poly(U)·poly(A)*poly(U) in the nucleotide phosphate and ε_a, ε_f and ε_b are the apparent, free and bound metal complex extinction coefficients,

respectively. K_b is the equilibrium binding constant in M^{-1}, C_t is the total metal complex concentration and s is the binding site size.

2.7. Thermal denaturation studies

Thermal RNA denaturation experiments were carried out with a Perkin-Elmer Lambda-25 spectrophotometer equipped with a Peltier temperature-control programmer (± 0.1 ^{o}C). The temperature of the solution was increased from 20 to 60 ^{o}C at a rate of 1 ^{o}C min^{-1}, and the absorbance at 260 nm was continuously monitored for solutions of the RNA Triplex (32.1 μM) in the absence and presence of different concentrations of either Ru**1** or Ru**2** in phosphate buffer. The thermal melting temperature (T_m) was taken as the midpoint of the melting transition as determined by the maximal of the first derivative plot.[15]

3. Theoretical calculations

The structural schematic diagrams of Ru**1** and Ru**2** are shown in Fig. 1. The general structure of each metal complex contains a Ru(II) ion, two ancillary ligands (bpy or phen) and a intercalative ligand btip. There is not any symmetry in these complexes. All calculations have been performed with the G98 quantum chemistry program-package,[34,35] at the DFT/B3LYP level using 6-31G* basis set on the carbon, nitrogen, sulfur and hydrogen atoms and a LanL2DZ basis set [36,37] with effective core potential on the ruthenium atom. The full geometry optimization computations for the ground states of these complexes with singlet state[38] were carried out. In order to vividly depict the detail of the frontier molecular orbital interactions, the stereographs of some related frontier molecular orbitals of the complexes were drawn with the Molden v3.7 program[39] based on the computational results.

3. Results and discussion

3.1. Electronic absorption spectral studies of the binding

The application of electronic absorption spectroscopy in triplexes–binding studies is one of the most useful techniques.[13–15] Aromatic small molecules binding with triplexes through intercalation usually result in obvious hypochromism and bathochromism, which is attributed to π-π stacking interactions between an aromatic chromophore of aromatic small molecules and the base pairs of triplexes.[40] The extent of the hypochromism commonly parallels the intercalative binding strength. Thus, the binding of Ru**1** and Ru**2** with poly(U)•poly(A)*poly(U) was first performed by absorption spectral measurements.

The absorption spectra of Ru**1** and Ru**2** in the absence and presence of the RNA triplex are given in Fig. 2. Fig. 2 indicates that obvious hypochromisms and red shifts in the MLCT bands (MLCT = metal-to-ligand charge-transfer) of the two complexes are observed upon progressively increasing the concentration of the RNA triplex. In the case of Ru**1**, the hypochromism at 463 nm reached about 16.7% with a red shift of 6 nm at a [UAU]/[Ru] ratio of 6.28 {UAU stands for poly(U)•poly(A)*poly(U), Ru stands for   Ru**1** and Ru**2**, respectively.}. For Ru**2**, the hypochromism at 464 nm was around 15.6%, with a red shift of 3 nm at a [UAU]/[Ru] ratio of 4.15. These spectral characteristics suggest that both complexes interact with the RNA triplex most likely through an intercalative mode, which involves a stacking interaction between the aromatic chromophore and the base pairs of the RNA triplex.

From the decay of the absorbance of the two complexes monitored at the MLCT bands, the intrinsic binding constants K_b of Ru**1** and Ru**2** were determined to be $(2.23 \pm 0.41) \times 10^6$ M^{-1} ($s = 1.31 \pm 0.03$) and $(1.59 \pm 0.46) \times 10^6$ M^{-1} ($s = 0.85 \pm 0.04$), respectively. The binding constants K_b and the binding site size s of Ru**1** are slightly higher than those of Ru**2**, which

suggests that Ru**1** binding to the triplex RNA is stronger than Ru**2**. In addition, the K_b values

of the two complexes are much higher than those of the compounds binding with the triplex

RNA through a partial intercalation, such as the metal complex PtPR $(1.3 \times 10^4 \text{ M}^{-1})$,[15]

alkaloid berberine $[1.6 \pm 0.40) \times 10^5 \text{ M}^{-1}]$[18] and palmatine $[(1.6 \pm 0.40) \times 10^5 \text{ M}^{-1}]$.[41] These

indicate that the sizes and shapes of small molecules have significant effects on the binding

affinities

3.2. Spectrofluorimetric and studies

Information regarding the binding affinity of the two complexes toward the triplex can also be

obtained form emission spectroscopy. Fig. 3 shows the emission spectra of Ru**1** and Ru**2** in the

absence and presence of the RNA triplex poly(U)•poly(A)*poly(U). The emission intensity of

either Ru**1** or Ru**2** increases steadily upon progressive addition the triplex RNA and eventually

reaches about 17 and 14 times in comparison with the triplex-free Ru**1** and Ru**2**, respectively.

In each case, a large emission change is indicative of a strong association of the complex with

the triplex, resulting presumably from an effective overlap of the bound complex with the base

triplets.[18] The result further indicates that the location of the bound Ru**1** and Ru**2** in a

hydrophobic environment similar to an intercalated state and Ru**1** protected by the triplex

RNA is more efficient compared with Ru**2**. Therefore, the accessibility of water molecules to

Ru**1** in the presence of the triplex is more difficult in comparison with Ru**1**, resulting in Ru**1**

displaying a greater emission increase than Ru**2** upon binding to the triplex in saturation state.

3.3. Determination of the binding mode by viscosity studies

Viscometric technique is a reliable hydrodynamic method to clarify the binding modes of

small molecules toward DNA/RNA.[42] The effects of Ru**1** and Ru**2** on the relative viscosity of

the RNA triplex poly(U)•poly(A)*poly(U) are presented in Fig. 4. Upon addition of either Ru**1**

or Ru**2** to the triplex, an initial decrease in the relative viscosity of the triplex solution was

observed and subsequent increases in the apparent molecular length of the triplex occurred.

The initial decrease in the apparent molecular length of the triplex may be indicative of a

conformational change in the triplex induced by either Ru**1** or Ru**2**. Such a conformational

change may be attributed to a kink or bend of the helix induced by the complex, which thereby

reduces the effective molecular length of the triplex.[43] Upon the ratios of [Ru]/[UAU] further

increasing, the subsequent extensions of the length of the triplex may arise from the

intercalation effect. In addition, the increase in solution viscosity observed in the contour

length profile of the triplex may simply reflect a binding-induced increase in the stiffness of

the triplex by Ru**1** and Ru**2**.

The above viscosity studies further confirm that Ru**1** and Ru**2** bind to the RNA triplex via

an intercalative mode. Notably, the effects of the two complexes on the viscosity of the RNA

triplex are obviously different from the metal complex PRPt.[15] Concerning PtPR, it binds the

triplex RNA poly(U)•poly(A)*poly(U) through a partial intercalative mode, due to the

platinum-containing residues prevent full penetration of the PR residue between base planes,

which results in no obvious changes of the viscosity of poly(U)•poly(A)*poly(U) upon

addition of PtPR. These data also indicates that the size and shape of metal complexes has a

significant effect on the binding modes.

3.4. Comformational change studies

To investigate the effect of Ru**1** and Ru**2** on the conformation of the RNA triplex, we also

recorded CD spectra of the triplex poly(U)•poly(A)*poly(U) modified by Ru**1** or Ru**2** (Fig.

5). Due to the stacking interactions between the base triplets and the helical structure of

the triplex strands, the intrinsic CD spectra of poly(U)•poly(A)*poly(U) (Fig. 5, a) shows

a large positive band in the 250-280 nm region and an adjacent weak negative band in the

wavelength range 230-250 followed by a small positive band below the 230 nm region.[31]

Notably, both Ru**1** and Ru**2** display no intrinsic CD signals because they are racemic

compounds. Furthermore, The RNA triplex displays no CD signs above 300 nm. Thus, for

the RNA triplex–Ru**1**/Ru**2** system, any CD signals above 300 nm may be attributed to the

interaction of the complex with the triplex, and any changes below 300 nm could be due

either to the triplex induced CD (ICD) of the metal complex or the metal complex induced

perturbation of the RNA triplex spectrum. Fig. 5 indicated that the conformation of the

triplex was found to be perturbed in the presence of either Ru**1** (Fig. 5, A) or Ru**2** (Fig. 5,

B). As the interaction progressing the characteristic positive band of the RNA triplex in the

250–280 nm regions displayed obvious red shifts and decreases in ellipticity. Furthermore,

obvious induced CD was observed in 300-500 nm regions. In the case of Ru**1**, three new

bands at around 327, 352 and 476 nm were observed, whereas only one broad negative

band occurred at about 344 nm in the presence of Ru**2**. The results suggested that

Ru**1**–induced conformational changes of the RNA triplex are more obvious than Ru**2**,

which is also consistent with the above results.

3.5. Thermal denaturation studies

A thermal denaturation experiment is an effective way to determine the stability of nucleic

acid duplexes and triplexes.[42] It's well established that a stacking interaction of intercalated

molecule as well as the neutralization of the phosphate charges through external binding may

increase the melting temperature of nucleic acid duplexes and triplexes. In particular, information regarding the specificity of binding of small molecules to the Hoogsteen base-paired third strand poly(U) or to the Watson–Crick base-paired duplex poly(U)•poly(A) of triplexes can be obtained by thermal denaturation experiment.[44] Concerning the triplex poly(U)•poly(A)*poly(U)-free Ru**1** and Ru**2**, the third strand poly(U) separation from the triplex occurs at about 37.5 $^{\circ}$C, while the duplex strand poly(U)•poly(A) separation occurs at around 45.6 $^{\circ}$C, which is in agreement with the literature data.[31]

To explore Ru**1** and Ru**2** effect on the stability of the triplex, the thermal denaturation experiments were carried out. The denaturation curves at different C_{Ru}/C_{UAU} ratios were presented in Fig. 6, and the quantitative data on the melting temperatures were summarized in Table 2. The results indicated that the binding stabilized the Hoogsteen base paired third strand by about 7.6 and 6.5 $^{\circ}$C for Ru**1** and Ru**2**, respectively. The stabilization temperature of the third strand enhanced by Ru**1** was slightly higher than that by Ru**2**, suggesting a delayed strand separation with bound Ru**1** under the same conditions. In addition, the results also suggest that both Ru**1** and Ru**2** tend to slightly destabilize the Watson–Crick base-paired duplex of the triplex, reflecting the two complexes binding more strongly to the Hoogsteen base-paired third strand than to the Watson–Crick base-paired duplex under the present experiments. Notably, the effects of Ru**1** and Ru**2** on the stabilization of the triplex are different from ethidium,[13] some alkaloids,[18] proflavine and its complex Pt-proflavine.[31] The melting experiments reveal that ethidium, PR and its metal complex PtPR, tend to strongly destabilize the third strand poly(U) and stabilize the duplex poly(U)•poly(A), whereas some alkaloids, such as berberine, palmatine and coralyne, stabilize the Hoogsteen base-paired third strand of the triplex with no obvious affecting on the stability of the duplex. Recent studies of

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the interaction mechanisms of coralyne with poly(U)•poly(A)*poly(U) confirm that coralyne tends to stabilize the triplex structure because of this small molecule being able to induce the triplex−to−duplex conversion and also the duplex−to−triplex conversion.[14b] These results further suggest that the effects of small molecules on the stabilization of RNA triplexes are complicated and sensitive to their structural features and interaction processes.

3.6. Theoretical explanation on the interaction difference between Ru1 and Ru2

The above experimental results confirm that the binding affinity for Ru**1** is slightly greater than that for Ru**2**, which results in Ru**1** displaying a stronger stabilization of the triplex poly(U).poly(A)*poly(U) in comparision with Ru**2**. Since the intercalative ligands of Ru**1** and Ru**2** are the same, the differences in the binding affinities for the two complexes may be attributed to the ancillary ligand effects. Previous investigations indicate that the hydrophobicity of a ancillary ligand is one of many important factors governing the binding affinities of Ru(II) polypyridine complexes to nucleic acids.[44] Obviously, concerning Ru**1** and Ru**2** here, the hydrophobicity of bpy and phen may not be the main factor effect on the triplex binding behaviors. Concerning Ru**1** and Ru**2** here, the hydrophobicity of the ancillary ligand bpy is weaker than that of phen. If the hydrophobicity of the ancillary ligand is a main factor governing the binding affinities for Ru**1** and Ru**2**, the binding affinity for Ru**1** should be smaller than that for Ru**2**, which is opposite to the experimental results. Therefore, we presume that the difference in the binding affinities of the two complexes can not be attributed to the ancillary ligand effects, although Ru**1** and Ru**2** contain the same the intercalative ligand btip and different ancillary ligands (bpy and phen). Herein, the situation of Ru**1** and Ru**2** is similar to Ru(II) complexes $[Ru(bpy)_2(actatp)]^{2+}$ and $[Ru(phen)_2(actatp)]^{2+}$ (actatp =

acenaphthereno[1,2-*b]*-1,4,8,9-tetraazariphenylene) binding with calf thymus DNA.[45] The result reported by Ji and his coworkers indicated that the binding affinity for $[Ru(bpy)_2(actatp)]^{2+}$ was bigger than that for $[Ru(phen)_2(actatp)]^{2+}$. This result suggest that the ancillary ligands governing Ru(II) complexes binding with nucleic acids can not t be absolute, even Ru(II) complexes contain the same intercalative ligand.

Notably, studies on the interactions of aromatic small molecules with DNA reflect that π-π stacking (intermolecular force) plays a dominant role in the intercalations and the binding affinities. In general, the base pairs of DNA are electron-donors and aromatic molecules as intercalators are electron-acceptors.[46,47] Based on the frontier molecular orbital theory, two factors play a critical role in the binding affinities of aromatic molecules with DNA.[48–50] One is the planarity area of intercalators because the larger planarity area is advantageous to the interaction between DNA and intercalators, another is the lowest unoccupied molecular orbital (LUMO) energies because lower LUMO energies of DNA intercalators are advantageous to accepting the electrons from the base pairs of DNA. In addition, the reported HOMO and NHOMO (the next HOMO) energies of the CG/CG stacking model calculated with the density function theory (DFT) methods are -1.27 and -2.08 eV, respectively.[47] As well-established, due to the Hoogsteen base pairings, the stability of RNA triplexes is much lower than DNA.[6,7] Thus, we may reasonably conclude that the HOMO energies of RNA triplexes are much higher than that of DNA, namely, the HOMO energies of the AU/UA stacking model of RNA triplexes calculated with DFT method should be higher than the value of -1.27 eV. If so, the trends in the interactions of Ru**1** and Ru**2** with the triplex could be also explained by DFT methods.

Although the crystal structures of Ru**1** and Ru**2** have not been obtained here, the

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DFT-optimized structures confirm that the planarities of the intercalative ligands btip in the two complexes are different: for btip in Ru**1** and Ru**2**, the dihedral angles of N(4)–C(5)–C(6)–S(7) are 0.2630 and 0.3751 $^{\circ}$ (Fig. 7 and Table 3), respectively. Therefore, the better planarity of btip in Ru**1** is more advantageous to the π-π stacking interaction between Ru**1** and the triplex RNA, which makes Ru**1** insert the bases of the triplex more deeply than Ru**2**. On the other hand, the DFT calculations indicate that the energies of frontier molecular orbitals of the two metal complexes changes by the change in the N(4)–C(5)–C(6)–S(7) dihedral angles for Ru**1** and Ru**2** (Fig. 8 and Table 3), respectively. From Fig. 8 and Table 3, we can clearly see that the energies of LUMO and L + x (L= LUMO; x = 1, 2, etc.) of Ru**1** are all smaller than those of Ru**2**. According to the frontier MO theory, [48–50] the lower energies of LUMO and L + x are more advantageous to Ru**1** accepting the electrons offered from the base pairs of the triplex RNA, which can weaken the electrostatic repulsion between the third-strand and the duplex of the triplex RNA. Synthetically considering the two factors, the slight difference in the binding affinity and increase of the triplex stabilization for Ru**1** and Ru**2** could be reasonably explained.

4. Conclusions

On the whole, the results from this work suggest that Ru**1** and Ru**2** can bind with the RNA triplex via intercalation and enhance the stabilization of the Hoogsteen base paired third strand. To some extent, the two complexes tend to destabilize the Watson–Crick base-paired duplex of the triplex, reflecting that both complexes are likely to bind with the Hoogsteen base-paired third strand more strongly than the Watson–Crick base-paired duplex under the present experiments. Combined with the DFT calculations, we presume that the slight binding

difference in Ru**1** and Ru**2** may be attributed to the planarity of the intercalative ligand btip and the LUMO level of Ru**1** and Ru**2**, instead of the polarity effect of the ancillary ligands bpy and phen. This study further advances our knowledge on the triplex RNA-binding by metal complexes, particularly Ru(II) complexes.

Acknowledgments

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Captions for Schemes and Figures

Fig. 1. Chemical structures of Ru**1**, Ru**2** and the base pairing scheme in poly(U)•poly(A)*poly(U) (symbols • and * represent Watson-Crick and Hoogsteen base pairing).

Fig. 2. Representative absorption spectral changes of Ru**1** (A) and Ru**2** (B) in phosphate buffer (6 mmol/L Na_2HPO_4, 2 mmol/L NaH_2PO_4, 1 mmol/L Na_2EDTA, 19 mmol/L NaC1, pH 7.0) at 20 °C in the absence and presence of poly(U)•poly(A)*poly(U). [Ru**1**] = [Ru**2**] = 20 μM, [UAU] = 0 –126 μM {UAU stands for poly(U)•poly(A)*poly(U)}. The arrows show the absorbance changes upon increasing poly(U)•poly(A)*poly(U) concentrations.

Fig.3. Representative fluorescence emission spectra of Ru**1** (A) and Ru**2** (B) treated with poly(U)•poly(A)*poly(U). [Ru**1**] = [Ru**2**] = 1.0 μM, [UAU] = 0–93 μM. The arrows show the emission intensity changes upon increasing poly(U)•poly(A)*poly(U) concentrations. Solution conditions are the same as those described in the legend of Fig. 2.

Fig. 4. Circular dichroism spectra of poly(U)•poly(A)*poly(U) (a, 50.0 uM) treated with Ru**1** (A) and Ru**2** (B), respectively. [Ru]/[UAU] = 0–0.35; Red lines: the Ru-free RNA triplex (Ru stands for Ru**1** and Ru**2**, respectively). The solution conditions are the same as those described in the legend of Fig. 2.

Fig. 5. Viscometric Ru**1** (■) Ru**2** (●) and titrations of poly(U)•poly(A)*poly(U) at 20 °C. [UAU] = 153 μM. Solution conditions are the same as those described in the legend of Fig. 2.

Fig. 6. Melting curves at 260 nm of poly(U)•poly(A)*poly(U) (32.1 μM) and its complexation with Ru**1** (A) and Ru**2** (B) at different [Ru]/[UAU] ratios. Solution conditions are the same as those described in the legend of Fig. 2, and [Na^+] = 35 mM.

Fig. 7. The DFT-optimized structures and visualization of the orbitals of Ru**1** (left) and Ru**2** (right).

Fig. 8 Some related frontier molecular orbital stereographs of complexes of Ru**1** and Ru**2**.

Table 1. Binding constants (K_b), average binding site size (s), hypochromicity (H) and bathochromic shifts of Ru**1** and Ru**2**.

Complexes	$\lambda_{max,\text{free}}$ (nm)	$\lambda_{max,\text{bound}}$ (nm)	$\Delta\lambda^a$ (nm)	H^b (%)	$K_b{}^c$ ($\times 10^6$ M^{-1})	s^d
Ru**1**	463	469	6	16.72	2.23 ± 0.41	1.31 ± 0.03
Ru**2**	464	467	3	15.60	1.59 ± 0.46	0.85 ± 0.04

[a] $\Delta\lambda$ represents the difference in wavelength of the MLCT band of the metal complex between free and completely bound the triplex states.

[b] $H = 100 \times (A_{\text{free}} - A_{\text{bound}})/A_{\text{free}}$ (A is the absorbance).

[c] K_b was determined by monitoring the changes of absorption at the MLCT bands.

[d] s is an average binding size.

Table 2. Melting Temperature (^{o}C) of poly(U)•poly(A)*poly(U) in the absence and presence of Ru**1** and Ru**2**, respectively. [Na^{+}] = 35 mM.

Complexes	C_{Ru}/C_{UAU}	T_{m1}[a] (^{o}C)	T_{m2}[b] (^{o}C)	ΔT_{m1}	ΔT_{m2}
poly(U)•poly(A)*poly(U)	0	37.5	45.6	–	–
poly(U)•poly(A)*poly(U)+ Ru**1**	0.01	40.1	46.3	2.6	0.7
	0.02	40.4	45.7	2.9	0.1
	0.05	44.6	–	7.1	–
	0.10	45.0	–	7.5	–
poly(U)•poly(A)*poly(U)+ Ru**2**	0.01	39.3	46.0	1.8	0.4
	0.02	41.6	45.5	4.1	-0.1
	0.05	43.8	–	6.3	–
	0.10	44.2	–	6.7	–

[a,b] T_{m1} and T_{m2} are the thermal melting temperatures corresponding to triplex to duplex and duplex to single strand transitions, respectively.

Table 3. Dihedral angles θ (°) and some frontier molecular orbital energies (ε_i/eV) of complexes Ru**1** and Ru**2**.

Title	θ (°)[a]	HOMO−2	HOMO−1	HOMO[b]	LUMO[c]	LUMO+1	LUMO+2	$\Delta\varepsilon_{L-H}$[d]
Ru**1**	0.2630	-10.7700	-9.7934	-9.4944	-7.4256	-7.3372	-7.1715	2.0688
Ru**2**	0.3751	-10.6786	-9.7483	-9.4509	-7.2673	-7.1720	-7.0858	2.1836

[a] N(4)–C(5)–C(6)–S(7).

[b] HOMO: the highest occupied molecular orbital; HOMO-1 = the next HOMO.

[c] LUMO: the lowest unoccupied molecular orbital; LUMO+1: the next LUMO.

[d] $\Delta\varepsilon_{L-H}$ = the energy difference between LUMO and HOMO.

poly(U)●poly(A)*poly(U)

Ru**1**

Ru**2**
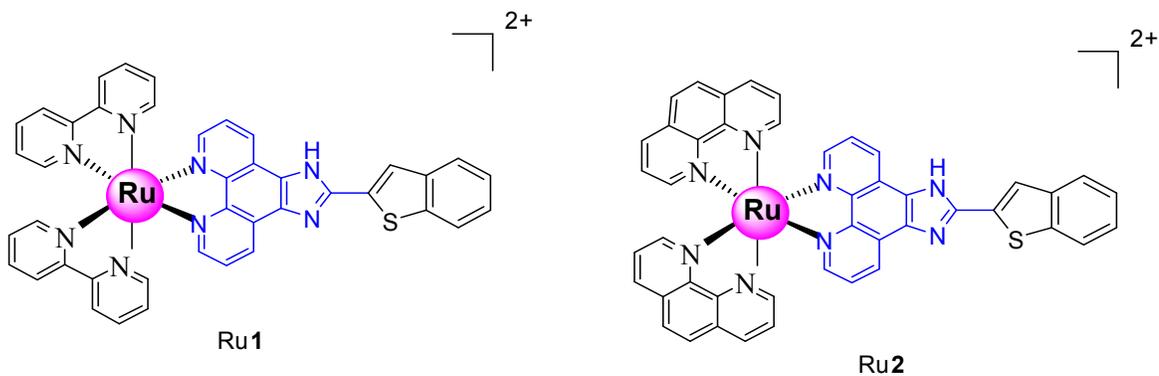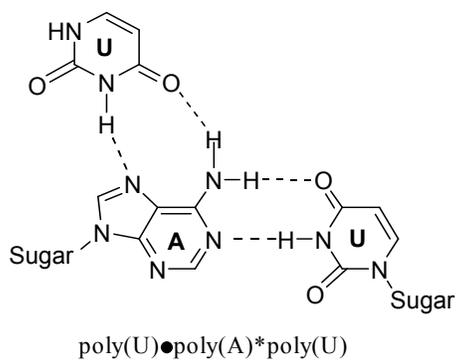
Fig. 1.
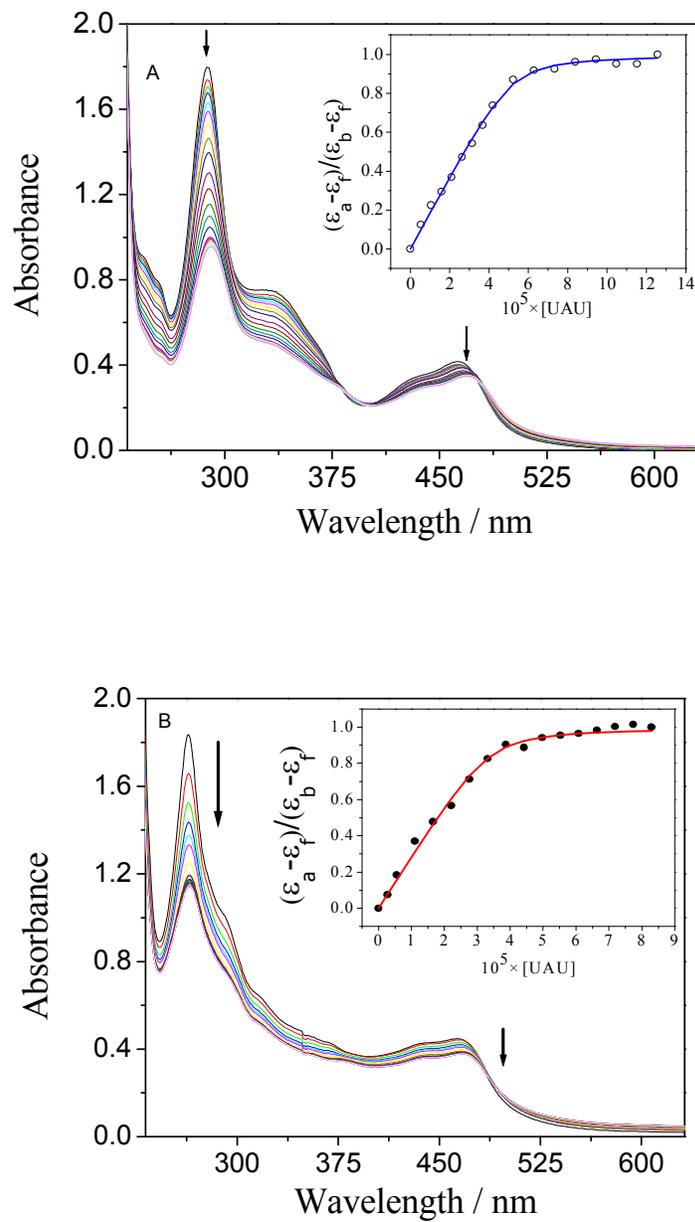
Fig. 2.
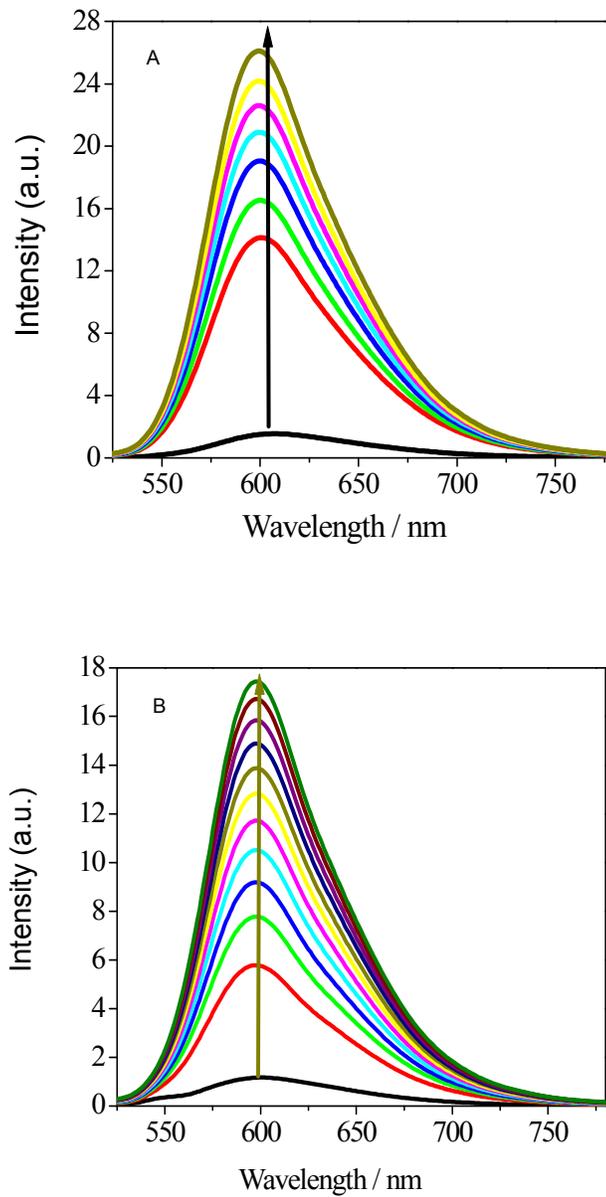
Fig. 3.

Fig. 4.

Fig. 5.

Fig. 6.

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Ru**1**   Ru**2**

Fig. 7.

Ru**1** HOMO-2   Ru**1** NHOMO-1   Ru**1** HOMO   Ru**1** LUMO   Ru**1** LUMO+1   Ru**1** LUMO+2

Ru**2** HOMO-2   Ru**2** NHOMO-1   Ru**2** HOMO   Ru**2** LUMO   Ru**2** LUMO+1   Ru**2** LUMO+2

Fig. 8.

poly(U)•poly(A)*poly(U)

Ru**1**

Ru**2**

In this work, two Ru(II) complexes, $[Ru(bpy)_2(btip)]^{2+}$ (Ru**1**) and $[Ru(phen)_2(btip)]^{2+}$ (Ru**2**), have been synthesized and characterized. The binding properties of the two metal complexes with the triple RNA poly(U)•poly(A)*poly(U) were studied by various biophysical and density functional theory methods. The main results obtained here suggest that the slight binding difference in Ru**1** and Ru**2** may be attributed to the planarity of the intercalative ligand and the LUMO level of Ru(II) complexes. This study further advances our knowledge on the triplex RNA-binding by metal complexes, particularly Ru(II) complexes.