Excited state behaviour of substituted dipyridophenazine Cr(III) complexes in the presence of nucleic acids

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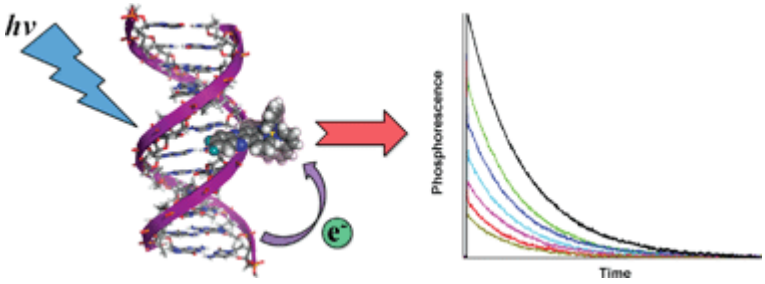
The photophysics and photochemistry of $[Cr(phen)_2(dppz)]^{3+}$ and its 11,12-substituted derivatives $[Cr(phen)_2(X_2dppz)]^{3+}$ {X = Me or F} have been studied in the presence of purine nucleotides or DNA using steady state and time-resolved absorption and luminescence spectroscopy. 5′-Adenosine monophosphate (5′-AMP) shows only a weak interaction with the excited states of each complex. By contrast they are efficiently quenched by 5′-guanosine monophosphate (5′-GMP), consistent with photo-induced electron transfer. Laser flash photolysis spectroscopy in the presence of 5′-GMP suggests that both forward and back electron-transfers are rapid. All complexes also display a strong affinity for DNA and evidence for both static and dynamic quenching mechanisms is provided.

Graphical Abstract:



Introduction

Metal heteroleptic complexes continue to attract attention because of their extraordinary excited state photophysical propertie which can be potentially exploited for applications such as solar energy converters and optoelectronics,[1–3] molecular light switches[4–6] and phototherapeutic agents.[7–9] d6 complexes such as those of ruthenium(II) or rhenium(I) have been extensively investigated as an advantage of these compounds is that their photochemical and photophysical properties can be readily tuned. For instance it is often possible to select a complex with an excited state having an appropriate redox potential or one which shows exquisite sensitivity to its environment.[10–18] These properties are particularly valuable for potential therapeutic and diagnostic applications involving the interaction of such complexes with nucleic acids or proteins.[19–20] For example, directed photooxidative damage of nucleic acids opens the possibility of designing a new class of selective antitumour drugs.[21–23]

Complexes with the ligand dipyrido[3,2-a:2¢,3¢-c]phenazine (dppz) are known to interact with double-stranded DNA by intercalation and the ruthenium complexes have been particularly well-studied.[24–27] More recently, chromium(III) complexes have also received attention, as these complexes (in which the central metal ion has a d3 configuration) exhibit long-lived, spectrally narrow room temperature phosphorescence and have strong excited state oxidizing power.[28–29] They are also water soluble and relatively non-labile, properties conducive to their application in biological systems. It should be emphasised that in contrast to the MLCT state of the analogous ruthenium complexes [26,30–31] the lowest excited state of the $[Cr(phen)_2(X_2dppz)]^{3+}$ is metalcentred and luminescent in aqueous solution. On binding to DNA the emission of the chromium complex is quenched, whereas that of the ruthenium is switched on. In an effort to modulate DNA binding constants and spectroscopic properties we have recently synthesised a family of chromium complexes $[Cr(phen)_2(X_2dppz)]^{3+}$ {X = H, Me, or F} and resolved each into their enantiomers.[32] In this previous study we showed that despite the energy of the metal centered excited state being unaffected by the substitution of the dppz ligand, the excited state of the difluoro species is

a significantly stronger oxidising agent than both the unsubstituted and the dimethyl species. We also provided evidence that the new complexes bind to calf thymus DNA (CT-DNA) via intercalation and that the dimethyl derivative has the higher binding affinity. The main aims of the current paper are to more fully understand the photophysical properties of these complexes and to reveal the role of electron transfer processes in controlling their photochemical behaviour in the presence of nucleic acids. For this purpose we have used nanosecond transient luminescence and absorption methods. As the lowest lying excited state is expected to be much longer lived than that of the more commonly studied ruthenium or rhenium complexes, it may be possible that the nature of the photoprocesses may be different. Furthermore, as this excited state is expected to be a doublet state, it is of particular interest to ascertain whether this feature will affect the rates of the forward and reverse electron transfer processes. (The role of the spin state in determining the rates of electron transfer reactions has been previously addressed by Verhoeven.[33])

Experimental

The chromium complexes were synthesised using previously reported methods.[32]
Briefly, $[Cr(phen)_2(CF_3SO_3)_2](CF_3SO_3)$wasprepared by reacting $[Cr(phen)_2Cl_2]Cl$ with trifluoromethanesulfonic acid under a stream of nitrogen. The pink triflato complex was precipitated out of solution and purified by repeated centrifugation. Complexes of the form $[Cr(phen)_2(X_2dppz)](CF_3SO_3)_3$ were synthesised by refluxing the triflato complex with a small excess of the X_2dppz ligand of choice for approximately 18 h. The yellow product was collected by suction filtration and purified by lipophilic and/or cation-exchange chromatography. Other chemicals used in this study – calf thymus DNA (CT-DNA), mononucleotides (5′-GMP, 5′-AMP), and $Na^2HPO^4{\cdot}12H^2O$, $NaH^2PO^4{\cdot}2H^2O$ (for phosphate buffer preparation) – were purchased from Sigma Aldrich and used without further purification. Concentrations of CT-DNA, 5′-GMP and 5′-AMP were determined spectrophotometrically using molar extinction coefficients of ε_{260} = 6600, 11800 and 15400 M^{-1} cm^{-1}, respectively. Samples for oxygen quenching studies were deoxygenated by nitrogen purging for approximately one hour in the dark.

Instrumentation

Absorption spectra were recorded on a Varian Cary 50 or Shimadzu 2401 UV/vis spectrometer. Emission spectra data were collected on a Perkin Elmer L55 (used in the phosphorescence mode) in air-saturated solutions at room temperature. The timeresolved emission and transient absorption measurements were performed using a commercially available nanosecond laser flash photolysis spectrometer manufactured by Edinburgh Instruments (model LP920). An R928 photomultiplier and Andor ICCD camera (model DH501-18F-13) were used for signal detection in the kinetic and spectroscopic modes, respectively. A xenon arc lamp (450 W) working in pulsed mode was used as a probe in transient absorption spectroscopy measurements. The samples were excited using the 308 nm line (pulse duration of ca. 20 ns) of a GAM excimer laser (model EX100). Samples were frequently checked for decomposition using standard UV/vis spectroscopy.

Results and Discussion

Absorption and emission spectra

The molecular structure and corresponding absorption spectra of the synthesised complexes are shown in Fig. 1. The electronic spectra exhibit strong ligand-centred absorption bands around 270–290 nm and dipyridophenazine p→p* transitions in the 350–400 nm region. The low intensity shoulders (centred at about 420 nm) may be attributed to d–d transitions. The positions of absorption band maxima are given in ESI Table 1. It is clear that while the positions of absorption bands for unsubstitiuted and difluoro species are similar, the dimethyl substitution causes a significant red shift of the bands particularly in the longer wavelength region.

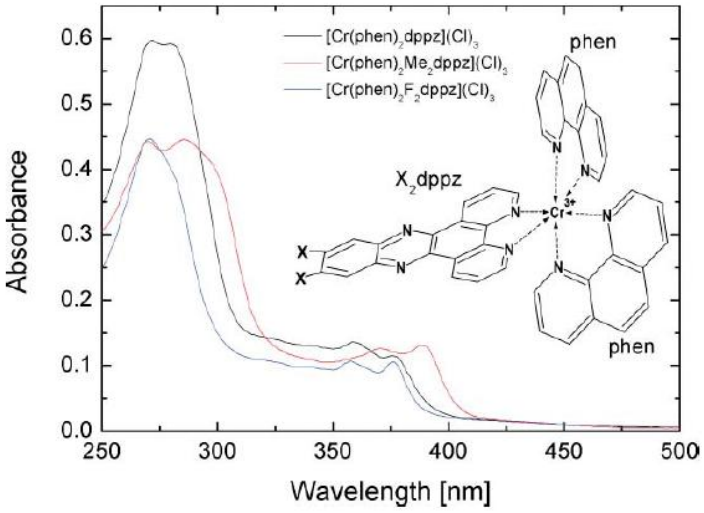


Fig. 1 Absorption spectra of 10 mM aqueous solutions of $[Cr(phen)_2(X_2dppz)](Cl)_3$ {X = H, Me, or F}.

One of the most important features of the synthesised complexes is their relatively strong room temperature emission in air-saturated aqueous solution. This behaviour contrasts with their counterparts $[Ru(phen)_2(dppz)]^{2+}$ [12,18] and $[Re(CO)_3(F_2dppz)(py)]+$.[10] The radiative transitions occur from the two lowest-lying doublet $2E_g$ and $2T_{1g}$ excited states to $4A_{2g}$ ground state (Oh symmetry) [34–35] giving well-resolved phosphorescence bands centred at about 730 nm and 698 nm, respectively (see Fig. 2). The shoulder at the red-side edge of the 730 nm band is probably due to radiative transitions to the higher-lying vibrational levels of the ground state. Phosphorescence spectra of all complexes were analyzed by means of least square multiple peaks nonlinear regression using the Origin 7.5 software package (Levenberg–Marquardt algorithm). Both the envelope and the background (constants) were fitted simultaneously.[36] The best fit in each case was obtained using a superposition of one Gaussian and three Lorentzian functions. The fit of the emission spectrum of $[Cr(phen)_2(F_2dppz)](CF_3SO_3)_3$ is presented in Fig. 2. The Gaussian nature of the peak at about 700nm–which is different than the others – is probably caused by the overlapping of more than one transition in this spectral region due to vibronic repetition. Modelling of the emission of analogous dppz andMe2dppz species gives rise to nearly identical line shapes and fitting parameters (area, full width at half maximum-FWHM, peaks center) (SI Table S2‡). This confirms the metal-centred nature of the excited state.

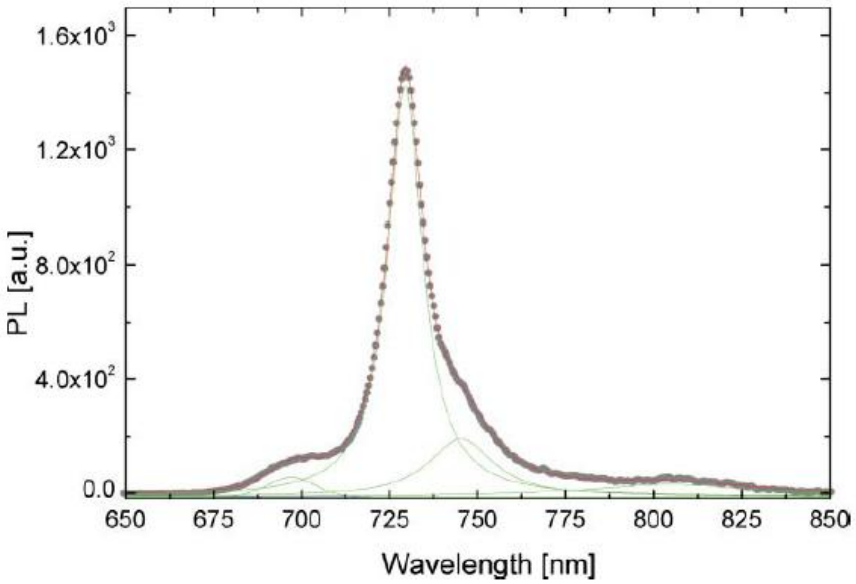


Fig. 2 Multipeak fitting of emission spectrum of an air saturated 100 mM phosphate buffer solution (pH = 7.4) of 45 mM $[Cr(phen)_2(F_2dppz)](CF_3SO_3)_3$ (λ_{exc} = 308 nm). The lineshape and resulting fitting parameters are collected in Table S2 (ESI‡).

Transient emission measurements

Phosphorescence decay monitored following excitation with a pulsed 308 nm excimer laser was found to follow excellent first order kinetics for the triflate and chloride salts of the three complexes (the example of the F_2dppz complex is depicted in SI Fig. S1). The phosphorescence decay rate was also found to be wavelength independent, as expected if the emitting doublet excited states $2E_g$ and $2T_1$ are thermally equilibrated. In confirmation the shape and position of time-resolved emission spectra (TRES) recorded using an ICCD camera are invariant with time. (A representative set of TRES for $[Cr(phen)_2(F_2dppz)](CF_3SO_3)_3$ is presented in SI Fig. S2).

	Aerated	N_2 purged		∞ dilution	
$[Cr(phen)_2(X_2dppz)]^{3+}$ (10 μM)	τ_a/μs	τ_{np}/μs	$k_q/\times10^7$ M^{-1} s^{-1}	τ_∞/μs	$k_g/\times10^7$ M^{-1} s^{-1}
$[X = H](CF_3SO_3)_3$	75 ± 5	177 ± 11	2.7 ± 0.5	84 ± 9	3.7 ± 0.9
$[X = Me](CF_3SO_3)_3$	67 ± 4	192 ± 12	3.4 ± 0.5	72 ± 2	2.6 ± 0.4
$[X = F](CF_3SO_3)_3$	68 ± 4	153 ± 8	2.9 ± 0.5	69 ± 3	0.7 ± 0.1
$[X = H](Cl)_3$	65 ± 5	128 ± 12	2.7 ± 0.7		
$[X = Me](Cl)_3$	60 ± 4	154 ± 13	3.6 ± 0.6		
$[X = F](Cl)_3$	59 ± 6	145 ± 15	3.5 ± 0.9		

Table 1 Emission lifetime values for aerated and N_2 purged 10 mM aqueous solutions of $[Cr(phen)_2(X_2dppz)][(Cl)_3$ or $(CF_3SO_3)_3]$ and corresponding quenching rate constants (*l*em = 730 nm), as well as emission lifetimes extrapolated to infinite dilution and the associated self-quenching constants.

The phosphorescence lifetimes for low concentration (10 mM) of aerated aqueous solutions of the triflate and chloride salts of all three complexes are collected in Table 1. In general the lifetimes of all complexes are relatively long (>59 ms) and only slightly dependent on the dppz substituent X. They more than double when the solution is nitrogen flushed, consistent with energy transfer quenching of the excited state by dissolved molecular oxygen ($3O_2$), as shown previously for other Cr(III) complexes.[37–38] Rate constants for quenching by oxygen (kq)[39] calculated using an oxygen concentration of 288 mM (20 °C) are collected in Table 1.[40] The lifetimes in both aerated and degassed solution are slightly longer for the triflate salt, possibly indicative of weak quenching by chloride as has been suggested by Neshvad et al.[41]

The emission lifetime of each complex in aerated solution was found to decrease when its concentration was increased. Fig. 3 shows the reciprocal lifetime for the triflate salts of each of the complexes as a function of their concentration in air-saturated solution. From the slope of the linear plots the rate constants for the quenching of the excited states by its ground states (kg) and the lifetimes at infinite substrate dilution (t_∞) have been evaluated (Table 1).[42] It may be observed that the self-quenching rate constant for the difluoro-complex is significantly less than that for the dimethyl and parent complexes. The self-quenching by $[Cr(phen)_2(X_2dppz)]^{3+}$ may be compared to that previously observed for $[Cr(bpy)_3]^{3+}$ and $[Cr(phen)_3]^{3+}$ whose excited state lifetime were found to be self-quenched in HCl and NaCl medium but not in water. In that case the mechanism of quenching was proposed to occur upon collision of Cl^--associated ground and excited state.[43–44]

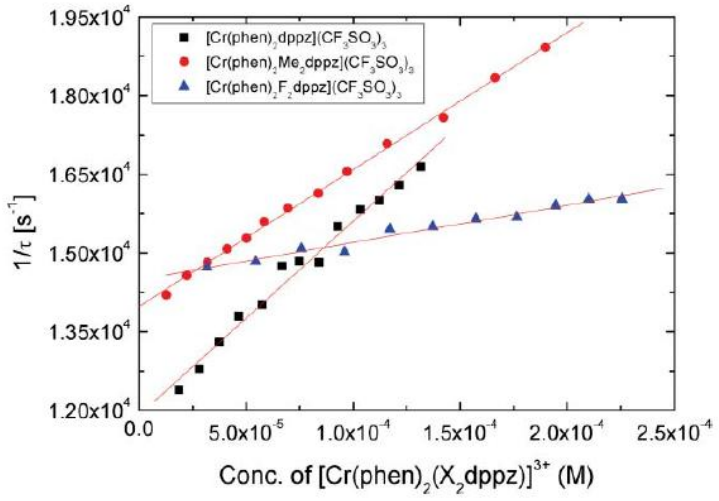


Fig. 3 Plot of 1/τ as a function of [substrate] for an air-saturated aqueous solution of $[Cr(phen)_2(X_2dppz)](CF_3SO_3)_3$ {X = H, Me, or F} (λ_{exc} =308 nm).

Emission quenching by mononucleotides and DNA

As previously reported, the phosphorescence of all three complexes is strongly quenched in the presence of double stranded DNA.[32,45] Since the excited state oxidizing power of all three complexes are relatively high (1.52 V, 1.49 V and 1.62 V versus NHE for X = H, Me and F, respectively) it was proposed that electron transfer is the most likely mechanism for the observed quenching. In order to obtain support for this hypothesis we have examined the behaviour of the excited states of all three complexes with guanosine-5′-monophosphate (GMP), adenosine- 5¢-monophosphate (AMP) and calf thymus deoxyribonucleic acid (CT-DNA).

Guanine is the most readily oxidized nucleobase and indeed the oxidizing power of all three complexes is far more than 1.29 V (vs NHE, pH 7) required for the direct one-electron oxidation of guanine.[46–47] Fig. 4(a) shows the lifetime emission data of $[Cr(phen)_2(F_2dppz)](CF_3SO_3)_3$ in the presence of increasing concentrations of GMP (see SI Fig. S3‡ for the steady state emission intensity quenching). It is evident that both the steady state phosphorescence and emission lifetime are strongly quenched by GMP. Moreover, the initial amplitude of these phosphorescence decay curves remains almost constant with increasing concentrations of GMP {Fig. 4(a)}. Such behaviour is characteristic of dynamic quenching.[48] It is also evident that for emission quenching by GMP {Fig. 5(a)} the lifetime and steady state Stern–Volmer plots deviate from each other only slightly, indicating that the quenching proceeds predominantly by a dynamic process.

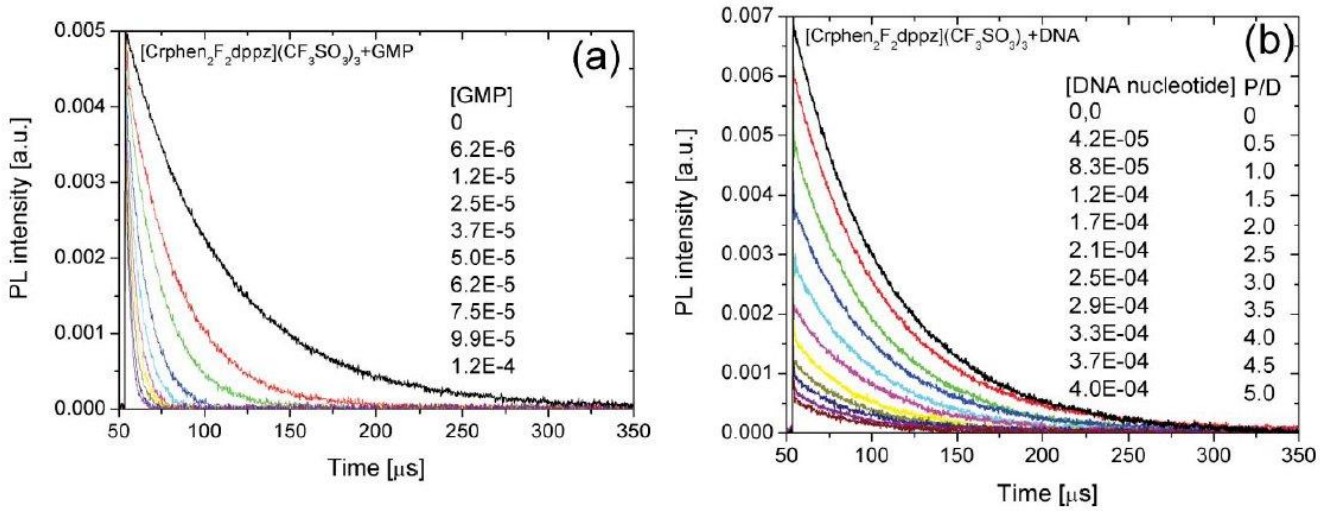


Fig. 4 Comparison of the phosphorescence lifetime quenching of an air saturated 100 mM phosphate buffer (pH = 7.4) solution of $[Cr(phen)_2(F_2dppz)](CF_3SO_3)_3$ in the presence of increasing concentration of (a) GMP, [Cr] = 45 mM and (b) CT-DNA, [Cr] = 80 mM. P/D = nucleotide/Cr, λ_{exc} =308 nm.

The analogous dppz andMe_2dppz complexes exhibit similar behaviour (see SI Fig. S4 and S5, respectively). The derived rate constants for each of the complexes is similar, in the range 2.3–2.8 × 10^9 M^{-1} s^{-1} (Table 2). The slight deviation of the intensity and lifetime Stern–Volmer plots may indicate a small amount of static quenching or possibly could be due to some photoaquation during the titration.[49] (The pH, temperature, and solvent dependence of the photoaquation quantum yield of these Cr(III) complexes will be the subject of a forthcoming publication.) The observed k_q values for each of the complexes are close to diffusion controlled values and to those reported for GMP quenching of the emission of $[Cr(phen)_3]^{3+}$ [29] and by others for $[Cr(phen)_2(dppz)]^{3+}$.[45]

	GMP	AMP	CT DNA
$[Cr(phen)_2(X_2dppz)](CF_3SO_3)_3$	$k_q/\times10^9$ M^{-1} s^{-1}	$k_q/\times10^6$ M^{-1} s^{-1}	$k_q/\times10^7$ M^{-1} s^{-1}
X = H	2.3 ± 0.3	7 ± 1	1.8 ± 0.5
X = Me	2.7 ± 0.3	7 ± 1	1.3 ± 0.6
X = F	2.8 ± 0.4	5.7 ± 0.9	2.8 ± 0.5

Table 2 Bimolecular quenching rate constants for emission lifetime quenching of $[Cr(phen)_2(X_2dppz)](CF_3SO_3)_3$ (X = H, Me, F) in the presence of GMP, AMP and CT-DNA

By contrast to the behaviour ofGMP, itwas found that emission quenching by AMP is very inefficient for each of the complexes (Fig. 5(a) and SI Fig. S6, S7 and S8‡). The fact that the derived rate constants are more than two orders of magnitude less than that for GMP can be attributed to the higher thermodynamic driving forces required to oxidize AMP.

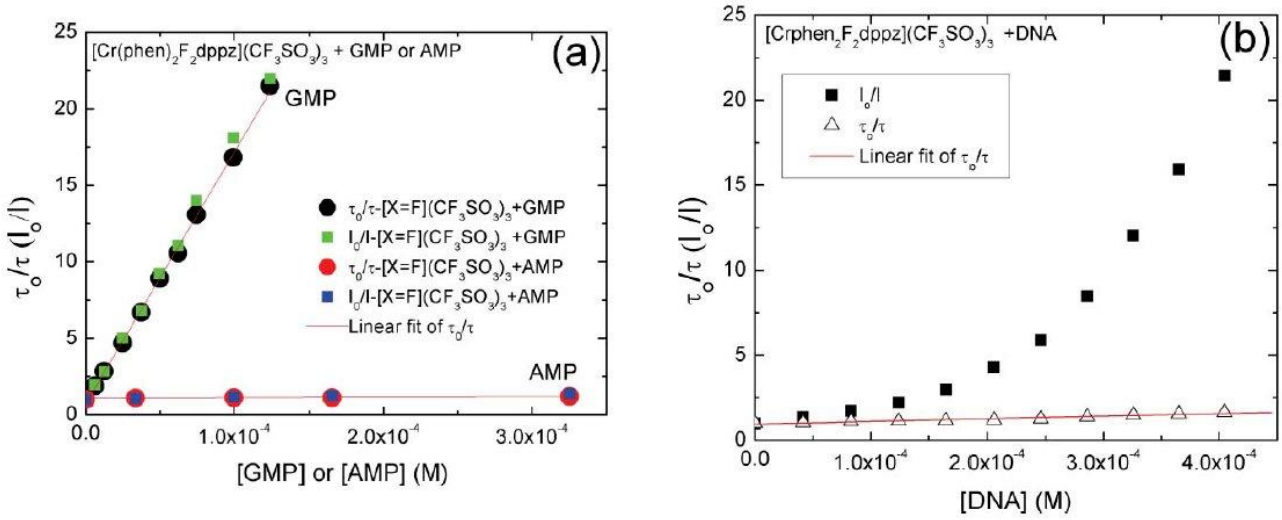


Fig. 5 Steady state and lifetime Stern–Volmer plots for emission quenching (at 730 nm) of an air saturated 100 mM phosphate buffer (pH = 7.4) solution of $[Cr(phen)_2(F_2dppz)](CF_3SO_3)_3$ in the presence of increasing concentration of (a) GMP, [Cr] = 45 mM; AMP, [Cr] = 36 mM and (b) CT-DNA, [Cr] = 80 mM (λ_{exc} =308 nm).

The Cr(III) dppz complexes are each strongly quenched in the presence of CT-DNA. However, in contrast to the GMP titration the initial phosphorescence amplitude is dramatically attenuated in the presence of DNA {Fig. 4(b)}. Moreover, the steady state Stern–Volmer plot shows an upward curvature consistent with static quenching {Fig. 5(b) and SI Fig. S9 and S10‡}. It is noteworthy that phosphorescence decays follows monoexponential kinetics even in the presence of DNA. This may be explained by assuming that the only complex which is emitting is that which is free in solution and not bound to DNA. The much smaller derived value for kq compared to that found with GMP (see Table 2) is consistent with this being the rate for the quenching of free molecule by the polynucleotide. Since the other mononucleotides (thymine and cytosine) have significantly higher oxidation potentials than AMP (and of course GMP), they are not expected to quench the Cr(III) complex excited state. We can therefore conclude that it is guanine bases in the DNA which act as efficient quenchers. Steady state emission experiments[32] show that the phosphorescence is more than 97% quenched at P/D > 12 for all complexes, consistent with a very low quantum yield of emission for bound complexes. However, only 42% of CTDNA base pairs contain guanine[37] so that one might expect to a first approximation that 34% of the intercalative binding sites would consist of only AT base-pairs and 67% have at least one GC base-pair. The near-total quenching upon binding to DNA may be explained in a number of ways: (a) that the complex binds preferentially to guanine-containing sites; (b) that the complex excited state being long-lived can leave a particular binding site and relocate within its lifetime; (c) that the oxidation potential of adenine is lowered dramatically upon base-pairing;[46] or (d) that quenching can occur through guanines in the DNA which are not immediately adjacent to the binding site of the complex.[50] These matters may be partly resolved by studies with synthetic polynucleotides such as $[poly(dG\text{-}dC)]_2$ and $[poly(dA\text{-}dT)]_2$ which are planned.

Transient Absorption Spectroscopy

To further probe the mechanism of the interaction of the [Cr(phen)2(X2dppz)]3+ complexes with nucleic acids we next used nanosecond laser flash photolysis. This technique should not only provide further information about the long-lived excited state but also allow the identification of any transient photoproducts, as was previously successfully achieved with ruthenium tetraazaphenanthrene complexes.11,51,52 In our laser flash spectrometer transient spectra are most conveniently recorded

using an ICCD camera, which allows one to reduce significantly the number of laser shots required to obtain the whole spectrum and hence minimise any problems due to possible decomposition due to photoaquation of Cr(III) complexes. A comparison of the excited state absorption spectra of aqueous solutions of all three complexes recorded using the ICCD camera over the initial 3 ms is presented in Fig. 6. They each exhibit a broad band at about 500 nm followed by a broad structureless shoulder extending out beyond 700 nm. There is also a positive absorption feature below 400 nm for both the parent and substituted species. However the exact positions of these short wavelength transient absorption bands remain uncertain due to lower sensitivity of the detector and strong overlapping of the transient and bleach in this spectral region.

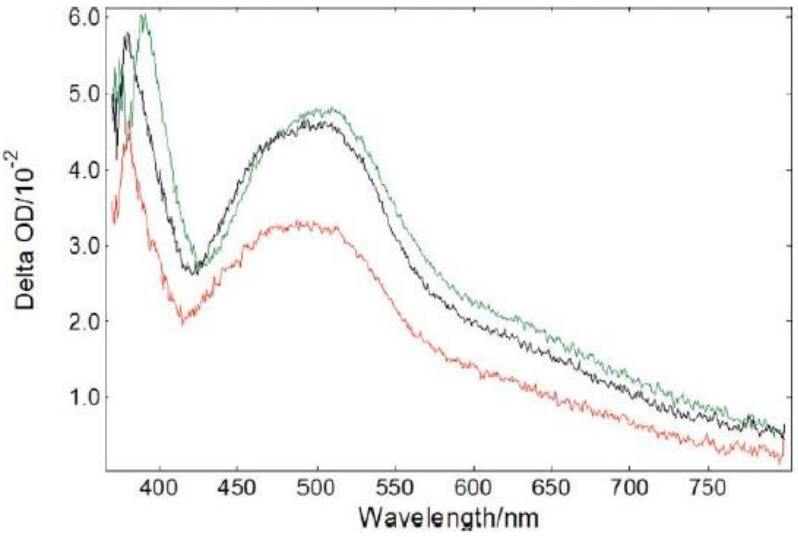


Fig. 6 Excited state absorption spectra recorded over the initial 3 ms (5 laser shots per spectrum) of 50 mM aerated aqueous solutions of $[Cr(phen)_2(X_2dppz)](Cl)_3$, X = H (black), Me (green), or F (red).

Time-resolved absorption spectra (TRAS) were recorded at different pump–probe delay times (for example, see SI Fig. S11‡ for the spectra of the Me_2dppz complex). For each complex it was found that the shape of the excited state absorption spectra did not depend on pump–probe delays and the decay of absorption bands is quite uniform. Decay kinetics were most conveniently obtained by measuring the absorbance changes at selected wavelengths. The signal decayed completely to the baseline obeying first order kinetics and the derived rate constant was wavelength independent. Lifetimes obtained are the same as those for the emission (see, for example, ESI Fig. S12 and S13 for representative decays of the Me_2dppz and dppz complexes, respectively).This confirms that the transient absorption is arising from the luminescent doublet states. The transient spectra are quite similar to that obtained from other $[Cr(diimine)_3]^{3+}$ and the observed transitions may be interpreted in terms of bothmetal-centered and charge-transfer doublet–doublet transitions.[43,53] The small red shift of the excited state absorption bands of the dimethyl-substituted complex (Fig. 6) is consistent with this interpretation.

Transient Absorption Studies in the presence of 5′-GMP

Transient absorption studies in the presence of 5′-GMP. Given that the excited state absorbs strongly in the visible spectral region, it should be possible to obtain evidence to support our photoinduced electron transfer hypothesis if the transient photoproducts are sufficiently long-lived. By analogy with the approach previously taken with ruthenium tetraazaphenanthrene complexes,[51–52] the transient absorption spectra (complemented by absorption decay kinetics) of $[Cr(phen)_2(dppz)](Cl)_3$ in the presence of GMP were examined. The excited state absorption spectra of $[Cr(phen)_2(dppz)](Cl)_3$ in the presence of GMP are shown in Fig. 7. The only signal shown can be attributed to the excited state of the complex and there is no evidence for extra transient bands which could be putatively associated with electron transfer products (oxidized GMP or reduced metal complex). The signal decay kinetics are fully consistent with this interpretation (see inset of Fig. 7). Similarly no evidence was seen for electron transfer products for the complex in the presence of CT-DNA (data not shown). The lack of products indicate that photoinduced electron transfer from guanine to the metal complex is followed by a rapid back electron transfer, all on a short timescale. It is intriguing to compare these results

obtained with the Cr(III) complexes with those from$[Ru(TAP)_2(dppz)]^{2+}$ where evidence for photoreduction of the metal complex and oxidation of the GMP was obtained. In the case of $[Ru(TAP)_2(dppz)]^{2+}$ the excited state is a triplet MLCT state with the electron located on the TAP ligand, whereas for $[Cr(phen)_2(dppz)]^{3+}$ the species is a doublet metal-centred state. It seems therefore unlikely that the spin state is responsible for the difference in reactivity. The Cr(III) complexes' excited states are all somewhat more oxidising (1.52 V, 1.49 V and 1.62 V versus NHE for X = H, Me and F, respectively) than that of $[Ru(TAP)_2(dppz)]^{2+}$ (1.44 V vs. NHE) and the quenching rate constants are slightly larger ($2.3–2.8 \pm 10^9$ M^{-1} s^{-1}) than that for the ruthenium complex 1.7 ± 10^9 M^{-1} s^{-1}. It has been proposed that the quenching of $[Ru(TAP)_2(dppz)]^{2+}$ and the subsequent back reaction proceed by proton-coupled electron transfer. It may be that the relatively slow rate for the back reaction with this complex allows for the separation of reaction products.

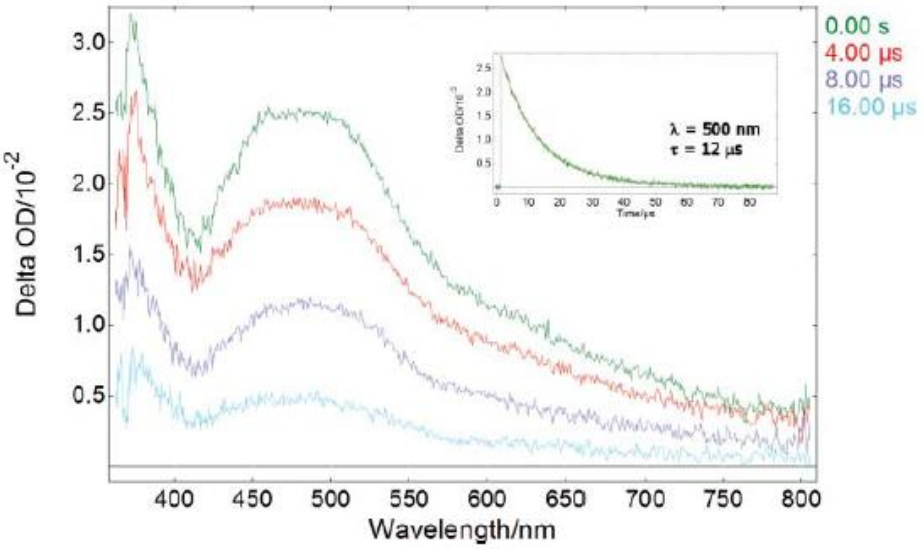


Fig. 7 Excited state absorption spectra of N_2 purged 10 mM phosphate buffer solution of 50 mM $[Cr(phen)_2(dppz)](Cl)_3$ in the presence of 14 mM GMP recorded at 0, 4, 8 and 16 ms using an ICCD camera (gate width 4 ms, 5 laser shots per spectrum). Inset: Monoexponential fit (red line) of transient absorption band decay (green line) at 500 nm.

Conclusion

In conclusion, these studies show that the excited states of these DNA-binding dppz Cr(III) complexes can be readily studied by both transient emission and absorption techniques. The lifetime of the excited state ismuch longer-lived than that of the analogous ruthenium complexes, which means that the complexes have potential as photophysical probes on the microsecond timescale. Unlike what is found for fac-$[Re(CO)_3(11,12\text{-}X_2dppz)(py)]$+ the photophysical properties (including the phosphorescence spectrum) do not depend strongly on variations of the X-substituent. The excited states of all complexes are dynamically and efficiently quenched by GMP, but only slightly by AMP, consistent with photoinduced oxidation of guanine. For DNA the quenching is predominantly static, as expected if rapid deactivation of the excited state occurs when the complexes intercalate into DNA. However, transient absorption spectra in the presence of GMP have so far not provided direct evidence for photoinduced electron transfer from guanine, suggesting that the back reaction is very rapid. This behaviour is significantly different than that observed for dppz ruthenium complexes and may be associated with different nature of the excited states (^{3}MLCT vs. 2MC). Picosecond or even femtosecond techniques using polynucleotides such as $[poly(dG\text{-}dC)]_2$ will be required to investigate this matter further.

Acknowledgements

SV and JAS are grateful to IRCSET for the award of postdoctoral fellowships. SFI (06/CHP036 and 07/CHEF437) are thanked for their financial support.

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