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Dinuclear Monointercalating Ru^{II} Complexes That Display High Affinity Binding to Duplex and Quadruplex DNA

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Abstract: The DNA duplex binding properties of previously reported dinuclear Ru^{II} complexes based on the ditopic ligands tetrapyrido[3,2-*a*:2′,3′-*c*:3″,2″-*h*:2″,3″-*j*]phenazine (tppz) and tetraazatetrapyrido[3,2-*a*:2′3′-*c*:3″,2″-*l*:2‴,3‴-*n*]pentacene (tatpp) are reported. Photophysical and biophysical studies indicate that, even at high ionic strengths, these complexes bind to duplex DNA, through intercalation, with affinities that are higher than any other monointercalating complex and are only equalled by DNA-threaded bisintercalating complexes. Additional studies at high ionic strengths using the 22-mer $d(AG_3[T_2AG_3]_3)$ [G3] human telomeric sequence reveal that the dinuclear tppz-based systems also bind with high affinity to quadruplex DNA. Furthermore, for these complexes, quadruplex binding is accompanied by a distinctive blue-shifted "light-switch" effect, characterized by higher emission enhancements than those observed in the analogous duplex effect. Calorimetry studies reveal that the thermodynamics of duplex and quadruplex binding is distinctly different, with the former being entirely entropically driven and the latter being both enthalpically and entropically favored.

Keywords: DNA · luminescence · ruthenium · sensors · telomeres

Introduction

The design of d^6 transition-metal complexes that bind to DNA is an area of burgeoning research activity.[1] Following the report of the DNA "light-switch" effect, observed when $[Ru(phen)_2(dppz)]^{2+}$ (dppz = dipyrido-[3,2-*a*:2′,3′-*c*]-phenazine, phen = 1,10-phenanthroline) intercalates into DNA,[2] complexes incorporating [M(dppz)] moieties (in which M = Ru,[3] Re,[4] and Os[5]) have attracted particular attention. Further studies on $[Ru(phen)_2(dppz)]^{2+}$ have revealed that while the intensity and lifetime of emission can be modulated by the structure and sequence of the target DNA,[6,7] emission energies are much less effected, for example, while the largest λ_{em} perturbations for duplex structures are around 10–15 nm, binding to triplex DNA produces no perturbation of emission wavelength.[8]

Although work on metallated porphyrins is well established, studies involving quadruplex DNA and complexes related to $[Ru(phen)_2(dppz)]^{2+}$ are rare. The Thorp group used $[Ru(bpy)_3]^{2+}$—a complex that displays low affinity, nonspecific interactions with DNA—to investigate redox damage in G-quartets,[9] while Barton, et al. probed G-quartet damage in short duplex/quadruplex conjugates using a Rh^{III} complex intercalated into the duplex structure.[10] The direct interaction between metal complexes and quadruplex has yet to be fully investigated. This is probably because quadruplexes are formed at high ionic strengths (e.g., [KCl] = 180 mM). In such conditions $[Ru(phen)_2(dppz)]^{2+}$ binding affinities are greatly reduced.[11]

Nonetheless, higher order G-quadruplex structures have considerable biological importance. Telomeric DNA, from a variety of species, has the consensus sequence $d(T_{1-3}(T/A)G_{3-4})$ and these G-rich sequence motifs are repeated for many thousands of bases in humans. The in vitro formation of quadruplex DNA in models suggests that telomeric DNA at the 3′ termini of chromosomes may be an important chemotherapeutic target for new antitumor agents.[12] In part, this strategy is based on telomerase inhibitory activity found for K^+, Na^+, and a number of small organic molecules, in which quadruplex stabilization is inferred to impede access to the telomeric DNA template.[13,14] It is also known that promoter regions of some genes contain G-rich sequences that have the potential to adopt a quadruplex secondary structure. Therefore, quadruplex formation may play a key

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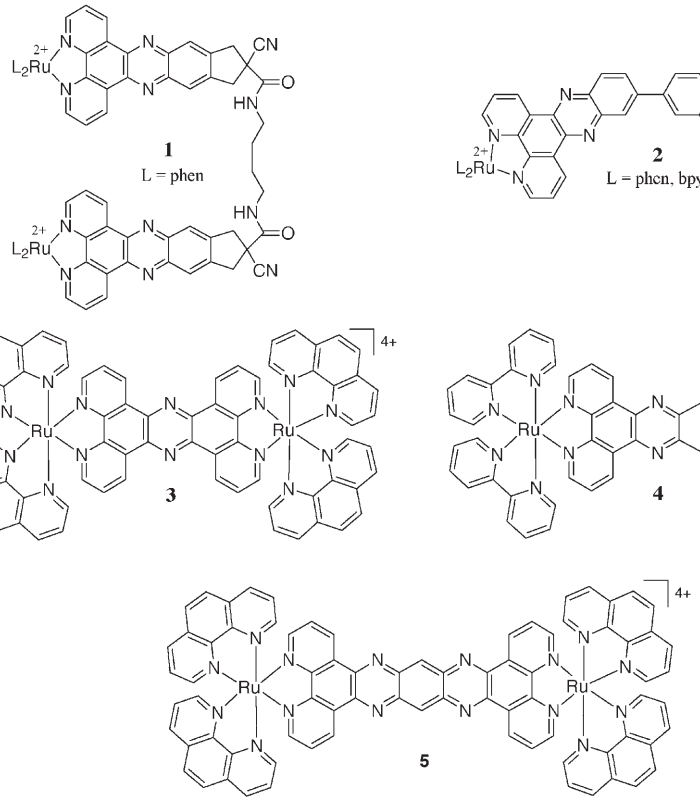
role in regulating gene expression. Indeed, it has been demonstrated that ligand-induced quadruplex formation in the *c-Myc* promoter leads to transcriptional down regulation.[15]

It is clear that high affinity binding to quadruplex by metal complexes will only be accomplished by systems whose binding properties are much less sensitive to changes in salt concentration. Recent work by the Nordén group has demonstrated that dinuclear potentially bisintercalating systems based on $[Ru(phen)_2(dppz)]^{2+}$ bind to duplex DNA with enhanced affinity, lower salt concentration dependence, and increased site sizes. For example, they have reported that, due to threading interactions, enantiopure diastereoisomers of the $[\{Ru(phen)_2\}_2\{\mu\text{-dppz}(11,11')\text{-dppz}\}]^{4+}$ ion (**1**) display binding affinities ($K_b > 10^8\,M^{-1}$) that are around two orders of magnitude higher than that of the mononuclear complex.[16] Similar nonthreading structures have been shown to bind to extended sequences, albeit with lower affinities.[17,18]

Much less work has been carried out on systems with ditopic ligand bridges. In 1993 Carson et al. showed that the binding affinity of dinuclear monointercalating systems can be highly dependent on the ancillary ligands of the metal centers.[19] More recently, Nordén, Lincoln and colleagues have described the extremely slow association and dissociation kinetics (several hours at 50 °C, 100 mM NaCl) of the threading dinuclear complex **2**,[20] while Wang and co-workers have reported on the DNA binding and pH-induced emission switching of a somewhat related dinuclear ligand bridged system.[21]

Tysoe et al.,[22] and others,[23] have investigated the interaction with DNA of a mononuclear complex of the ditopic ligand tetrapyrido[3,2-*a*:2′,3′-*c*:3″,2″-*h*:2″,3″-*j*]phenazine (tppz) and shown that it binds with affinities that are entirely comparable to those of $[Ru(phen)2(dppz)]^{2+}$. However, the DNA binding properties of the previously reported related dinuclear complexes **3**,[24] **4**,[25] and their analogue **5**,[26,27] based on the tetraazatetrapyrido[3,2-*a*:2′3′-*c*:3″,2″-*l*:2‴,3‴-*n*]pentacene (tatpp) ligand have yet to be investigated. Herein we report on the interaction of these complexes with duplex and quadruplex DNA.



Experimerntal Section

Materials: Commercially available materials were used as received. The complexes $[Ru(phen)2(dppz)]^{2+}$, **3**, **4**, and **5** were synthesized by using adapted literature procedures.[24–27] All reactions were carried out under an inert argon atmosphere. Calf thymus DNA (CT-DNA) was purchased from Sigma chemical company. It was purified by phenol extraction until Abs 260 nm/Abs 280 nm was >1.9. The 22-mer oligonucleotide sequence 5′-AGGG-$(TTAGGG)_3$ (G3) was supplied as a lyophilized solid by Eurogentec (Liege, Belgium). Following dialysis into the appropriate buffer and an annealing step for G3 (heating to 95 °C for 5 minutes followed by slow cooling to room temperature and storage at 4 °C for 48 h), concentrations of DNA solutions were determined spectroscopically by using the extinction coefficient of CT-DNA, $\varepsilon = 6600\,mol\,(nucleotides)^{-1}\,m^3\,cm^{-1}$ at 260 nm,[28] and $2.16\times10^5\,mol\,(quadruplex)^{-1}\,m^3\,cm^{-1}$ at 260 nm for G3.

Luminescence titrations were carried out using CT-DNA or G3 DNA and nitrate salts of the relevant complex in 25 mM NaCl, 5 mM TRIS pH 7.0 or 200 mM KCl, 10 mM KH_2PO_4/K_2HPO_4, 1 mM K_2EDTA, pH 7.0 buffers made with doubly distilled water (Millipore).

Instrumentation: Electronic absorption spectra were recorded on a Carey Bio-3 UV/Vis spectrophotometer. Emission spectra were recorded on a Hitachi F4500 spectrophotometer.

The luminescence lifetime measurements were collected using the Edinburgh instrument 199 spectrometer operating under single-photon-counting conditions. The MHz repetition-rate excitation source was an IBH nanoLED-05 (450 nm excitation). Fluorescence emission was isolated through the use of appropriate narrow band (±10 nm) interference filters (600 nm as appropriate).

Thermal denaturation experiments for CT-DNA were preformed by using a Cary 3-bio UV/Vis spectrophotometer at low salt buffer conditions. All samples were run in a 1 cm path length Teflon-stoppered quartz cuvette. Absorbance changes at 260 nm versus temperature were collected at a heating rate of $1\,°C\,min^{-1}$, over the temperature range of

25–98 °C by using a block temperature controller. Thermal denaturation experiments for G3-DNA were preformed using a Jasco J-810 spectropolarimeter at high salt buffer conditions. All samples were run in a 0.1 cm path length, stoppered quartz cuvette. CD [mdeg] signal changes at 295 nm versus temperature were collected at a heating rate of 1 °C min^{-1} by using a Peltier controller. G3-DNA concentrations of 20 μM and complex concentration of 15 μM were used.

ITC experiments were conducted by using a VP-ITC from MicroCal LLC (Northampton MA, USA) interfaced to a Gateway PIII PC. Data acquisition and analysis was performed by using Origin 5.0 (MicroCal LLC) and all titrations were performed at 25 °C in either low salt (25 mM) or high salt (200 mM) buffers as appropriate.

Viscosity data was obtained by using a Cannon–Fenske capillary viscometer submerged in a water bath at 27 °C. CT-DNA. Samples were first sonicated for 30 mins. CT-DNA solutions were approx 1 mM bp (bp = base pair) and flow times were recorded in triplicate using a digital stopwatch. All solutions were in a Tris buffer.

Results and Discussion

Duplex binding studies: The complexes were synthesized as hexafluorophosphate salts and then converted to nitrate salts by anion metathesis. As has been described before,[26,27] it was confirmed that complex **5** is nonemissive in all solvents. However, **3** and **4** display photophysical properties that are related to dppz-based systems: although they are luminescent in nonaqueous solutions they are effectively nonemissive in water. The interaction of these complexes with duplex DNA was then investigated.

Thermal denaturation experiments: As a preliminary screen, UV spectroscopy was used to assess induced melting point (T_m) shifts of CT-DNA in the presence of complexes **3**, **4**, and **5** (Table 1).

Table 1. T_m and ΔT_m for CT-DNA in 25 mM NaCl, 5 mM TRIS pH 7.0.

	T_m [°C]	ΔT_m [°C]
CT-DNA	73.3	–
+[**3**][$(NO_3)_4$]	78.9	+5.6
+[**4**][$(NO_3)_4$]	77.5	+4.2
+[**5**][$(NO_3)_4$]	76.8	+3.5

It was found that all three complexes stabilize duplex DNA to varying degrees, with **3** producing the highest ΔT_m value of +5.6 °C. No hysteresis is observed in any of these experiments. These observations are consistent with a reversible noncovalent interaction between the complexes and CT-DNA.

Viscosity studies: It is known that dinuclear complexes such as **2** can, at least initially bind to DNA through groove binding interactions.[20] It is well-established that intercalation results in a lengthening of DNA, thus producing increases in relative specific viscosity of solutions of DNA.[29,30] Therefore, to probe the nature of the interaction between the dinuclear complexes and DNA, the effect of the addition of the complexes on the viscosity of aqueous CT-DNA solutions was investigated. It was found that viscosity increases on addition of **3**, **4**, or **5** confirming that all these complexes are intercalators (Figure 1). It should be noted that although

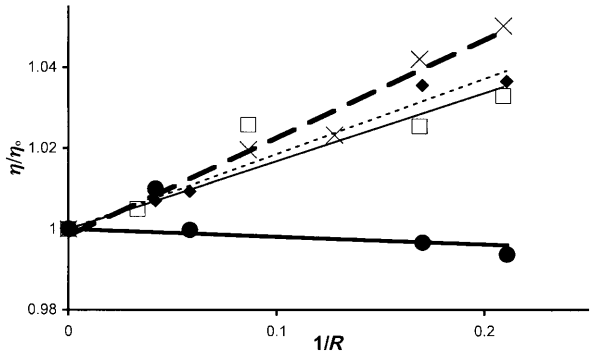


Figure 1. Plot of relative viscosity $(\eta/\eta_0)^{1/3}$ of CT-DNA versus $1/R$ for [**3**][$(NO_3)_4$] (□, dotted line), [**4**][$(NO_3)_4$] (◆, solid line) and [**5**][$(NO_3)_4$] (×, broken line) and the established groove binder Hoechst 33258 (●, thick line) in a Tris buffer (5 mM Tris, 25 mM NaCl, pH 7).

the change in viscosity is small compared to many metallointercalators, there is a clear increase when compared to the typical groove binder Hoechst 33258.

Absorption spectroscopy studies: Addition of CT-DNA to buffered aqueous solution of the complexes produces distinctive changes in their absorption spectra. In all three cases, $\pi\rightarrow\pi^*$ and $Ru^{II}\rightarrow L$ metal-to-ligand charge-transfer ($MLCT^3$) bands display pronounced hypochromicity, with several bands shifting in energy and shape (Figure 2).

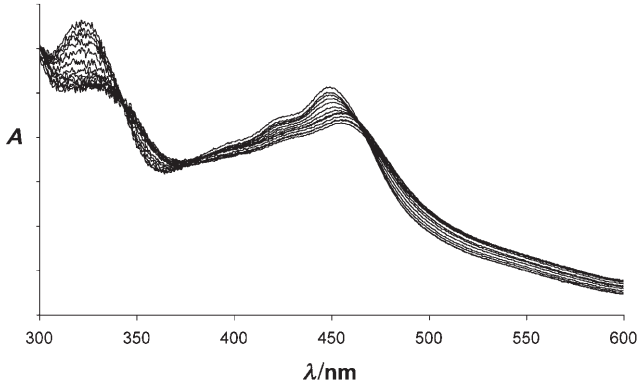


Figure 2. Changes in the UV/Vis spectra of [**5**][$(NO_3)_4$] in the presence of titrated CT-DNA using 25 mM NaCl, 5 mM TRIS pH 7.0 at 25 °C.

Fitting data from titrations to the McGhee–von Hippel model for non-cooperative binding to an isotropic lattice[31] consistently gave sites sizes of around 2–3 base pairs, which are comparable to mononuclear intercalators; however, fits of binding affinities for all three complexes gave values much greater than 10^6 M^{-1}. Given these results the effect of DNA on the emission properties of the complexes was investigated.

Luminescence studies involving duplex DNA: Complex **5** shows no evidence of emission, even in the presence of excess DNA. In contrast, addition of CT-DNA to 25 mM NaCl solutions of **3** and **4** result in large steady-state luminescent enhancements (>60 times; Figure 3).

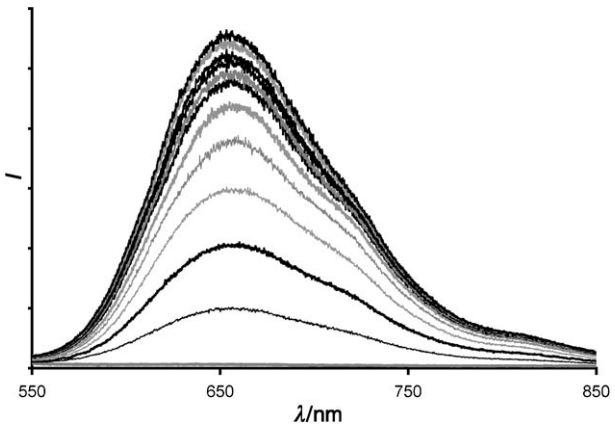


Figure 3. Changes in the emission of **[3]**[$(NO_3)_4$] on addition of titrated CT-DNA using 25 mM NaCl, 5 mM TRIS pH 7.0 at 25 °C.

This effect has been reported for the monomeric analogues of **3**[22,23] and **4**[25] and has been rationalized by using the same arguments employed to explain the light-switch effect of $[Ru(phen)_2(dppz)]^{2+}$. This analogy with dppz systems has been confirmed by computational studies on Ru^{II} complexes of dppz, tppz, and other related ligands.[32] However, in contrast to the behavior of the monomeric analogues, emission from **3** ($\lambda_{em}=658$ nm) and **4** ($\lambda_{em}=637$ nm) is blue-shifted relative to their emission in acetonitrile in which $\lambda_{em}(\mathbf{3})=710$ nm[24] and $\lambda_{em}(\mathbf{4})=671$ nm.[22]

For each titre of CT-DNA, luminescent enhancements occur within minutes of DNA addition, indicating that association rates are relatively rapid. These observations confirm that in contrast with systems such as **1** and **2**, in which emission enhancements may take days to saturate, the rigid planar structures of complexes **3–5** result in conventional nonthreading intercalation.

Estimates of binding parameters obtained from McGhee–von Hippel fitting of the luminescent data again reveal that while the site sizes are consistent with those obtained for analogous mononuclear systems, the binding affinities of the complexes are higher than those reported for any other monointercalating complex, and are comparable to those found for threading complexes such as **1** in similar conditions[16] (Table 2).

Given these affinities it seemed likely that appreciable binding would be observed at salt concentrations representative of in vivo conditions. Consequently, analogous studies were carried out using 200 mM KCl, 10 mM KH_2PO_4/K_2HPO_4, 1 mM K_2EDTA, buffered solutions of **3** and **4**—conditions that are commonly employed to promote the formation of quadruplex structures. As might be expected from polyelectrolyte theory,[33,34] there is some reduction in overall affinities, although values of K_b for **3** and **4** are still high ($>10^5\,M^{-1}$) and again are comparable to threading bisintercalator complexes in such conditions.[16] These affinities are particularly striking as it has been shown that, due to hydration effects, binding constants of cationic complexes measured in phosphate buffers are significantly lower than those obtained in tris buffers at the same cation concentrations[35]

Table 2. Estimates of binding parameters for **[3]**[$(NO_3)_4$], **[4]**[$(NO_3)_4$] and **[5]**[$(NO_3)_4$] binding to CT-DNA obtained by fits of luminescence data to the noncooperative McGhee–von Hippel model for binding to an isotropic lattice.[a]

	25 mM NaCl aqueous buffer		200 mM KCl aqueous buffer	
Complex	K_b	S	K_b	S
[3][$(NO_3)_4$]	$1.1\ x10^7$	2.6	3.1×10^6	2.0
[4][$(NO_3)_4$]	3.3×10^8	2.2	6.0×10^6	1.8
[5][$(NO_3)_4$][b]	1.1×10^7	1.1	3.7×10^5	1.3

[a] Averaged values of several titrations. [b] Nonluminescent, therefore parameters obtained represent an upper limit estimated from fits of absorption data.

The estimates of binding site sizes for **3** and **4** at higher ionic strengths were a little lower than values obtained at low salt concentrations. To investigate binding stoichiometry at high salt concentration in more detail, changes in luminescence signal were used to construct continuous variance Job plots[36] (Figure 4).

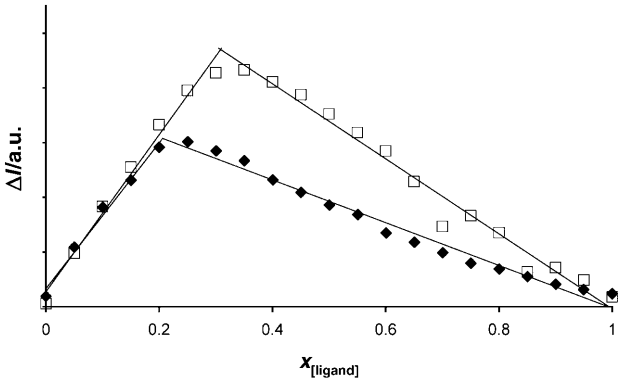


Figure 4. Job plots using luminescence data for **[3]**[$(NO_3)_4$] (◆) and **[4]**-[$(NO_3)_4$] (□) with CT-DNA at 10 μM final using 200 mM KCl, 10 mM KH_2PO_4/K_2HPO_4, 1 mM K_2EDTA, pH 7. x=mole fraction of complex added to DNA.

For each complex one major inflection point was revealed; for **[3]**[$(NO_3)_4$] this occurred at $x=0.22$, which is consistent with a stoichiometry approaching 1:3 bp, while for **[4]**[$(NO_3)_4$] an inflection point at $x=0.32$ indicates a binding ratio of 1:2 bp. These studies indicate stoichiometries that are slightly higher than those obtained from fits of the titration data at high salt concentrations, but corresponds well the data obtained by using lower salt concentration, and are consistent with the nearest neighbor exclusion model of intercalation.

Isothermal titration calorimetric (ITC) studies of 3 and 4 with CT-DNA: To further probe the thermodynamics of the interaction of these complexes with CT-DNA at high salt concentrations, and dissect the observed binding free energy into enthalpic and entropic components, we have conducted ITC experiments. These titrations were conducted in a buffer containing 200 mM KCl; CT-DNA was used as the substrate. Complex **5** was insufficiently soluble at these concentrations to obtain interpretable data. Figure 5 shows typical ITC data for the interaction of **4** with CT-DNA.

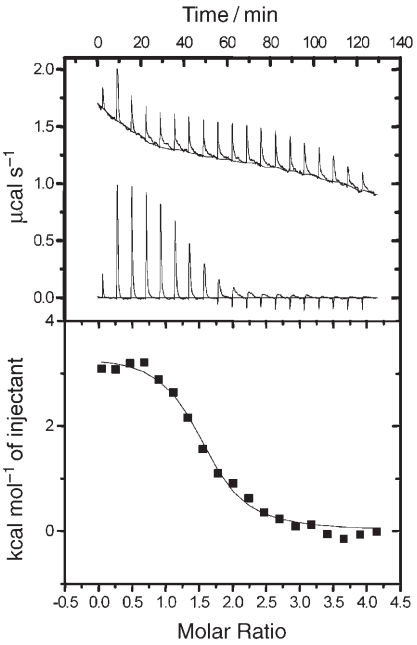


Figure 5. Sample ITC data for the interaction of [**4**][$(NO_3)_4$] with CT-DNA in 200 mM KCl, 10 mM KH_2PO_4/K_2HPO_4, 1 mM K_2EDTA, pH 7.0 at 25 °C. Upper panel: addition of ligand into buffer and titration of complex into DNA. Lower panel: binding isotherm obtained from integration of upper panel data after correcting for molar concentration of reactants.

The ITC data were then fit to a single set of identical binding sites model to yield the parameters shown in Table 3. Comparison of the data in Tables 2 and 3 shows some discrepancies between calorimetrically determined binding constants and those obtained from spectroscopic titrations. There are several explanations for this observation.

Table 3. Calorimetrically measured thermodynamic parameters for the interaction of **3** and **4** with CT-DNA in 200 mM KCl, pH 7.0 at 25 °C.[a]

Complex	K_b [M bp^{-1}]	ΔG_{obs} [kJ mol^{-1}]	ΔH_B [kJ mol^{-1}]	$T\Delta S$ [kJ mol^{-1}]
[**3**][$(NO_3)_4$]	$2.3(\pm0.8)\times10^5$	-30.7 ± 0.9	11.3 ± 0.3	42.0 ± 1.0
[**4**][$(NO_3)_4$]	$3.8(\pm0.7)\times10^5$	-31.8 ± 1.5	14.1 ± 0.4	45.9 ± 0.6

[a] Enthalpy values were measured directly using ITC and the standard relationship $\Delta G=\Delta H-T\Delta S$ was used to compute changes in entropy.

One difficulty is that the binding parameters are obtained from techniques in which data are fitted to different models. For absorption/luminescence titration, the binding isotherms are fitted by using the neighbor exclusion model, based on the observation that these ligands bind by intercalation. However ITC data is fitted by using the more simplistic single set of identical binding sites model. The reason for the simplistic treatment of the ITC data is that the raw data are straightforward sigmoidal curves and hence there is no statistical justification for using more complex models. In addition ITC data analysis that incorporates cooperativity is a nontrivial task. Significant discrepancies between spectroscopic and calorimetric binding constants are regularly reported in the literature. One source of the discrepancy is the different concentration regimes employed in the different techniques, that is, millimolar quantities in ITC and micromolar in luminescence. The way in which these concentration differences can lead to observed differences in binding constant has been previously discussed in full.[37] Briefly the binding constant can be determined accurately only if the ligand (titrant) is added to a fixed and constant concentration $[S_o]$ of DNA (titrate) such that $[S_o]\ll 1/K_b$. If $[S_o]\gg 1/K_b$ then $[S_o]$ must be greater than the ligand concentration in order to obtain a binding isotherm that will yield a reliable value for the binding constant. In our ITC experiments $[S_o]$ was typically ~0.25 mM binding sites (assuming a site size of 2 bp); this value is clearly larger than $1/K_b$, which is $\sim3\times10^{-7}$. However, the principal motivation for conducting ITC experiments was not to obtain another estimate of binding affinity, but to directly measure binding enthalpies.

Direct and model-independent determinations of the binding enthalpies for **3** and **4** show that the interaction of both complexes with duplex DNA is entropically driven. In both cases, enthalpy changes are positive and hence make a net unfavorable contribution to the binding free energy. This is a similar thermodynamic profile to practically all transition metal compounds that we have examined to date.[11,17,38] The conventional explanation for this pattern of thermodynamic parameters is that the favorable entropy, typical of hydrophobic interactions,[39,40] derives from large changes in solvation of the ligands and the DNA grooves that accommodate phenanthroline or bipyridyl ligands. In addition these compounds are tetracationic and hence there is a significant release of condensed counterions from the DNA lattice upon binding. This phenomenon also serves as a source of favorable entropy. The profile of such interactions is in contrast to the thermodynamics of binding of many other proven intercalators, such as ethidium or propidium, which typically have large favorable enthalpy driven interactions.[41] However, the intercalator actinomycin is also reported to bind to DNA with a near zero enthalpy.[42] It has been pointed out previously that actinomycin shares some structural features in common with $[Ru(phen)_2(dppz)]^{2+}$,[43] which are also displayed by complex **3**; these compounds possess planar intercalating chromophores to which bulky ancillary groups are attached. In the case of actinomycin these are cyclic peptide groups, while for[$Ru(phen)_2$-

$(dppz)]^{2+}$ and **3** these are phenanthroline ligands. Clearly accommodating these bulky constituents into a groove results in similar energetic costs and a similar pattern of expelling site specifically bound water and counterions that normally reside in the grooves of duplex.

The overall trend in binding thermodynamics for **3** and **4** determined by ITC is entirely consistent with absorptions and luminescence data obtained at correspondingly high salt concentrations. For example, we find that compound **4** binds to duplex DNA with an affinity that is approximately twice that of compound **3**. The interaction of **4** with CT-DNA is also associated with a larger unfavorable enthalpy as compared to **3** and the slightly larger favorable free energy observed for **4** comes from ~4 kJ mol^{-1} more favorable entropy as compared to **3**. These data demonstrate the importance of the ancillary ligand, since **3** and **4** have the identical intercalating chromophores and differ only in the composition of the ligands attached to the metal center.

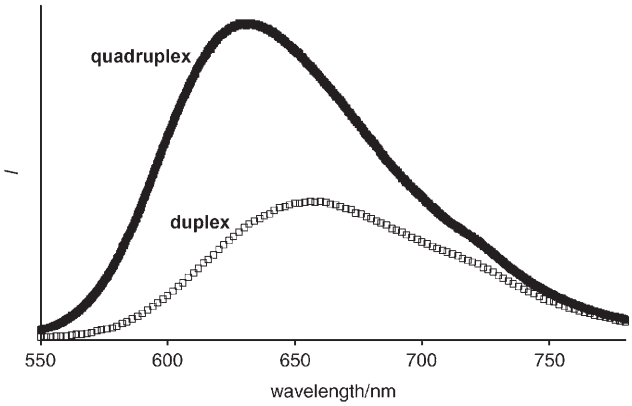


Figure 6. Comparison of emission observed for 5 μM solution of **3** in the presence of G3 quadruplex and calf-thymus duplex DNA. $\lambda_{Ex} = 450$ nm. Buffer: 200 mM KCl, 10 mM KH_2PO_4/K_2HPO_4, 1 mM K$_2$EDTA, pH 7.00 at 25 °C.

Quadruplex binding studies: Having established that **3–5** bind to duplex DNA at high ionic strengths, their interaction with quadruplex structures in these conditions was then investigated.

The complexes incorporate ligands with planar, electron-deficient aromatic ring systems that are structurally reminiscent of molecules known to bind quadruplexes, such as trisubstituted acridines, porphyrins, and pentacyclic quinoacridinium salts.[44] While these molecules have the structural characteristics of intercalators, and are known to intercalate into duplex DNA, their binding mode with quadruplex structures is less well-defined, with NMR and crystallographic studies revealing nonintercalative stacking interactions, such as end pasting.[45]

Thermal denaturation experiments: Initial experiments involved the 22-mer d($AG_3[T_2AG_3]_3$) [G3], human telomeric sequence, which folds into a intramolecular antiparallel quadruplex structure in the presence of K^+. CD spectroscopy was used to confirm that the DNA had folded into a quadruplex conformation, and also as an initial screen to assess T_m shifts in the presence of subsaturating (0.75:1 ligand/quadruplex) amounts of complex. Comparisons with known quadruplex binding agents, suggested that **5** would bind with the highest affinity to quadruplex DNA. Contrary to these expectations, while **3** and **4** induced positive T_m shifts of +3.8 and +5.4 °C, respectively, **5** led to quadruplex *destabilization* by −1.0 °C. Under the same conditions, $[Ru(phen)_2(dppz)]^{2+}$ produced a T_m shift of +0.8 °C. As a consequence of this screen, the quadruplex binding properties of complexes **3** and **4** were further investigated through luminescent titrations studies involving the telomere sequence.

Luminescence studies involving quadruplex DNA: Addition of G3-DNA to aqueous buffered solutions of both complexes also results in a light switch effect, but in this case an emission enhancement, about 2.5 times larger than that observed for duplex DNA occurs (i.e., emission enhancements $\approx \times 150$), and the luminescence is also blue-shifted by up to 32 nm (Figure 6).

These observations imply that the complexes are more inaccessible to water molecules and that there is a greater overlap between the aromatic surfaces of the metal complexes and the bases when bound to quadruplex as opposed to duplex DNA. Further evidence for this hypothesis is provided by initial studies on the emission lifetimes of the bound complexes (Table 4).

Table 4. Photophysical data for complexes **3** and **4** bound to CT-DNA and G3-DNA quadruplex.[a]

	Duplex binding[b]		Quadruplex binding[c]	
Complex	λ_{em} [nm]	τ [ns]	λ_{em} [nm]	τ [ns]
3	658	84	631	129
4	637	92	605	123

[a] Buffer: 200 mM KCl, 10 mM KH_2PO_4/K_2HPO_4, 1 mM K$_2$EDTA, pH 7.00. [b] Data obtained from a 3.1 [bp]/[complex] mixing ratio. [c] Data obtained from a 1.1 [quadruplex]/[complex] mixing ratio.

Surprisingly, it was found that for both complexes data fitted best for a single luminescence lifetime. Whereas lifetimes for both **3** and **4** when bound to CT-DNA are almost identical to the previously reported value of 90 ns measured for **4** in acetonitrile, lifetimes for the quadruplex bound complexes are noticeably longer. Again, this is consistent with lowered solvent accessibility when bound to quadruplex as opposed to duplex DNA; more complete π-overlap, and hence more optimized stacking interactions on binding to G3 relative to duplex. would give rise to greater shielding from solvent and hence lengthen lifetimes and blue-shift luminescence.

Binding stoichiometries with quadruplex were then investigated through luminescence based Job plots (Figure 7). Again, two major inflection points for both complexes, at

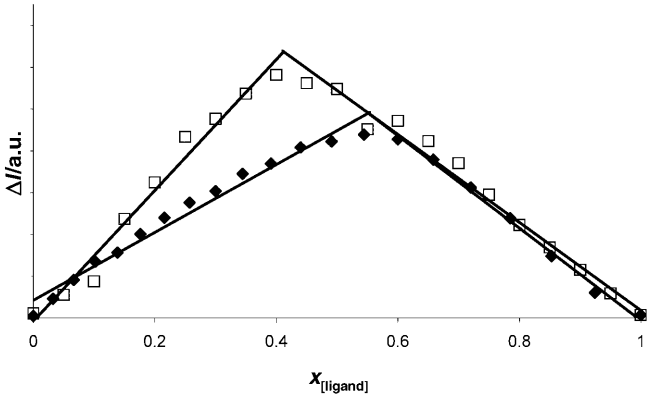


Figure 7. Job plot using luminescence data for [**3**][$(NO_3)_4$] (◆) and [**4**]-[$(NO_3)_4$] (□) with G3-DNA at 10 μM final using 200 mM KCl, 10 mM KH_2PO_4/K_2HPO_4, 1 mM K_2EDTA, pH 7. x = mole fraction of complex added to DNA.

$x = 0.55$ for [**3**][$(NO_3)_4$] and at $x = 0.42$ for [**3**][$(NO_3)_4$], were observed. These data are consistent with a 1:1 [quadruplex]/[complex] binding mode.

Quadruplex binding affinities: Based on the luminescence emission enhancement observed for the binding of the complexes with G3 DNA, equilibrium binding experiments were conducted in order to quantify the binding interactions at 25 °C. The concentrations of **3** and **4** were fixed at 5 μM and the concentration of DNA quadruplex was incrementally increased until saturation emission. These data were used to construct binding curves, which best fitted to a simple one set of sites binding model revealing that **3** binds to G3 with an affinity of 4.4×10^6 M (quadruplex)$^{-1}$, while **4** binds with a slightly higher affinity of 9.5×10^6 M (quadruplex)$^{-1}$. Hence, both **3** and **4** bind to quadruplex with affinities that are higher than to duplex DNA.

Calorimetry studies with quadruplex: To further probe the affinities observed in luminescence titration experiments, we conducted isothermal titration calorimetry experiments at 25 °C. Due to solubility problems associated with **4** at the concentrations required for such studies, calorimetry experiments were restricted to the interaction of **3** with G3 quadruplex. Figure 8 shows primary ITC data for the interaction of **3** with G3 quadruplex DNA.

It was found that the stoichiometry of this interaction was 1:1, confirming the luminescence titration data (Table 3). The overall thermodynamic profile for the binding of **3** to quadruplex DNA is summarized in Table 5.

For reasons similar to those outlined in the duplex studies above, the binding constant determined from calorimetry for the interaction of **3** with G3-DNA is less than the value determined from luminescence titrations. Again, it should be emphasized that the main intention of conducting ITC experiments in this study is to dissect the binding free energy into enthalpic and entropic components. The overall picture that emerges from this thermodynamic analysis is that, in contrast to the entirely entropically driven binding

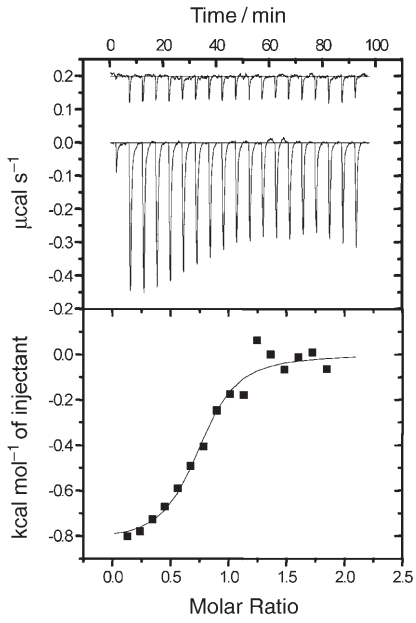


Figure 8. ITC data showing 18 15 μL injections of 0.96 mM [**3**][$(NO_3)_4$] titrated into 80 μM(quadruplex) G3-DNA in 200 mM KCl, 10 mM KH_2PO_4/K_2HPO_4, 1 mM K_2EDTA, pH 7.0 at 25 °C.

Table 5. Calorimetrically derived thermodynamic profile for the interaction of **3** with G3 DNA using 200 mM KCl, 10 mM KH_2PO_4/K_2HPO_4, 1 mM K_2EDTA, pH 7.0 at 25 °C.[a]

	K_b [M^{-1}]	ΔH_B [kJ mol^{-1}]	$-G_{obs}$ [kJ mol^{-1}]	$T\Delta S$ [kJ mol^{-1}]	N
[**3**][$(NO_3)_4$]	$3.0(\pm 1.1) \times 10^5$	-3.5 ± 0.1	-31.3 ± 0.9	27.8 ± 0.9	0.7

[a] Enthalpy values were measured directly using ITC and the standard relationship $\Delta G = \Delta H - T\Delta S$ was used to compute changes in entropy. All values per quadruplex.

to duplex, there is also a small favorable enthalpic contribution to the interaction of **3** with G3 quadruplex, with a ΔH value of -3.5 kJ mol^{-1}, which is consistent with favorable stacking interactions with G-tetrads.

Given the lateral loop conformations delineated in a recent X-ray structure of the G3 quadruplex,[46] intercalation of **3** and **4** in between successive G-tetrads of the conformation would seem to be an unlikely binding mode, although, stoichiometry and binding thermodynamics are consistent with interactions such as "end-pasting" or threading through the lateral loops. However, it should also be pointed out that a very recent report[47] has revealed that in solution the G3 quadruplex structure is conformationally dynamic suggesting that a variety of alternative interactions, including intercalation, cannot be ruled out. Studies designed to resolve this issue are currently underway.

Conclusion

In summary, we report an almost complete thermodynamic profile for dinuclear, monointercalating Ru^{II} complexes that,

even at high ionic strengths, binds to duplex DNA with very high affinities that are only equalled by threaded metallo–bisintercalators. Furthermore, compounds **3** and **4** bind to quadruplex DNA with affinities that are comparable, or higher, than those obtained for duplex binding. Indeed, ITC data for compound **3** clearly show that binding to quadruplex is about 5 kJ mol^{-1} more favorable than binding to duplex DNA. In addition, binding to quadruplex DNA is accompanied by a distinctive "quadruplex light-switch" effect, in which emission is blue-shifted and considerably more enhanced relative to duplex binding.

The properties of these systems indicate that related compounds may have potential as in vivo quadruplex probes: if systems that display a "quadruplex light-switch" effect sufficiently blue-shifted away from the normal duplex phenomenon so that "cross-talk" between emission signals can be eliminated, only modest differentials in binding preference for quadruplex over duplex will be required.

It should be pointed out that this initial work the complexes studied are present as a mixture of diasteromers. However, methodologies for the syntheses of enantiomerically pure isomers of **3**[24] and **5**[26] have been reported. Consequently, future biophysical and photophysical studies will investigate the interaction of enantiopure isomers of these complexes with duplex and G3-DNA as well as other quadruplex structures. Ongoing synthetic studies, targeting systems that display enhanced differential binding preferences and/or luminescent emission for quadruplex over duplex DNA, will be reported in future publications.

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