Chiroptical, ESMS and NMR spectroscopic study of the interaction of enantiopure lanthanide complexes with selected self-complementary dodecamer oligonucleotides †

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The interaction of Δ- and Λ-Eu and Yb cationic complexes bearing an *N*-methylphenanthridinium chromophore with $[(CG)_6]_2$, $[(AT)_6]_2$ and $[CGCGAATTCGCG]_2$ has been interrogated by ESMS, ^{1}H NMR, absorption, difference circular dichroism, fluorescence quenching and Ln luminescence emission spectroscopy. Stepwise complexation occurs with up to 3 : 1 limiting stoichiometry for $[(CG)_6]_2$ and 2 : 1 for $[CGCGAATTCGCG]_2$, as indicated by absorption measurements and direct ESMS observation of the non-covalent duplex adducts. Binding to $[(CG)_6]_2$ occurred with an affinity of 8.7×10^6 M^{-1} $duplex^{-1}$ for the Δ-Eu isomer which was 50 times greater than to $[(AT)_6]_2$. A primary component of the free energy of binding has been linked to an intercalative interaction, supported by absorption, ICD and fluorescence quenching characteristics. The lanthanide coordination environment and local helicity remain unchanged but the oligonucleotide underwent distinctive changes in local helicity and pitch which were sensitive to the handedness of the Ln complex and in certain cases to the nature of the Ln ion (Yb *vs.* Eu). With $[CGCGAATTCGCG]_2$ ^{1}H NMR TOCSY and NOESY analysis in the presence of increasing concentrations of the Gd analogues revealed that the most avid complex binding site was located in the centre of the oligonucleotide, with the Δ-isomer binding more strongly.

Several chiral, octahedral complexes of the late d-block metals have been studied as structural and reactive probes for nucleic acids.[1] Typical examples include the Δ- and Λ-isomers of the ubiquitous $Ru(bpy)_3^{2+}$, which binds externally to the anionic backbone,[2] and $Ru(phen)_3^{2+}$, which is believed to be bound by the minor groove.[3] A large series of mixed ligand dipyridophenazine (dppz) complexes of ruthenium, rhodium and osmium has also been examined, *e.g.* $[Os(bpy)(dppz)]^{2+}$. These complexes are reputed to bind to DNA by preferential intercalation of the phenazine moiety from the minor groove.[4,5] For each of these systems, the perturbation of the metal–ligand charge transfer transitions that accompanies DNA binding, provides a sensitive spectroscopic handle to study the nature of the bound complex.

There are no examples, however, of well-defined water-soluble, chiral lanthanide complexes which are suitable for studying their interaction with the polyanionic nucleic acids. Such complexes should be available in both enantiomeric forms and be amenable to being addressed by chiroptical and luminescence methods at excitation wavelengths in excess of 340 nm. In addition, they should be kinetically stable with respect to racemisation and ligand dissociation pathways. In general, the literature reports that define lanthanide–nucleic acid interactions have been dominated by the interactions of the aqua ions themselves.[6] Such work has often focused on the selective sensitisation of selected ions, *e.g.* Tb^{3+},[7] and advantage has been taken of this effect in several assays directed at the detection of selected nucleic acids.[8–11] Another theme of much current activity relates to the ability of certain lanthanide ions to induce nucleic acid cleavage[8,12] and attempts have been made to target certain sequences using an anti-sense strategy.[13]

Recently, we have defined a series of axially symmetric, cationic lanthanide complexes in which the configuration of a remote carbon stereogenic centre determines the helicity of the overall complex.[14,15] In the prototypical example, $[Ln\cdot\mathbf{1}]^{3+}$, the complex exists in solution as one major isomeric species with a square-antiprismatic geometry about the lanthanide centre. An (*S*)-configuration at carbon gives rise to a complex with a positive sign for the four NCCO torsion angles (Δ) and a λ configuration (negative sign for each C_4-related N–C–C–N torsion angle) for each of the four 12-N_4 chelate rings. Complexes of Yb, Eu and Tb have been shown to be configurationally stable on the NMR and lanthanide emission timescale,[16,17] suggesting that such complexes may be useful as chiral probes. The complexes are also kinetically robust in water with respect to lanthanide dissociation over the pH range 2 to 10. Given these promising features, we resolved to study analogues of these complexes as NMR and luminescent probes of nucleic acid structure. An attractive feature is the ease of Ln^{3+} substitution allowing the definition of systems with distinctive function. Thus, following in the footsteps of pioneering work by Williams,[18] NMR shift (Dy, Tm) and relaxation (Gd) agents may be obtained. Long-lived visible (Eu, Tb) and near-IR (Yb,

† Electronic supplementary information (ESI) available: examples of data sets and analysis. See http://www.rsc.org/suppdata/p2/b1/b104796p/

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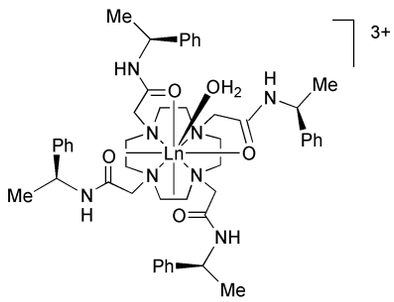
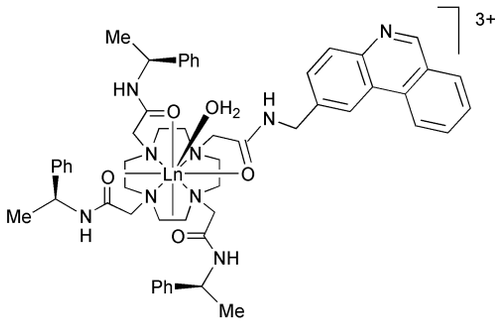


(*SSSS*)-Δ-[Ln•**1**]$^{3+}$



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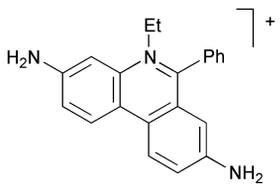

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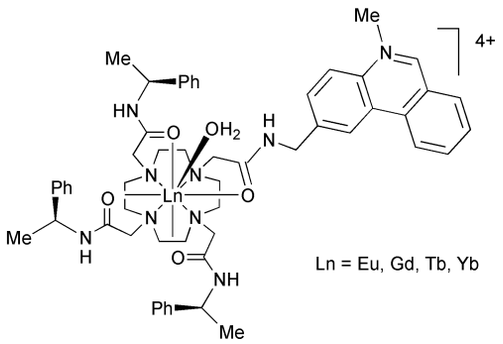

(*SSS*)-Δ-[Ln•**4**]$^{4+}$

Nd) emissive probes are readily accessible,[19] whilst redox active (Ce^{4+}) and singlet oxygen precursors (Tb^{3+}) may also be envisaged—the latter requiring an efficient back-energy transfer process to repopulate the intermediate sensitising aryl triplet state.

Recently, we reported the synthesis and excited state reactivity of the Eu and Tb complexes, [Ln•**2**]$^{3+}$, containing a simple or *N*-alkylated phenanthridine moiety to sensitise lanthanide luminescence. Perturbation of the intermediate phenanthridinyl singlet or triplet excited states by changes in pH, pO_2 and pCl^- was reported by variations in lanthanide luminescence intensity or lifetime,[19,20] following excitation in the range 350 to 375 nm. The tricyclic, alkylated phenanthridinium moiety is well-known to intercalate between DNA base pairs—a good example being the case of its diamino derivative, ethidium bromide, **3**. With this in mind, the Δ- and Λ-*N*-alkylated phenanthridinium complexes [Ln•**4**]$^{4+}$ were targeted for our initial studies of oligonucleotide and nucleic acid binding.

Results and discussion

Complex characterisation

The synthesis of the *N*-alkylated complexes, [Ln•**4**]$^{4+}$, has been reported previously.[20] ^{1}H NMR spectra for each complex revealed the presence of one major isomer (≥95%) in solution, both in D_2O (295 K) and in CD_3OD over the temperature range 230–310 K. Chemical shift values for the paramagnetically shifted resonances, *e.g.* the ring CH_2N protons, closely resembled those found for the related parent series[15,21] [Ln•**1**],$^{3+}$ which have also been characterised by X-ray crystallography. This suggested that each complex adopts a common, monocapped square-antiprismatic coordination geometry with a twist angle between the N_4 and O_4 places of about 40°.[22] Mirror image CD spectra were measured for the Δ- and Λ-complexes (Fig. 1). The longest wavelength bands at 320 and 370 nm gave rise to weak CD spectra, with $g_{abs}^{320} = +1.1 \times 10^{-3}$ and $g_{abs}^{370} = -9 \times 10^{6}$ for the Δ-Eu complex.

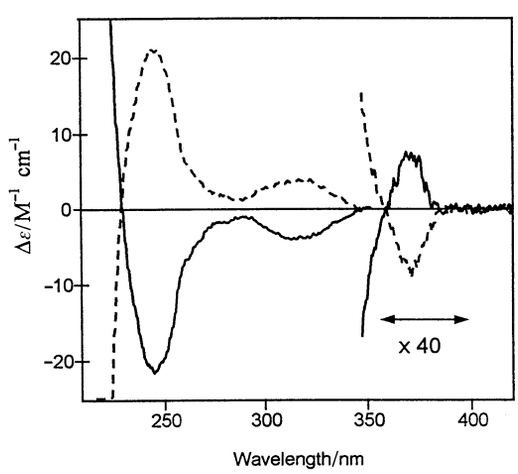

Fig. 1 Circular dichroism spectra for (*SSS*)-Δ-[Eu•**4**]$^{4+}$ (dotted line) and (*RRR*)-Λ-[Eu•**4**]$^{4+}$ (bold) (298 K, 10 mM complex, pH 7.4 HEPES).

Circularly polarised luminescence (CPL) spectroscopy is the emission analogue of CD and probes the chirality of the excited state.[23] The metal-based CPL emission spectra for the enantiomeric Eu, Tb and Yb complexes were recorded in aqueous media. Details of the near-IR CPL spectra from these and related Yb complexes have been discussed elsewhere.[16,22] Equal and opposite CPL spectra were observed in water or aqueous methanol, following excitation of [Tb•**4**]$^{4+}$ at 330 nm: the (*SSS*)-Δ-[Tb•**4**]$^{4+}$ complex for example gave values associated with the $\Delta J = -1$ ($^5D_4$–$^7F_5$) transition of $g_{lum}^{544} = -0.17$ and $g_{lum}^{547} = +0.25$ (Fig. 2). Strong CPL was also observed in the magnetic-dipole allowed $\Delta J = 1$ transitions of the analogous Eu complexes (Fig. 3). The emission and CPL spectra were very similar in form to those found for the parent complexes

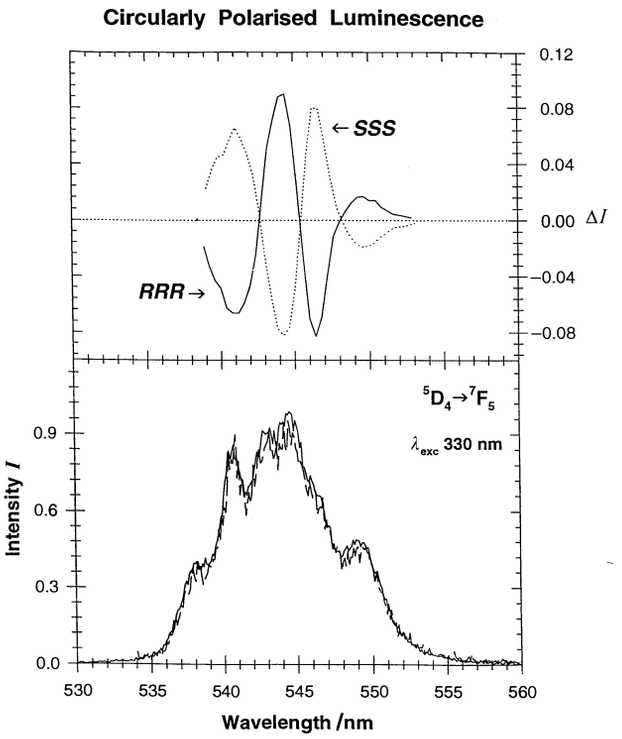

Fig. 2 Circularly polarised luminescence emission (upper) and total emission spectrum, showing the $\Delta J = -1$ transition, for (*SSS*)-Δ-[Tb·**4**]$^{4+}$ (dotted line) and Λ-[Tb·**4**]$^{4+}$ (line), following excitation at 330 nm (295 K, pH 6).

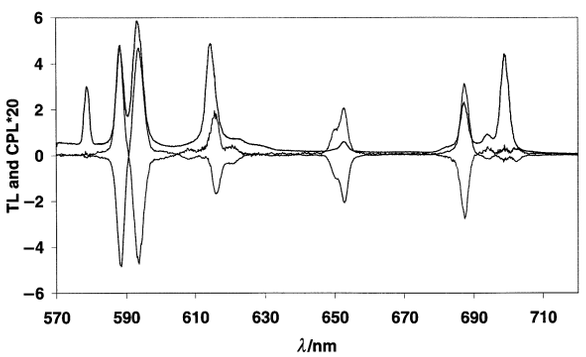

Fig. 3 Total luminescence emission (upper) and circularly polarised emission (×20) spectra for (*SSS*)-Δ-[Eu·**4**]$^{4+}$ (positive at 588 and 616 nm) and (*RRR*)-Λ-[Eu·**4**]$^{4+}$ (λ_{exc} 365 nm, H_2O, pH 5.5).

[Eu·**1**]$^{3+}$. Given that, for a common axial donor and a common ligand donor type, the intensity of a lanthanide CPL transition is likely to be determined by the local helicity at the metal centre, the similarity in *g* values observed for [Ln·**1**]$^{3+}$ and [Ln·**4**]$^{4+}$ complexes is consistent with a similar degree of twist in each case. It is this twisting which controls the extent of mixing of the magnetic and electric dipole transition moments giving rise to 'rotatory strength'.[24] The commonality of a regular square-antiprismatic structure suggested by these CPL studies agrees with the conclusion derived from the NMR solution studies described above.

Interaction of chiral lanthanide complexes with $[(CG)_6]_2$, $[(AT)_6]_2$ and $[CGCGAATTCGCG]_2$

The nature, stoichiometry and consequences of the interaction of Δ- and Λ-[Ln·**4**]$^{4+}$ complexes with selected oligonucleotides were studied by absorption, CD and emission spectroscopy in conjunction with selected ^{1}H NMR and ESMS studies. The binding interaction was studied with the self-complementary oligonucleotides $[(CG)_6]_2$, $[(AT)_6]_2$ and the 'Drew–Dickerson' dodecamer $[CGCGAATTCGCG]_2$. The last nucleotide was chosen as it not only possessed a 2 : 1 mixture of base-pair types but also it has been the subject of detailed structural and NMR analyses over the last 20 years.[25,26]

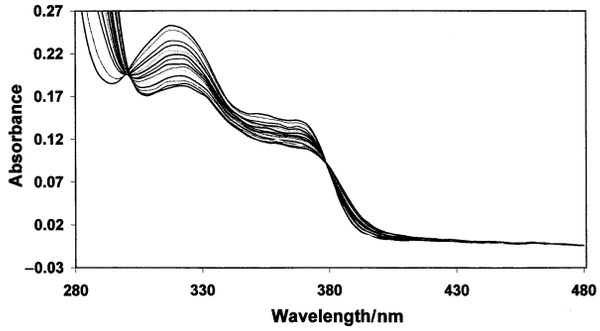

Fig. 4 Absorption spectral changes for Δ-[Eu·**4**]$^{4+}$ following incremental addition of $[CGCGAATTCGCG]_2$ (pH 7.4 HEPES, 10 mM NaCl, 33 μM complex, 298 K), showing the hypochromism at 320 and 370 nm.

Absorption spectroscopy

Changes in the absorption spectrum of Δ- and Λ-[Ln·**4**]$^{4+}$ complexes (Ln = Eu, Yb) were monitored following incremental addition of each oligonucleotide at pH 7.4 (HEPES), under low added salt conditions ($I = 0.02$ M). With $[(CG)_6]_2$, fairly well-defined isosbestic points were observed for the europium complexes at 302 and 378 nm. A pronounced hypochromism was evident in the bands at 321 and 370 nm, accompanied by a small red-shift and the appearance of a tailing absorption band beyond 400 nm. Changes in the absorbance value at 321 nm reached a limit at a ratio of complex : oligo of 3 : 1, beyond which no significant differences were observed. The overall behaviour of the Δ and Λ europium complexes was very similar (Table 1) to that of $[(CG)_6]_2$. The complexes with $[(AT)_6]_2$ showed much smaller changes in intensity and the isosbestic point at 378 nm was absent. With the Drew–Dickerson dodecamer (DD), changes in the absorption spectrum resembled those observed with $[(CG)_6]_2$ (Fig. 4). Plots of the change in absorbance at 320 nm *versus* concentration of added oligo tended to a saturation limit for a complex : oligo stoichiometry of 2 : 1. This pattern of behaviour—a small red-shift and presumed hypochromism in the phenanthridinium chromophore for oligos containing (CG) base-pairs—suggested that there was a significant charge-transfer interaction between the electron-poor phenanthridinium moiety and the relatively electron-rich (CG) base-pairs. This interaction was not apparent with (AT) base pairs (no red-shift, little or no tail) suggesting that the Δ and Λ europium complexes may be binding to the CG base-pairs *via* an intercalative interaction.[27]

A rather different pattern of behaviour was observed with the corresponding ytterbium complexes. The Λ-isomer showed pronounced hypochromism and a red-shift with both $[(CG)_6]_2$ and (to a lesser degree) $[(AT)_6]_2$, while the Δ-isomer gave rise to very small (<5%) hypochromism at 320 and 370 nm. An absorption tail to the red was apparent but no isosbestic points were defined. With the Drew–Dickerson dodecamer, the Λ-isomer gave slightly larger hypochromism values although the overall changes in spectral form were similar to those with the Δ-isomer. In comparison to the Eu series, a striking feature is the effect of complex helicity: with Eu more pronounced shifts and hypochromism were associated with the Δ-complex possibly binding to (CG) base-pairs; with Yb, the Λ-isomer gave rise to the larger spectral changes for both CG and AT binding (Table 1).

Circular dichroism studies

The interaction of each complex with the oligonucleotides was also examined by circular dichroism difference spectroscopy,

Table 1 Absorption spectral changes following addition of oligonucleotides to Δ- and Λ-$[Ln\cdot\mathbf{4}]^{4+}$ (pH 7.4, 10 mM HEPES, 10 mM NaCl)

Diastereoisomeric complex	% Hypochromism at 320 nm	% Hypochromism at 370 nm
Δ-$[Eu\cdot\mathbf{4}]^{4+}$–$[(CG)_6]_2$	35	27
Λ-$[Eu\cdot\mathbf{4}]^{4+}$–$[(CG)_6]_2$	33	27
Δ-$[Eu\cdot\mathbf{4}]^{4+}$–$[(AT)_6]_2$	13	10
Λ-$[Eu\cdot\mathbf{4}]^{4+}$–$[(AT)_6]_2$	15	12
Δ-$[Eu\cdot\mathbf{4}]^{4+}$–$(DD)_2$	34	28
Λ-$[Eu\cdot\mathbf{4}]^{4+}$–$(DD)_2$	32	26
Δ-$[Yb\cdot\mathbf{4}]^{4+}$–$(DD)_2$	30	24
Λ-$[Yb\cdot\mathbf{4}]^{4+}$–$(DD)_2$	31	27
Δ-$[Yb\cdot\mathbf{4}]^{4+}$–$[(CG)_6]_2$	[a]	[a]
Λ-$[Yb\cdot\mathbf{4}]^{4+}$–$[(CG)_6]_2$	36	30
Δ-$[Yb\cdot\mathbf{4}]^{4+}$–$[(AT)_6]_2$	[b]	[b]
Λ-$[Yb\cdot\mathbf{4}]^{4+}$–$[(AT)_6]_2$	25	22

[a] A red shift was observed with a pronounced long wavelength tail absorption (>400 nm), but ≤5% hypochromism. [b] Significant hyperchromism was observed. [c] In a control experiment *N*-Me- and *N*-Et-phenanthridinium chloride (and iodide) exhibited limiting hypochromism values of 32 and 11% at 320 nm upon binding to $[(CG)_6]_2$ and $[(AT)_6]_2$. [d] DD = $[CGCGAATTCGCG]_2$.

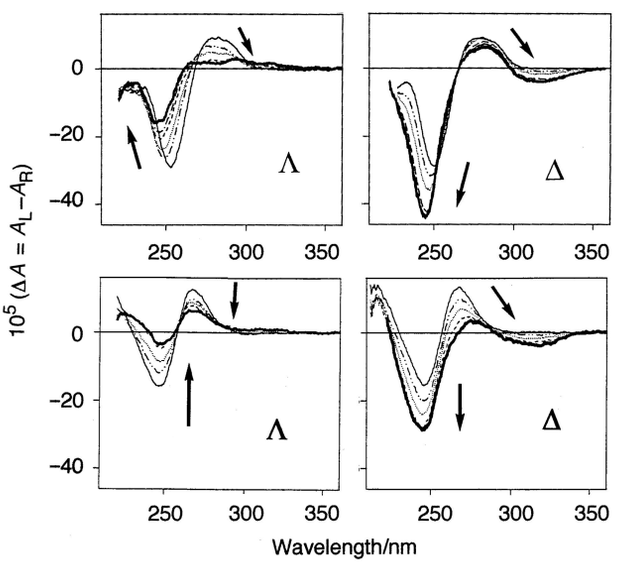

Fig. 5 Near-UV CD difference spectra for $[(CG)_6]_2$ (upper) and $[(AT)_6]_2$ (lower) in the presence of increasing ratios of Λ- (0–7.4, left) and Δ- (0–4.7, right) $[Eu\cdot\mathbf{4}]^{4+}$ (295 K, 10 mM HEPES, pH 7.4, 10 mM NaCl). Near identical behaviour was exhibited by the Yb complexes.

adding the lanthanide complex to a fixed concentration of the oligonucleotide. The spectrum due to the free complex at the same concentration was subtracted to allow examination of the bound species alone. Analysis of the near-UV region of each oligonucleotide revealed the features characteristic of B-DNA, *e.g.* for $[(CG)_6]_2$ a strong negative band was evident at 250 nm with a positive band at 275 nm.[28] Following addition of an excess of complex, these gross features persisted negating the possibility of a B⟶Z transition which can occur in the presence of certain highly charged metal complexes.[29]

In the 220–260 and 290–340 nm region, the observed changes for a given oligonucleotide were markedly dependent upon complex chirality. For example, with $[(CG)_6]_2$ addition of Λ-$[Eu\cdot\mathbf{4}]^{4+}$ led to a decrease in intensity of both bands and a shift of 5 nm to lower wavelength for the 250 nm band (Fig. 5). The positive band almost disappeared upon saturation with the complex. With Δ-$[Eu\cdot\mathbf{4}]^{4+}$ the 250 nm band *increased* in intensity and shifted by 6 nm, the 275 nm band decreased slightly in intensity and a new negative induced CD band appeared around 320 nm which reached a limiting intensity value at a ratio of 3 complexes per duplex. Very similar behaviour was exhibited by the Yb complexes.

In the binding to $[(AT)_6]_2$, addition of Δ-$[Eu\cdot\mathbf{4}]^{4+}$ led to a 40% increase in intensity but to little shift of the 250 nm band, while the 270 nm band shifted 5 nm to the red and was reduced in intensity. Again a new negative band at *ca.* 320 nm appeared, reaching a saturation limit at a stoichiometry of close to 2 : 1 (Fig. 5).[30] Changes were very different following addition of the Λ-isomer: the 250 nm was reduced in intensity by a factor of 3 and the 270 nm band decreased by 50%.

The Δ-Eu complex, but not the Λ-isomer, exhibited a negative band at 320 nm associated with an induced CD into the 'short axis' transition of the phenanthridinium chromophore. This feature was also not significant in the difference CD spectra obtained for titrations of both the Δ- and the Λ-Eu complex with the Drew–Dickerson dodecamer. With this 'mixed' oligo, the Δ-isomer gave rise to very small changes in intensity (10% decrease at 282 nm at an oligo : complex ratio of 1 : 2). With the Λ-isomer markedly different behaviour was apparent. A 50% reduction in intensity at 282 nm was observed (oligo : complex ratio 1 : 2) and a significant decrease in intensity was observed at 252 nm (Fig. 6). With $[CGCGAATTCGCG]_2$ the Λ-Eu isomer only showed CD changes that resembled those obtained with $[(AT)_6]_2$ rather than $[(CG)_6]_2$ (Fig. 5).

The ytterbium analogues exhibited different behaviour in binding to the Drew–Dickerson oligonucleotide: the Λ-Yb complex behaved almost identically to the Λ-Eu complex whereas the Δ-Yb complex gave rise to CD spectral changes which were similar to those shown with $[(CG)_6]_2$. A 6 nm shift of the 250 nm band to lower frequency was observed and a decrease and shift to longer wavelength for the 280 nm band, together with the appearance of a negative ICD band around 320 nm.

The phenanthridinium chromophore in the free complex $[Ln\cdot\mathbf{4}]^{4+}$ shows a weak ICD band at 370 nm which is positive in sign for the Λ-isomer and negative for the Δ-isomer (Fig. 7). Upon binding to $[(CG)_6]_2$ and $[(AT)_6]_2$, difference CD spectra revealed an increase in the *positive* intensity of this band which was independent of lanthanide complex chirality. Closer inspection of this phenomenon for both $[(CG)_6]_2$ and $[(AT)_6]_2$ revealed that this induced CD band was made up of two components—both positive in sign—at 360 and 380 nm with the former being the more intense. This may indicate that there are two (or more) different binding sites for the complex in which the phenanthridinium group is oriented in the same sense, with respect to the oligonucleotide helical axis. With $[CGCGAATTCGCG]_2$, the Λ-Eu and Yb complexes again behaved similarly, with well-defined positive ICD bands apparent at 360 and 377 nm in each case. However with the Δ-isomer both the Yb and Eu complex gave rise to a positive CD band at 370 nm and a *negative* one of equal intensity at 384 nm. Thus, uniquely, the Δ-isomer binds with the phenanthridinium electric dipole transition moment vector oriented in 2 very different directions with respect to the oligonucleotide helical axis. Such

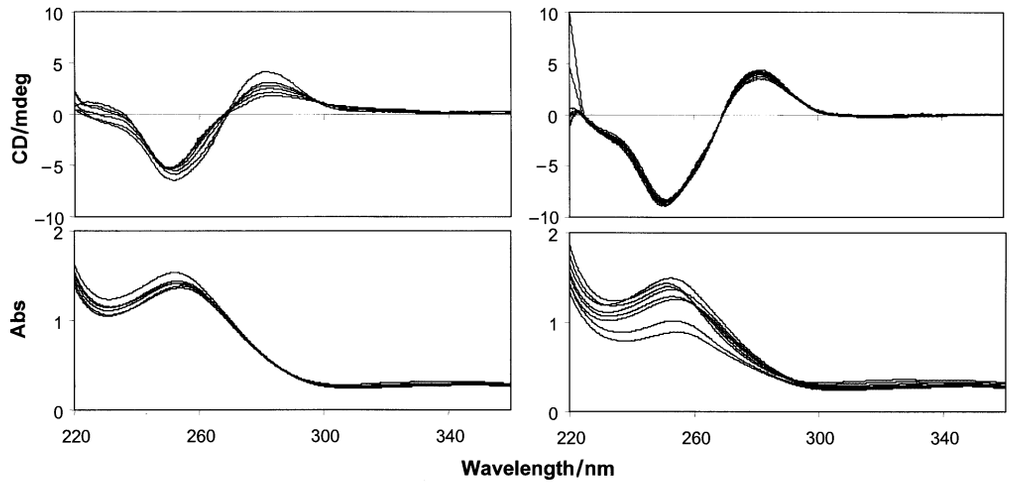

Fig. 6 Absorption and difference CD spectra for $[CGCGAATTCGCG]_2$ in the presence of Λ-$[Eu{\cdot}\mathbf{4}]^{4+}$ (left; up to 1 : 3) and Δ-$[Eu{\cdot}\mathbf{4}]^{4+}$ (right, up to a ratio of complex/oligo of 1 : 4.1) (295 K, pH 7.4, 10 mM HEPES, 10 mM NaCl).

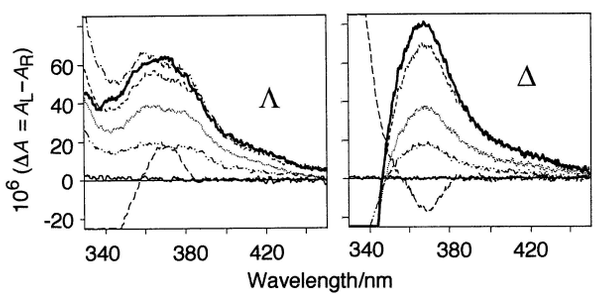

Fig. 7 Near-UV/visible CD difference spectra for $[(CG)_6]_2$ in the presence of increasing concentrations of Λ- (left) and Δ- (right) $[Eu{\cdot}\mathbf{4}]^{4+}$. CD spectra of the complexes alone are shown for reference (dotted line): (295 K, pH 7.4, 10 mM HEPES, 10 mM NaCl).

behaviour suggests that the local chirality of the phenanthridinium moiety is being determined primarily by the right-handed helicity of each nucleic acid oligomer and only for one binding mode of the Δ-isomer with $[CGCGAATTCGCG]_2$ was this not the case.

That the phenanthridinium moiety is an important determinant in defining the nature (CG *vs.* AT) and stereoselectivity (Δ *vs.* Λ) of the binding interaction was corroborated by certain control experiments. Circular dichroism difference spectral titrations, probing the binding of Δ- and Λ-$[Eu{\cdot}\mathbf{1}]^{3+}$ with each oligonucleotide were examined. These tripositive complexes—lacking the phenanthridinium moiety—revealed only very small changes (<5%) in molecular ellipticity. Separate examination of *N*-Me-phenanthridinium halide binding with $[(CG)_6]_2$ revealed a very weak *negative* induced CD band at 350 nm. Thus, the observed changes (Fig. 5–7) are a function of the helicity and structure of the complexes themselves and do not correspond to the additive effect of the separate components.

Luminescence studies

The fluorescence emission of the *N*-methylphenanthridinium group at 408 nm was monitored as a function of added oligonucleotide, following excitation at the isosbestic point, *i.e.* at *ca.* 378 nm for $[(CG)_6]_2$ and $[CGCGAATTCGCG]_2$ and at *ca.* 308 nm for $[(AT)_6]_2$. Significant quenching of the singlet excited state was observed, particularly for the former two oligonucleotides, presumably arising from a charge-transfer catalysed non-radiative deactivation process. This is likely to be promoted by the interaction of the electron-poor phenanthridinium group with the electron-rich base-pairs. The long-lived europium luminescence was also quenched and changes in Eu emission intensity echoed those observed in phenanthridinium

Table 2 Approximate intrinsic binding constants, *K*, and site sizes, *n′*, for complexes of $[(CG)_6]_2$ and $[(AT)_6]_L$[a] with Δ- and Λ-$[Ln{\cdot}\mathbf{4}]^{4+}$ (Ln = Eu or Yb; 298 K; 10 mM HEPES, pH 7.4, 10 mM NaCl)

Diastereoisomeric complex	$10^5 K/M^{-1}$ duplex^{-1}	n'/duplex^{-1}
Δ-$[Eu{\cdot}\mathbf{4}]^{4+}$–$[(CG)_6]_2$	87[b]	3.98
Λ-$[Eu{\cdot}\mathbf{4}]^{4+}$–$[(CG)_6]_2$	36	3.96
Δ-$[Eu{\cdot}\mathbf{4}]^{4+}$–$[(AT)_6]_2$	1.6[b,d]	1.52
Λ-$[Eu{\cdot}\mathbf{4}]^{4+}$–$[(AT)_6]_2$	8.0[d]	1.82
Λ-$[Yb{\cdot}\mathbf{4}]^{4+}$–$[(CG)_6]_2$	57	4.1
Δ-$[Yb{\cdot}\mathbf{4}]^{4+}$–$[(CG)_6]_2$	15	4.8
Λ-$[Yb{\cdot}\mathbf{4}]^{4+}$–$[(AT)_6]_2$	30	4.3
Δ-$[Yb{\cdot}\mathbf{4}]^{4+}$–$[(AT)_6]_2$	13	0.5

[a] Typically $[Ln{\cdot}\mathbf{4}]^{4+}$ was between 20 and 30 μM; the estimated binding value and site sizes *n′* showed some variation (±40%) with initial complex concentration; values represent the mean of up to three independent measurements. [b] An approximate non-linear least squares analysis of the variation of CD intensity *vs.* added Eu complex, assuming a 1 : 1 binding model, gave values for an apparent affinity constant of 10^6 M^{-1} for Δ-Eu/$[(CG)_6]_2$ and 10^5 M^{-1} for Δ-Eu/$[(AT)_6]_2$. A rather limited Job plot revealed discontinuities at 0.5 and 0.67 consistent with stepwise 1 : 1 and 2 : 1 complexation. [c] *N*-Ethylphenanthridinium iodide bound to $[(CG)_6]_2$ and $[(AT)_6]_2$ with affinities (sizes) of 6.8×10^5 (3.9) and $<0.5 \times 10^5$ M^{-1} duplex^{-1} respectively. In comparison, ethidium bromide was bound to $[(CG)_6]_2$ with an affinity of 31×10^5 (2.3). [d] At 25 μM added oligonucleotide, the I_o/I ratio was 54 for $[(CG)_6]_2$–Δ-Eu, 43 for $[(CG)_6]_2$–Λ-Eu, 1.8 for $[(AT)_6]_2$–Δ-Eu and 3.6 for $[(AT)_6]_2$–Λ-Eu.

fluorescence intensity. No change in the form of the europium emission spectrum and its CPL spectrum was observed on oligonucleotide binding.[31] In addition, the lifetime of the metal-based emission did not change significantly from its value of 0.55 ms, measured in the absence of added oligonucleotide. Such behaviour tends to negate the possibility of any quenching of the metal excited state by photoinduced electron transfer from the DNA bases. Moreover, the lack of change in the total and circularly polarised emission spectrum suggests that the europium complex retains an axially bound water[31] and that its local helicity is not perturbed much by the oligonucleotide interaction.[32]

The variation of phenanthridinium fluorescence emission intensity with added oligonucleotide was analysed using intrinsic methods. Both Scatchard and McGhee–von Hippel methods were used to analyse apparent (intrinsic) binding constants, *K*, and deduce site sizes per duplex, *n* (see ESI).[33] Such parameters are primarily dependent here upon the free energy of binding associated with the putative charge-transfer interaction of the electron-poor phenanthridinium moiety. Values are collated in Table 2 and revealed several trends. Firstly, for

Table 3 Apparent Stern–Volmer quenching constants for the effect of added NaI on phenanthridinium fluorescence in $[Ln\cdot\mathbf{4}]^{4+}$ bound to $[(CG)_6]_2$ and $[(AT)_6]_2$ (pH 7.4, 10 mM HEPES, 10 mM NaCl, λ_{em} 405 nm)

Diastereoisomeric complex	$10^{-3}K_{sv}^{-1}/M^{-1}$	$10^5 K/M^{-1}$ duplex^{-1}
Δ-$[Eu\cdot\mathbf{4}]^{4+}$–$[(CG)_6)]_2$	24	87
Λ-$[Eu\cdot\mathbf{4}]^{4+}$–$[(CG)_6]_2$	16	36
Δ-$[Yb\cdot\mathbf{4}]^{4+}$–$[(CG)_6]_2$	23	15
Λ-$[Yb\cdot\mathbf{4}]^{4+}$–$[(CG)_6]_2$	≈30 (107)[a]	57
Δ-$[Yb\cdot\mathbf{4}]^{4+}$–$[(AT)_6]_2$	16	13
Λ-$[Yb\cdot\mathbf{4}]^{4+}$–$[(AT)_6]_2$	22	30

[a] A clear discontinuity in the Stern–Volmer plot was observed in this case only beyond ≈30 mM added NaI; beyond this value the fluorescence was rather insensitive to increase in $[I^-]$.

the Eu series, binding was stronger with $[(CG)_6]_2$ compared to $[(AT)_6]_2$ by a factor of up to 50. The Δ-isomer bound slightly more strongly than the Λ in the former case, but the reverse was true in the latter case. With the Yb analogues, again binding of the Λ-isomer to $[(CG)_6]_2$ was slightly stronger than to $[(AT)_6]_2$, but the Λ-isomer was bound the more strongly in each case. Values obtained with $[CGCGAATTCGCG]_2$ for both Eu and Yb analogues (Δ or Λ) were rather similar (±80%). The apparent binding affinities were *ca.* 5×10^6 M^{-1} duplex^{-1} in each case.

The overall free energy of binding is likely to be made up from electrostatic and intercalative contributions. With such highly charged species, a predominantly electrostatic interaction is likely to be significantly reduced at higher ionic strength, while a predominantly intercalative binding mode is characterised by a less steep dependence on I.[34] The quenching of the fluorescence of Λ-$[Eu\cdot\mathbf{4}]^{4+}$ upon binding to $[(CG)_6]_2$ was studied at $I = 0.02$ (Na^{-1}), 0.06 and 0.11 M. No changes in absorbance spectral behaviour were noted and the estimated affinity constants decreased from $\log K = 6.55$ ($\log I = -1.7$), to $\log K = 6.36$ ($\log I = -1.22$) and $\log K = 5.84$ ($\log I = -0.96$). At the highest ionic strength, the use of added NaCl and NaOAc gave the same values of K. The modest change with ionic strength ($\log K$/$\log I$ plot has a slope of -0.9) suggested that an intercalative binding mode predominated, in this case.

Competitive quenching experiments

Halide ions quench the *N*-alkylphenanthridinium excited state effectively with the quenching efficiency following the ease of electron transfer: $I^- > Br^- > Cl^- >> F^-$.[20] Competition between charge transfer quenching by nucleobase interaction and quenching involving electron transfer from X^- may give useful comparative information. Reference points for such an analysis are the estimated Stern–Volmer quenching constants for $[Ln\cdot\mathbf{4}]^{4+}$ with I^-, Br^- and Cl^-.[20] Measurements were focused on I^- quenching as the K_{sv} value of 1.9 mM^{-1} for $[Eu\cdot\mathbf{4}]^{4+}$ meant that variations of ionic strength as $[I^-]$ increased would be minimised. Apparent Stern–Volmer quenching values were measured for selected complexes (Table 3). In each case, binding to the oligonucleotide increased the K_{sv}^{-1} value, by an order of magnitude or more *i.e.* the binding to the oligonucleotide shielded the phenanthridinium singlet state. Furthermore, the order of quenching inhibition followed the order of apparent stability constants for the binding interaction that had been determined earlier (Table 2). If nothing else, this behaviour lends support to this order of the estimated binding affinities.

Electrospray mass spectroscopic measurements

Electrospray ionisation mass spectrometry has been used to analyse oligonucleotides of up to 25–30 base pairs.[35,36] The stability of a given duplex under ESMS conditions is not only dependent on the base sequence[37] but also on the local solvation environment and the nature of the counterions. Ion clusters are commonly formed with Na^+ and Mg^{2+} giving rise to broad peak envelopes which reduce sensitivity and complicate spectral interpretation. For the parent oligonucleotides and their non-covalent adducts, the importance of determining the most effective sample preparation has been highlighted.[38] Although a quantitative analysis of relative spectral form and intensity in screening putative non-covalent adducts is considered not to be reliable, information on binding stoichiometry has been obtained that is in accord with NMR solution based methods.[39] Thus, the binding of the distamycin family of antibiotics to $[CGCGAATTCGCG]_2$ has been reported.[40]

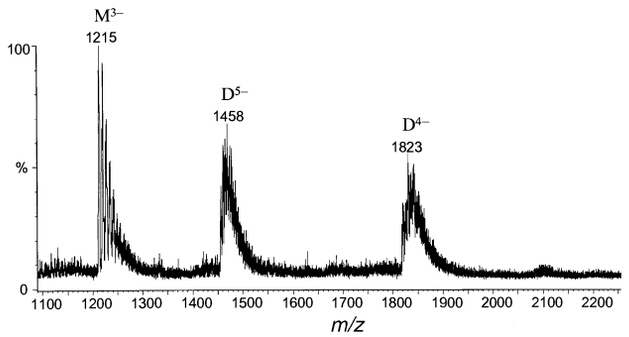

Fig. 8 Electrospray mass spectrum of $[CGCGAATTCGCG]_2$ (13 μM, 0.1 M NH_4OAc, 80 V) revealing the negatively charged monomeric (M) and duplex species (D).

It was found that in the presence of 0.1 M ammonium acetate solution, 10 μM solutions of $[CGCGAATTCGCG]_2$ (MW = 7292) gave rise to the most intense duplex (D) peaks when the instrument was operated in negative ion mode with an 80 V cone voltage, while examining the mass range from 200–2800. Under these conditions, the monomer $[M]^{3-}$ gave rise to the base peak at 1215, with $[D]^{5-}$ at 1458 (70%) $[D]^{4-}$ at 1823 (60%) and $[M]^{4-}$ at 912 (30%). Spectral assignment of self-complementary oligonucleotides is complicated by the fact that monomer ions of charge n have the same m/z value as duplex ions of charge $2n$. The sets of peaks are readily distinguished however. Expansion of the peak envelopes revealed a series of ammonium ion adduct peaks (*e.g.* $[M]^{3-}$, $[M + NH_4]^{3-}$, $[M + 2NH_4]^{3-}$ ⋯) which were separated by $18/2n$ for duplex ions and by $18/n$ for monomeric species. The peak envelope for a monomeric species revealed maximal intensity for the peak of lowest m/z (no NH_4 bound) whereas in the duplex series, the intensity of successive ammonium adducts rose to a maximum for two or three added ammonium ions before tailing off (Fig. 8).

Mass spectral analysis of the adducts with Δ- and Λ-$[Eu\cdot\mathbf{4}]^{4+}$ revealed little difference between the isomeric species and similar spectra were obtained with the Yb analogues. Spectra were analysed as a function of the mole ratio of the components and the most intense duplex–complex $[DC]^{n-}$, peaks and highest signal–noise spectra were obtained at relatively low oligo : Eu ratios (Table 4). The quintuply charged adduct comprising duplex plus two europium complexes was apparent for each ratio examined, from 1 : 2 to 1 : 8. The most intense peak observed was due to the 1 : 1 monomer–complex adduct as a triply charged ion (Fig. 9). No significant peaks were observed for europium adducts with a 3 : 1 ratio (complex : oligo).

With $[(CG)_6]_2$ (MW 7288), rather poorer signal–noise was evident in the negative ion ES spectra obtained, and the 'free' oligonucleotide revealed peaks at 1215 ($[M]^{3-}$, 80%), 1458 ($[D]^{5-}$, 50%), and 1822 ($[D]^{4-}$, 40%). For a 1 : 4 ratio of oligo : Δ-$[Eu\cdot\mathbf{4}]$, a relatively intense peak was observed at 1498 (90%), corresponding to $[DC_3]^{7-}$ in addition to a peak ascribed to duplex bound to two Eu complexes at m/z 1884 ($[DC_2]^{5-}$, 85%). In the analysis of $[(CG)_6]_2$ with the Δ- and Λ-$[Yb\cdot\mathbf{4}]$ complexes, evidence for a species corresponding to duplex bound to 3 lanthanide complexes was also obtained: peaks were observed at 2110 ($[DC_3]^{5-}$, 75%) and at 2637 ($[DC_3]^{4-}$, 15%), in addition

Table 4 Negative ion electrospray mass spectral analysis of adducts of Δ-[Eu·**4**]$^{4+}$ and $[CGCGAATTCGCG]_2$ (80 V, 0.1 M NH_4OAc, 10 μM oligonucleotide)[a]

Oligo : complex ratio	Base peak	Other major peaks (%)
1 : 2	$[MC]^{3-}$	$[M]^{3-}$ (60); $[DC_2]^{5-}$ (40); $[DC]^{5-}$ (20); $[D]^{5-}$ (20)
1 : 4	$[MC]^{3-}$	$[CNH_4]^-$ (55); $[M]^{3-}$ (40); $[DC_2]^{5-}$ (40); $[MC_2]^{3-}$ (20)
1 : 5	$[MC]^{3-}$	$[M]^{3-}$ (50); $[DC_2]^{5-}$ (30)

[a] M = monomer (3646); D = duplex (7292); C = [Eu·**4**] (1066).

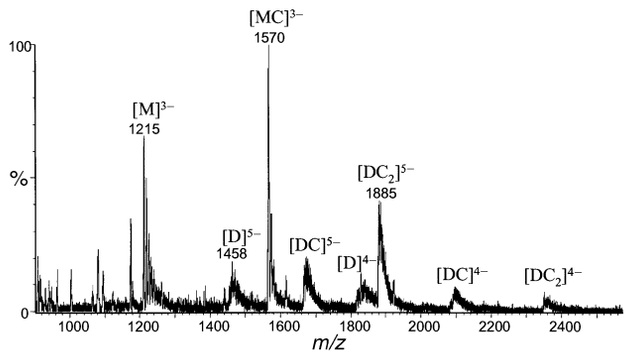

Fig. 9 Electrospray negative ion mass spectrum of a 1 : 2 mixture of $[CGCGAATTCGCG]_2$ and Δ-[Eu·**4**]$^{4+}$ (80 V, 10 μM oligo, 0.1 M NH_4OAc).

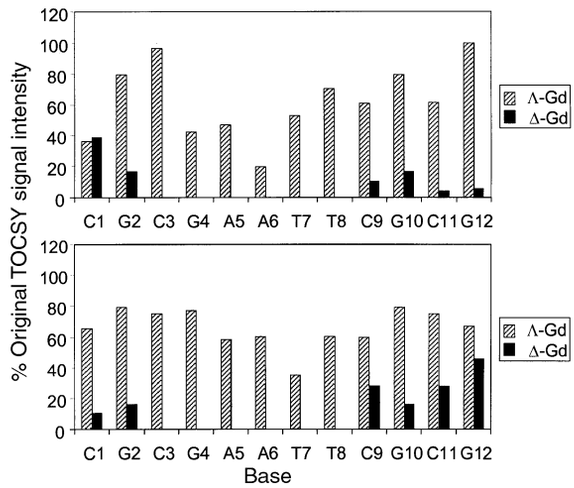

Fig. 10 Relative intensity of selected ^{1}H NMR TOCSY signals following addition of 1% Δ- or Λ-[Gd·**4**]$^{4+}$ (D_2O, pD 7.3, 0.7 mM oligonucleotide): upper: C-1′H/C-2′H; lower: C-1′H/C-2″H.

to the base peak at 1577 ($[MC]^{3-}$, 100%) and a strong peak at 1893 ($[DC_2]^{5-}$, 70%).

In summary, clear evidence for 3 : 1 complex : duplex stoichiometry was obtained with $[(CG)_6]_2$ but with $[CGCGAATTCGCG]_2$, only distinct 2 : 1 species were evident for both Eu and Yb complexes in a manner which was independent of complex chirality.

^{1}H NMR studies

The palindromic oligonucleotide [CGCGAATTCGCG] has been shown to exist preferentially as a duplex in aqueous solution at concentrations above 0.1 mM,[26,41,42] and it exhibits a simple monophasic duplex/manner transition (with no hair-pin) around 65–75 °C depending on salt concentration. ^{1}H and ^{31}P NMR analysis of the oligonucleotide (500 MHz, 0.7 mM oligo, 295 K) including standard TOCSY and NOESY pulse sequences were used to confirm the original spectral assignments.[26]‡ Incremental addition of Δ-(*SSS*)-[Eu·**4**]$^{4+}$ was monitored by ^{1}H NOESY methods: following addition of 0.1 equivalents of complex, the NOESY signal intensity ascribed to G4, A5, A6, T7, T8 and C9 had almost completely disappeared. Addition of up to one equivalent of the europium complex increased the degree of line-broadening in the one dimensional ^{1}H spectra but most cross-peaks remained strong (>50% of original intensity) for C-1, G-2, G-10, C-11 and G-12. No significant shifts in the ^{31}P spectra were apparent over this range, any small changes being masked by significant line-broadening.

As the introduction of the paramagnetic complex was giving rise to 'site-selective' relaxation of certain nucleobase resonances but no shift (possibly because free/bound complex exchange was not fast on the NMR time-scale), a parallel set of experiments was undertaken using the analogous Δ- and Λ-[Gd·**4**]$^{4+}$ complexes. ^{1}H NMR TOCSY and NOESY spectra were examined as a function of the gadolinium complex concentration. The paramagnetic relaxation rate enhancement at each proton is inversely proportional to $1/r^6$ (r = Gd–^{1}H separation) so that addition of low concentrations of the Gd complex induces relaxation (suppressing NOESY/TOCSY cross peaks) of protons nearest to the lanthanide complex binding site.[43] Addition of only 0.01 equivalents of Δ-[Gd·**4**]$^{4+}$ to the oligonucleotide led to loss of the TOCSY cross-peaks for G-4, A-5, A-6, T-7 and T-8. When 0.02 equivalents were added some signals in C-9 also began to be lost, while even after addition of 0.1 and 0.25 equivalents, residual signals for C-1, C-3, C-9 and C-11 were clearly evident. The parallel experiment using Λ-[Gd·**4**]$^{4+}$ showed that the onset of the changes only occurred beyond addition of 0.02 equivalents. Indeed only after addition of 0.05 equivalents of Λ-[Gd·**4**]$^{4+}$ was the effect as marked as for addition of 0.01 equivalents of the Δ-enantiomer. Again loss of signal intensity occurred first at G-4, A-5, A-6, T-7 and T-8 and C-9 (Fig. 10). Parallel regio-selectivity was apparent by monitoring changes in NOESY spectra, but the effect now was only apparent at higher concentrations of added complex.

This behaviour—clearly indicating that the highest affinity binding site occurs in the AATT region or at the junction with the G-4 C-9 base pair—provides complementary information to that obtained by CD, probing oligo helicity, and to absorbance and luminescence spectroscopy highlighting the phenanthridinium environment.

Conclusions

The Δ- and Λ-enantiomers of [Ln·**4**]$^{4+}$ exhibit very distinctive behaviour in their binding to the self-complementary oligonucleotides examined. Absorption and mass spectroscopic studies suggest that the limiting stoichiometry of binding is 3 : 1 for $[(CG)_6]_2$, *i.e.* one complex per four base pairs in the duplex. With $[CGCGAATTCGCG]_2$ the stoichiometry appeared to be not more than 2 : 1; if higher complex : oligo ratios are present their formation constants are likely to be significantly less than β_{ML} and β_{ML_2}. Analyses of luminescence quenching using the McGhee–von Hippel approximation seemed to overestimate the number of bound complexes per oligonucleotide—this

‡ Details of the effect of added [Ln·**4**]$^{4+}$ (Ln = Eu, Gd) on the observed TOCSY or NOESY cross peaks for the assigned resonances of each base are given in the ESI.

could be related to the presence of a non-specific binding mode in which an electrostatically bound complex (*e.g.* at the surface) is quenched by the more electron-rich base-pairs.[44] Apparent binding affinities to $[(CG)_6]_2$ were up to 50 times greater than to $[(AT)_6]_2$ and the small reduction with increasing added salt concentration and the inhibition of competitive I^- quenching suggested that the *N*-alkylphenanthridinium moiety was intercalating between (CG) base-pairs. This hypothesis was corroborated by the marked hypochromism, red-shift and long-wavelength tail observed in absorbance spectral titrations, and the positive CD induced into the phenanthridinium (long axis) chromophore which was the same sign for Δ- and Λ-Eu complexes with $[(CG)_6]_2$ and $[(AT)_6]_2$.

Complexes of the strongly helical Δ- and Λ-lanthanide complexes are diastereoisomeric and possess different free energies. Modest but distinctive selectivity was observed in binding affinity (Table 2) and the complexes bound with little change to the local metal ion helicity (CPL). Differences were dependent upon the nature of the oligonucleotide *and* the complex lanthanide *e.g.* Eu *vs*. Yb. These differences were also revealed by the distinctive difference CD spectra that characterised each diastereoisomeric complex. In this respect, the form of the CD changes in the Δ-/Λ-$[Eu\cdot\mathbf{4}]^{4+}$ complexes with $[CGCGAATTCGCG]_2$ resembled those observed with $[(AT)_6]_2$ only for the Λ-isomer. Taken together with the ^{1}H NMR analysis showing clearly that the highest affinity binding site was close to the AATT region of the Drew–Dickerson dodecamer, the complex appears to bind to this region with a significant change in local oligonucleotide helicity.

Overall this behaviour illustrates the considerable scope that exists for probing oligonucleotide binding using well-defined chiral lanthanide complexes. Obviously the complexity of the system requires a multidisciplinary approach to be adopted. In the following paper, we discuss our preliminary work examining the interaction with selected polymeric nucleic acids.

Experimental

The lanthanide complexes used in this study were prepared as described elsewhere.[20] HPLC-purified self-complementary oligonucleotides were purchased from Oswel Ltd. (Southampton, UK) and were used as received. Gold-label ammonium acetate was purchased from Fluka for use in ESMS studies. For absorbance and luminescence titrations, the oligonucleotide solution (typically 0.1 to 0.3 mM per monomer) was titrated in 10 or 20 μL increments into a fixed concentration of complex in 1 cm quartz cells. Complex concentration (2.5 mL, $\varepsilon = 8000$ M^{-1} cm^{-1} at 320 nm) was adjusted to give an absorbance of *ca.* 0.2 (320 nm) in a pH 7.4 HEPES buffer containing 10 mM NaCl, unless otherwise stated. Occasionally a minimal volume of methanol was added (<5% total volume) to aid complex solubilisation. Luminescence spectra were recorded at 295 K using an Instruments S.A. Fluorolog-3 spectrometer with excitation wavelengths of 378 or 304 nm, using a 375 nm cut-off filter as needed, with excitation and emission slits set at 1 and 1.5 nm respectively. Points were recorded at 1 nm intervals with an integration time of 0.5 s. For Eu emission spectra, corrected spectra were obtained, allowing for the response characteristics of the Hamamatsu R928 in the range 650–750 nm. Absorbance spectra were measured using a Unicam UV2-100 spectrometer. Details of other luminescence and CPL instrumentation are given elsewhere.[14,15,20,24]

For the difference CD studies, the complex solution was titrated into a fixed concentration of oligonucleotide (typically 3 μM in monomer) contained in 1 cm quartz cells prepared by dilution of stock solutions with salted HEPES buffer. The complex solution (0.16 mM) was added to the oligonucleotide solution in 20 μL aliquots. Spectra were recorded in 4 successive scans using a JASCO J-810 spectropolarimeter at 295 K and the spectrum due to the aqueous buffer subtracted, followed by subtraction of the complex at the same corrected concentration, allowing for the small dilution effects.

For mass spectral studies, stock solutions of oligonucleotide (0.13 mM) were prepared in 0.1 M NH_4OAc. Solutions were heated to 70 °C for 15 minutes and were allowed to cool overnight. Prior to mass spectral analysis, solutions were diluted by addition of Purite water to give a concentration of *ca.* 15–20 μM ($duplex^{-1}$) in 200 μL total sample volume with varying ratios of oligo : complex (1 : 2 to 1 : 8). Mass spectra were recorded in negative ion mode using a Fisons Platform II spectrometer using a 10 μL loop with 10 μL min^{-1} flow rate and an ion source temperature of 60 °C. Cone voltages of 60, 80 and 100 V were investigated.

^{1}H (499.824 MHz) and ^{31}P (202.332 MHz) NMR spectra were recorded using a Varian Unity 500 instrument (11.8 T) at 295 K in D_2O (99.8% D from Sigma Chem. Co.) solutions. For the ^{1}H NMR spectra, the HOD signal was used as internal reference, set at δ 4.75 ppm and was suppressed by a presaturation pulse. TOCSY and NOESY spectra were acquired employing standard VNMR software. Oligonucleotide concentrations of 0.725 mM in degassed D_2O (600 μL) were employed and the successive additions of D_2O solutions containing 0.01, 0.02, 0.05, 0.1 and 0.25 molar equivalents of Δ- or Λ-$[Ln\cdot\mathbf{4}]^{4+}$ were carried out such that the total added volume added did not exceed 50 μL. The pH of the solutions was adjusted with DCl and CO_2-free NaOD (from Sigma Chem. Co.) using a Crison MicropH 2002 pH-meter with an Ingold 405-M5 combined electrode.

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