Differentiating quadruplexes: binding preferences of a luminescent dinuclear ruthenium(II) complex with four-stranded DNA structures†

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The interaction of luminescent dinuclear ruthenium(II) complexes containing the bridging ligand tetrapyrido[3,2-a:2′,3′-c:3″,2″-h:2″,3″-j]phenazine with several intramolecular and intermolecular quadruplex DNA structures has been explored. It was found that these interactions produced distinctly different luminescence signatures. Binding curves constructed from these optical changes reveal that binding affinities for the quadruplex structures vary by over two orders of magnitude. The differences in quadruplex binding affinity and optical signature are rationalized through a consideration of the structural features of the quadruplexes. In particular we conclude large blue shifted emission enhancements are only observed on binding to quadruplexes containing lateral loops that are at least three base pairs long.

Introduction

Although it has been established for some time that G-rich oligonucleotides form four-stranded structures *in vitro* through non-Watson–Crick hydrogen bonded G-quartet motifs,[1] these structures continue to inspire a great deal of research attention.[2] For example, G-rich single-stranded overhangs of telomeres are capable of folding into intramolecular quadruplexes, and—through inhibition of anomalous telomerase activity—substrates designed to stabilize the dynamic human telomere sequence (HTS) in such structures are being targeted as novel anticancer drugs.[3] The activity of the *c-myc* oncogene also appears to be modulated by quadruplex formation, with evidence from various studies suggesting that the active oncogene is a duplex structure while its silent form is folded into a quadruplex.[4] Again, substrates that facilitate quadruplex folding of the oncogene are being investigated.[5]

A number of other genomic sequences associated with disease states have been connected to various quadruplex structures.[6] Furthermore, whole genome analyses reveal the presence of a large number of putative quadruplex structures, which appear to be selected out of coding sequences.[7]

One of the notable features of quadruplex DNA is its structural diversity: one, two, or four oligonucleotides can be folded together into a quadruplex and individual folded strands within these structures can be parallel or antiparallel to each other.[1–3,8]

In the context of this diversity in structure and postulated function, the identification of small molecules that can act as markers for quadruplex DNA has recently become an attractive research target.[9–14] In 2001, Mergny and colleagues reported on specific ethidium derivatives that bind with high affinity to quadruplex DNA accompanied by a ten-fold emission enhancement.[9] More recently, the Che group has reported on a Pt^{II}-based system that shows a very large emission enhancement of a [Pt (π*(dppz)] ^{3}MLCT-based luminescence, with an output at 477 nm, on binding to several different quadruplex structures.[10] In very recent work, Luedtke and co-workers reported a guanidinium modified phthalocyanine that displays a large binding-induced emission enhancement and huge binding preference for quadruplex over duplex structures ($\Delta K_b \approx 5000$). However, for this latter system, binding affinities for different quadruplex sequence are of the same order of magnitude and, again, no differences in luminescence output is observed on binding to the different quadruplex stuctures.[11]

With the aim of producing systems that can optically differentiate between oligonucleotide structures, we have reported on the interaction of octahedral metal complexes that display distinctive luminescent[12,13] or colorimetric[14] changes on binding to duplex and quadruplex DNA structures.

In particular, we found that dinuclear ruthenium(II) complexes $\mathbf{1}^{4+}$ and $\mathbf{2}^{4+}$, based on the tetrapyrido[3,2-a:2′,3′-c:3″,2″-h:2″,3″-j]phenazine, tppz, bridging ligand (Scheme 1), bind with high affinity to both duplex and quadruplex DNA. More interestingly, although they are virtually non-luminescent in water, they display a distinctly different emission signature relative to duplex DNA when bound to the HTS quadruplex ($d[AG_3(T_2AG_3)_3]$) in K^+ solutions: apart from a blue shift of ~30 nm on binding to quadruplex compared to duplex, $I_{bound}/I_0(quad) \approx 150$ as opposed to only $I_{bound}/I_0(duplex) \approx 50$.[12] In recent *in cellulo* work we have found that these complexes stain nuclear DNA, where similar, non-colocalized, emission maxima are observed.[13]

Given these initial results, we now report on an investigation into the interaction of these complexes with other quadruplex structures *in vitro*, with the aim of discovering what specific structural features prompt observed emission changes.

We chose three other structures to compare with basket folded HTS–Fig. 1: a bimolecular structure from the *Oxytricia* telomere sequence, OTS ($[d(G_4T_4G_4)]_2$), composed of two antiparallel hairpins containing four stacked tetrads and diagonal loops,[15] the thrombin binding aptamer sequence, TBA

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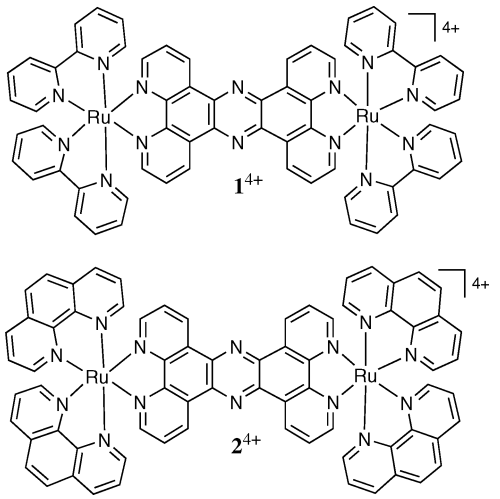

Scheme 1

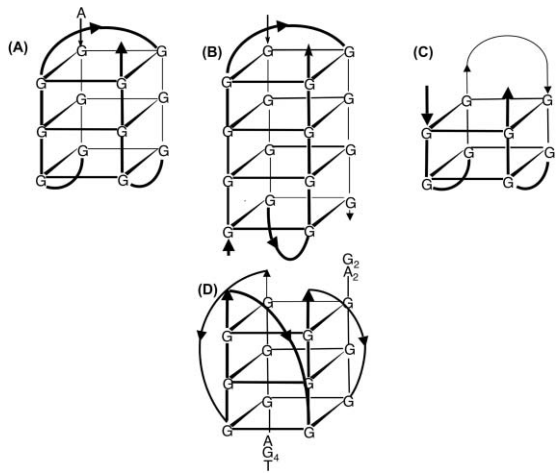

Fig. 1 Schematic structures of relevant DNA quadruplexes. (A) Basket structure of HTS. (B) Double hairpin structure of OTS. (C) Chair structure of TBA. (D) Predominant propeller loop structure of Pu27.

($d(G_2T_2G_2TGTG_2T_2G_2)$), which folds into an antiparallel chair quadruplex containing two stacked tetrads where the external loops are all lateral,[16] and a second single oligonucleotide intramolecular quadruplex: the purine rich sequence found in the promoter of *c-myc*, Pu27 ($d(TG_4AG_3TG_4AG_3TG_4A_2G_2)$), a dynamic mixture of isomers containing three tetrads with short internal propeller loops and two extended overhangs.[17]

Results and discussion

Before any experiments were carried out, the secondary structures of the folded quadruplexes were confirmed by comparison of their CD spectra with published data. In the conditions used for our experiments it was clear that HTS also took up a predominantly antiparallel structure with the distinctive features being a positive maximum centred at 295 nm peak and a negative peak centred around 260 nm. This agrees with previous studies on this quadruplex carried out in analogous conditions[18] and is consistent with the hypothesis that molecular crowding is required for parallel structure formation.[19]

Emission based fluorescence studies were then used to assess the quadruplex binding properties of $[\mathbf{1}]Cl_4$.

Emission Titrations

As has been previously reported for studies with HTS, titration of the quadruplex structures into aqueous buffered solutions of $\mathbf{1}^{4+}$ induced characteristic increases in luminescent intensity. For example, addition of OTS results in a broad ^{3}MLCT based emission centred at 651 nm—Fig. 2. However the enhancement of luminescence and the energy of λ_{max} are both dependent on individual structures.

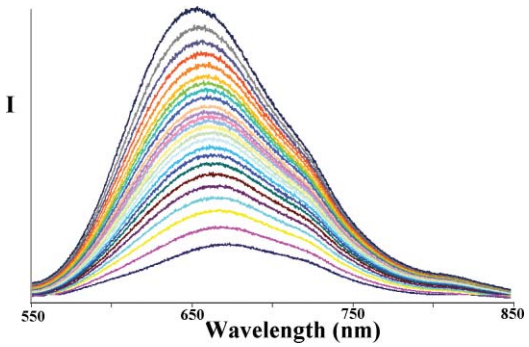

Fig. 2 Changes in the luminescence of aqueous buffered solution of $[\mathbf{1}]Cl_4$ on progressive addition of OTS.

While HTS, OTS and Pu27 produced enhancements of $I_{bound}/I_0 > 50$, TBA induced a much smaller change. Furthermore, addition of the different quadruplexes resulted in λ_{max} values ranging from 635 nm for HTS to 670 nm for TBA—Table 1, Fig. 3.

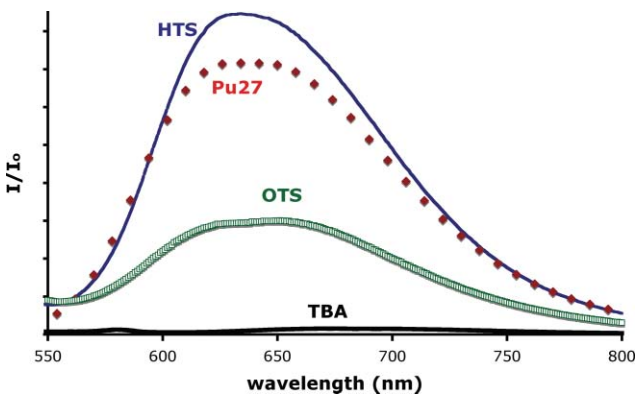

Fig. 3 A comparison of luminescence from buffered aqueous solutions of $[\mathbf{1}]Cl_4$ on addition of excess quadruplex. Binding ratio in all cases: [**1**]:[quadruplex] = 1 : 9.

Table 1 Summary of luminescence changes for complex $\mathbf{1}^{4+}$ on binding with selected DNA quadruplex structures

Quaduplex	λ_{em}/nm	I_{bound}/I_0
HTS	635	138
Pu27	639	120
OTS	651	61
TBA	670	≈ 3

Table 2 Summary binding data for complex $\mathbf{1}^{4+}$ with selected quadruplex structures[a]

Quaduplex	K_b / M^{-1}
HTS	9.5×10^6
Pu27	9×10^5
OTS	5.6×10^6
TBA	5.5×10^4

[a] Fit to a single set of identical binding sites model.

Indeed, the λ_{max} values correlate well with the observed emission enhancements; larger emission gains are associated with blue shifting of λ_{em}—Fig. 4. In previous studies on a variety of emissive systems, higher energy luminescence and larger emission enhancements have been associated with metal complex binding sites that are more solvent inaccessible.[20]

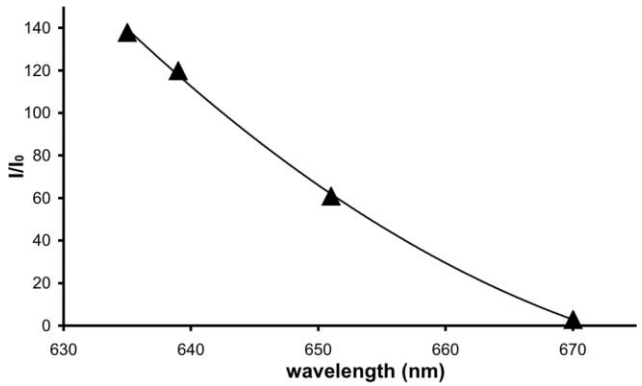

Fig. 4 Relationship between luminescence enhancement and emission wavelength for the binding of complex $\mathbf{1}^{4+}$ with studied quadruplexes.

The enhancements in luminescence observed for $\mathbf{1}^{4+}$ were used to construct binding curves for the interaction of the complex with each quadruplex structure. All titrations resulted in curves that displayed binding saturation, Fig. 5 and Table 2, and were fit to a simple one set of identical binding sites model.

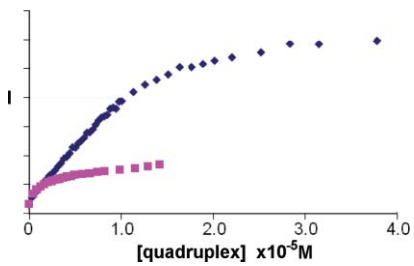

Fig. 5 Saturation binding curves constructed from luminescence data for the interaction of complex $\mathbf{1}^{4+}$ with Pu27 (♦) and TBA (■).

In all cases, the data fit well to a 1 : 1 binding model. This ratio is consistent with the expected end stacking interaction observed for extended aromatic systems and the G-tetrad motif of quadruplexes. However binding affinities for the quadruplex structures vary by over two orders of magnitude and, interestingly, the affinities do not entirely correlate with λ_{em} or magnitude of the emission. The highest affinity binding and largest emission enhancement is seen for HTS. In contrast, although Pu27 produces the next largest increase in emission, the binding affinity for this structure is lower than that for OTS. These trends in binding parameters and emission profiles can be explained through a consideration of the variation in quadruplex structure.

The binding-induced light switch effect and blue shifting of the λ_{max} observed in Fig. 3 occurs due to the transfer of a complex from a polar aqueous environment into a less polar environment[20] and thus indicates how isolated the complex is from the bulk solvent. The largest enhancement in the emission of $\mathbf{1}^{4+}$ occurs for HTS, thus suggesting that the binding site for this structure isolates the complex from the bulk solvent more effectively than the sites on the other folded quadruplexes. Given the 1 : 1 binding stoichiometry, end stacking while threaded through the TTA diagonal loop seems the most likely binding mode, as this would offer the most hydrophobic pocket. This binding mode has been observed by crystallography for acridine derivatives bound to the lateral loops of OTS.[21] Indeed, it is noticeable that $\mathbf{1}^{4+}$ binds to the other structure with diagonal loops, OTS, with a comparable affinity. However, binding to OTS induces a lower luminescent enhancement; again this is consistent with $\mathbf{1}^{4+}$ threading through a diagonal loop, since the longer TTTT diagonal loops of OTS may be expected to provide less protection for the threaded complex from solvent access compared to the shorter loops of HTS.

Surprisingly, although K_b for Pu27 is an order of magnitude lower than that for HTS, binding-induced emission enhancements and λ_{em} values are very similar. In the solid state, external binding to the propeller loops of the related bimolecular parallel quadruplex based on the HTS has been observed,[22] however such an interaction is not likely to protect $\mathbf{1}^{4+}$ sufficiently for the observed enhancement in its photo-emission, and furthermore a recent combined experimental and theoretical study indicates that end-stacking is always an energetically preferred binding mode to quadruplexes.[23] Therefore, it seems likely that $\mathbf{1}^{4+}$ is bound through stacking interactions at the top end of the G-tetrad core as has been observed for a number of other cationic extended aromatic systems.[24] Structural studies on this binding mode using a 24-base sequence derived from Pu27 and the well-studied TMPyP4 porphyrin have revealed that on binding, the single stranded overhang of the quadruplex folds over the end-stacked substrate to produce a binding pocket structurally related to a diagonal loop. Such a binding mode is consistent with the data for $\mathbf{1}^{4+}$ obtained in this study. It is known that Pu27 is structurally dynamic and previous studies have indicated that small molecule binding substrates can produce dramatic conformational changes trapping out highly specific structures.[4,5,17] The possibility that binding of $\mathbf{1}^{4+}$ causes similar effects can be discounted as the CD spectrum of Pu27 is virtually unchanged upon addition of the complex with no evidence of the characteristic CD changes that accompany conformational switching.

Analogous titrations with [**2**]Cl_4 resulted in binding parameters that were very close, but slightly lower, than the values obtained for [**1**]Cl_4 (*e.g.*; complex $\mathbf{2}^{4+}$ binds to OTS with $K_b = 1.3 \times 10^6\ M^{-1}$, compared to $K_b = 5.6 \times 10^6\ M^{-1}$ for $\mathbf{1}^{4+}$). Emission enhancements for the two compounds are virtually identical. This indicates that $\mathbf{2}^{4+}$ does not have a distinctly different binding target to $\mathbf{1}^{4+}$ and suggests that both complexes bind to each quadruplex structure by similar modes. The slightly lower binding affinities observed for $\mathbf{2}^{4+}$ is consistent with the increased steric demand provided by the ancillary ligands in a through loop binding geometry.

Conclusions

In summary, for the first time as far as we aware, we describe a system whose wavelength and intensity of emission is dependent on the structure of the DNA quadruplex to which it binds. Using this effect, details on interaction of the complexes with several well-characterized quadruplexes have been explored and distinct binding preferences for specific structures have been revealed. In particular, we have found that blue shifted luminescence and high affinity binding is observed when the complex binds, through end-stacking, to antiparallel quadruplex structures containing external loops at least three bases in length. The presence of shorter lateral loops limits binding affinities by several orders of magnitudes and results in negligible emission changes.

It should be pointed out that although this initial study involves complexes as an unresolved mixture of diastereomers, ongoing studies on the interaction of resolved complexes—and other related systems—with specific DNA structures designed to delineate further biophysical and structural details will form the basis of future full reports. In particular the synthesis of complexes that function as luminescent markers for individual quadruplex structures is being targeted.

Experimental section

Materials

The synthesis of $\mathbf{1}^{4+}$ and $\mathbf{2}^{4+}$ as hexafluorophosphate salts was carried out through reported procedures.[25,26] The complexes were converted to chloride salts by treating the PF_6 salt with $^{n}Bu_4Cl$ in dry acetone. The chloride salts were collected by filtration or centrifugation, washed copiously with acetone, and dried at 70 °C under vacuum for 8 h. Quadruplex DNA sequences were purchased from Eurogentech or Sigma–Aldrich company as their HPLC purified sodium salts. Samples were dissolved in buffer (10 mM KH_2PO_4/K_2HPO_4, 1 mM K_2EDTA in 50–200 mM KCL (pH 7.0)) Samples for binding studies were annealed at 95 °C for 10 min, and then cooled at 5 °C for 24 h. Circular dichroism spectra were recorded on a Jasco J–810 Spectropolarimeter at 25 °C.

Solutions were quantitated by UV spectroscopy at 260 nm using extinction coefficients calculated by the nearest neighbour method. Values used: $\varepsilon_{260} = 228500\ M^{-1}$(Strands) cm^{-1} HTS. $\varepsilon_{260} = 143300\ M^{-1}$(Strands) cm^{-1} TBA. $\varepsilon_{260} = 115200\ M^{-1}$(Strands) cm^{-1} OTS. $\varepsilon_{260} = 279900\ M^{-1}$(Strands) cm^{-1} Pu27.

Luminescence titrations were carried out using a Hitachi F-4500 Fluorescence spectrophotometer. Protocol used: 1–3 mL of buffer was loaded into a 1 cm path length luminescence cuvette. A volume of buffer was removed and replaced with the same volume of a stock solution of complex, to give a final concentration of 2–7 mM. The cuvette was loaded into the spectrophotometer and kept at 25 °C. After equilibrium, the emission spectrum of the solution was recorded at 450 nm. Typically 0.5–2 mL of a concentrated stock solution of DNA was added to the cuvette and mixed. The emission spectrum was recorded, typically showing an enhancement in emission. This procedure was continued until the emission became constant. Data were analysed using the one-set-of-sites binding model.

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