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COMMUNICATION

Pushing the limit: synthesis, photophysical and DNA binding studies of a NIR-emitting Ru(II)-polypyridyl probe with 'light switch' behaviour†

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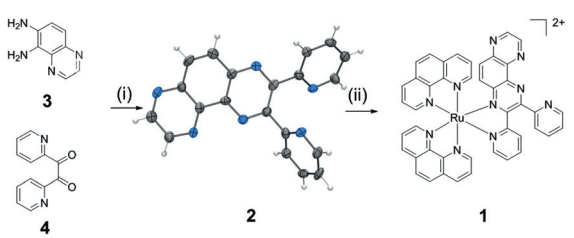
The new Ru(II) polypyridyl complex 1 was synthesised using microwave irradiation from the new polypyridyl ligand 2 'DipyTAP', and its photophysical properties, and DNA binding abilities were investigated using various spectroscopic techniques; and 1 was shown to act as a 'NIR molecular light switch' for DNA with an emission window between 680 and 860 nm.

Luminescent transition metal coordination complexes that possess DNA binding abilities have been the subject of growing interest in recent times by virtue of their potential use as DNA structure probes and cellular imaging agents.[1,2] In particular, complexes such as Ru(II) and Cr(III)-polypyridyl complexes have been intensively studied owing to their tuneable photophysical and photochemical properties, which are governed by the nature of the polypyridyl ligands employed.[3] Moreover, complexes that function as molecular "*light* switches" for DNA, *i.e.* being non-luminescent in aqueous media but intensely luminescent upon binding to DNA, hold particular potential as biological imaging agents.[4,5] However, most Ru(II)-polypyridyl complexes suffer from short wavelength absorption, with the metal-to-ligand charge transfer (MLCT) absorption maximum being shorter than 500 nm, and MLCT centred emission, usually shorter than 650 nm; a drawback for biological applications and for their potential use as cancer photo-therapeutics.[6,7] Recently, we have initiated a research programme into the development of new polypyridyl ligands for application in biology,[8] and we have developed several examples that have been used in Ru(II)-polypyridyl complexes, as DNA targeting binders and imaging agents.[9] We have also employed these for conjugation to gold nanoparticles,[10] and have shown them to be excellent luminescent imaging probes, and formed mixed-lanthanide (Yb(III) and Nd(III)) transition-metal (f–d) cyclen-Ru(II) complexes as dual visible- and near-infrared (NIR)-emitting DNA sensors.[11] With the view of developing this area even further, we have set out to generate novel polypyridyl ligands that could be used to generate long-wavelength excitation and emitting complexes. Inspired by the work of Meyer *et al.* and Zhou *et al.* who have recently developed a number of Ru(II)-polypyridyl complexes which exhibit ^{1}MLCT absorbance maxima as long as 550 nm and a singlet oxygen quantum yield as high as 0.43,[12] we set out to develop **1** based on **2**, a new polypyridyl ligand, which we have named '**DipyTAP**' as it contains two well established polypyridyl ligands within a single structure. We foresaw that through the use of a delocalized π-system the ^{1}MLCT absorption of **1** may be shifted to longer wavelengths as has been reported by Zhou *et al.* Herein, we show that the inclusion of ligand **2** in the Ru(II) polypyridyl complex **1** $[Ru(phen)_2(DipyTAP)]^{2+}$, results in a ~100 nm red shift of the MLCT absorption in aqueous solution in comparison to its parent complex $[Ru(phen)_3]^{2+}$. Moreover, we observe that the ^{3}MLCT emission is fully quenched in aqueous media, similar to that seen for $[Ru(phen)_2(dppz)]^{2+}$ and related structures,[13,14] but upon interaction of **1** with DNA the quenching process is perturbed resulting in an increase in the observed luminescence. To the best of our knowledge this is the first example of a Ru(II)-polypyridyl complex that can both act as a light switch for DNA, absorbing between 500 and 600 nm; emitting at long wavelengths within the NIR region, with an emission window between 700 and 850 nm; of significant importance, particularly for applications in imaging and photo-therapy.[2,6,10]

The synthesis of the Ru(II) complex **1** and that of the ligand **DipyTAP**, **2** is shown in Scheme 1. The synthesis of **2** was achieved by condensation of 5,6-diaminoquinoxaline,[8] **3** with 2,2′-dipyridil, **4** by reflux in EtOH yielding **2** as a beige solid in 92% yield. Crystals suitable for X-ray diffraction analysis were grown by recrystallisation from hot EtOH, and the structure of **DipyTAP** is shown in Scheme 1.‡ Subsequent reaction of **2** with $Ru(phen)_2Cl_2$ for 40 min under microwave radiation, followed



Scheme 1 Synthesis of ligand **2** and complex **1** including the X-ray crystal structure of **2**. (i) EtOH, Δ; (ii) $Ru(phen)_2Cl_2$, EtOH : H_2O, Δ.

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† Electronic supplementary information (ESI) available: Characterisation and figures. CCDC 859051 (**2**). For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c2dt00020b

by precipitation from H_2O using excess NH_4PF_6, yielded **1**, which was further purified by column chromatography using silica (eluent: 40 : 4 : 1 $CH_3CN/H_2O/NaNO_3$-(sat), giving **1** in 42% yield. The complex was fully characterised (see ESI†); the ^1H NMR is shown in Fig. 1, while MALDI HRMS gave m/z = 399.0763 for the M^{2+} ion, where the observed isotopic distribution pattern matched that of the calculated one (see ESI†).

The Cl^- salt of **1** was reformed by stirring the PF_6^- form in Amberlite resin (Cl^- form) and was found to be fully soluble in both aqueous solution and many organic solvents. The characteristic absorption spectra of **1** and **2** together with the excitation and emission spectra of **1** in MeCN are shown in Fig. 2. The absorption spectrum of **2** showed λ_{max} at 220 and 305 nm whereas **1** had characteristic transitions at *ca.* 220, 262, 295, 370, 420 and at 535 nm. The absorbance spectra were also measured in MeOH, EtOH, acetone and CH_2Cl_2, however, very little variation was observed between these solvents.

In pH 7.4 phosphate buffered aqueous solution the absorbance spectrum remained largely unchanged where the band at 262 nm (ε = 71 900 cm^{-1} M^{-1}) was characteristic of π–π* transitions of the ancillary phen ligands while the band at 295 nm (ε = 36 700 cm^{-1} M^{-1}) was attributed to π–π* transitions localised on ligand **2**. The less intense band at 370 nm (ε = 20 700 cm^{-1} M^{-1}) was attributed to transitions within the phenazine part of the ligand, while the transitions at 420 nm (ε = 9950 cm^{-1} M^{-1}) and 545 nm (ε = 8600 cm^{-1} M^{-1}) were characteristic of MLCT transitions from the Ru(II) centre. Interestingly the latter MLCT band at 545 nm was seen to be red shifted by ~10 nm in comparison to that in organic solutions suggesting a stabilisation of the MLCT state in aqueous media.

Upon excitation into each of the absorbance maxima, luminescence was observed at long wavelength, which when recorded in aerated MeOH, EtOH, acetone and MeCN solution, within an emission window of *ca.* 200 nm, with a λ_{max} at *ca.* 765 nm. While in CH_2Cl_2, the λ_{max} was blue shifted, occurring at *ca.* 720 nm, for which a quantum yield of luminescence of Φ_F < 0.001 was determined, no significant changes were seen in degassed solution, except for a slight increase in Φ_F. Similarly, the excitation of **1** (either at 765 or 720 nm) yielded emission spectra that were structurally identical to the absorption spectra in the same solvents (Fig. 2). As had been anticipated, no significant MLCT based emission was detected from **1** in aqueous solution suggesting a quenching pathway caused by the polar protic solvent as previously been seen for dppz based Ru(II) complexes, which are structurally related to **1**.[13] However, as such complexes are also known to give rise to large emission enhancements upon binding to DNA,[14] we next investigated the ability of **1** to bind to DNA to assess its suitability as a potential luminescence '*switch on*' probe.

Considering the positively charged nature and the propensity of Ru(II) complexes to bind to DNA,[7,9–11] we expected **1** to interact with DNA through a combination of electrostatic and π-stacking or groove binding interactions, which typically results in changes in their electronic spectra. To determine the binding affinity of **1** for DNA, a series of DNA titrations were carried out with salmon testes DNA (stDNA) at pH 7.4 (10 mM phosphate buffer), using absorption and emission spectroscopy. As can be seen the absorption spectrum, Fig. 3, was significantly affected, in particular, the MLCT centred transition at 536 nm underwent a 27% hypochromism as well as being red shifted to 545 nm. Unfortunately, we were unable to determine accurately Φ_F but it was found to be <0.001. Moreover, such changes were found to be only marginally affected by increasing NaCl concentration whereby 70% of the complex remained bound to DNA even after the addition of 300 mM NaCl (see ESI†). This suggests that although electrostatic interactions were prominent in the binding of **1** to stDNA, this was not the sole mode of interaction. From these UV/Vis absorption titrations we were able to determine the binding affinity of **1** for stDNA, by fitting the above changes in the MLCT absorption using the method of McGhee and Von Hippel.[15] This gave the binding constant K_b as 3.2 × 10^4 M^{-1} (±0.12) (average of several titrations) with a binding site size of n = 7.05 (±0.23). Concomitantly, the emission spectrum of **1** was greatly affected upon binding of **1** to

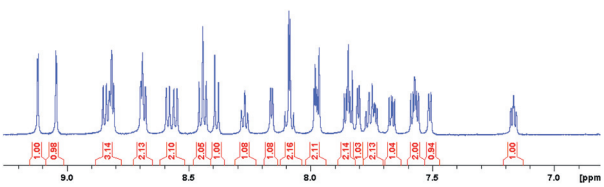

Fig. 1 The ^1H NMR spectra of **1** (600 MHz, CD_3CN).

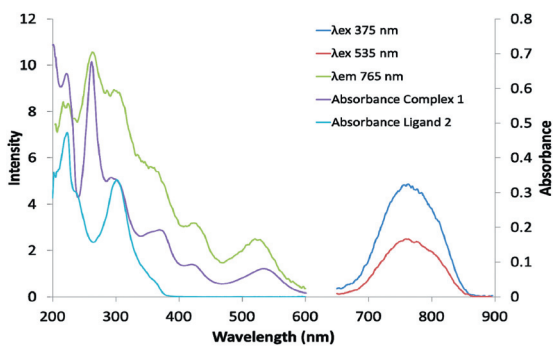

Fig. 2 The UV/Visible absorption spectra the excitation (λ_{em} = 765 nm) and emission spectra (λ_{ex} = 370 nm and 535 nm) of **1** (10 μM) in MeCN.

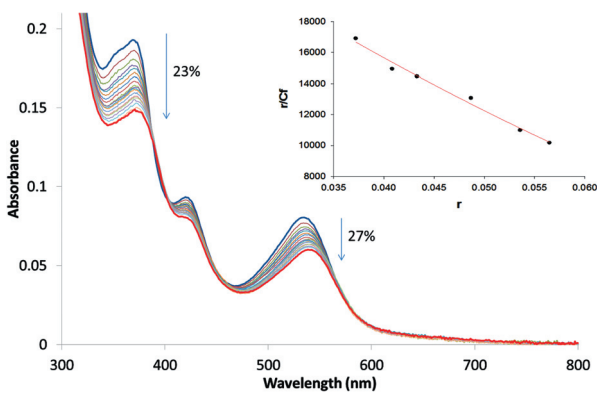

Fig. 3 Changes in the absorption spectrum of **1** (9.4 μM) with increasing concentration of stDNA (0–670 μM). Inset: The fit of the changes in the MLCT band using the McGhee and Von Hippel equation.

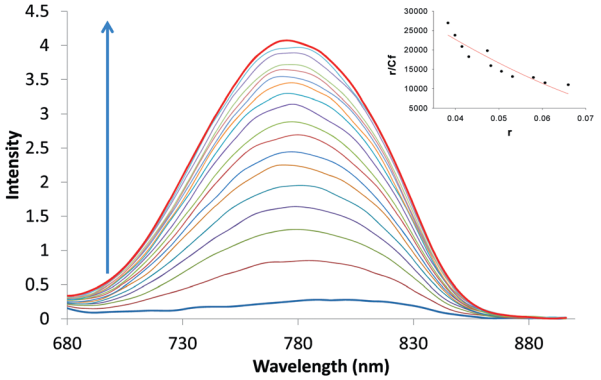

Fig. 4 Changes in the luminescence emission spectrum of **1** (9.4 μM) with increasing concentration of stDNA (0–670 μM) (λ_{ex} 545 nm). The blue and the red spectra indicate the beginning and the end point of the titrations. Inset: The fit of the changes in the MLCT band using the McGhee and Von Hippel equation.

stDNA where, in particular, the MLCT centred emission, occurring within the window of 680–860 nm, was '*switched on*' with an excess of two order of magnitude enhancement as demonstrated in Fig. 4. This enhancement was accompanied by a blue shift of *ca.* 20 nm (see Fig. 4) from *ca.* 800 nm to 780 nm for the fully bound complex. Similar effects were seen in the emission spectrum upon excitation at other wavelengths such as 420 nm and 370 nm. Again, analysis of these binding interactions by fitting the changes in the emission spectra using the above binding model of McGhee and Von Hippel, gave $K_b = 5.3 \times 10^4$ M^{-1} (±0.58) and a binding site size of $n = 8.0$ (±0.56), which is consistent with the results seen in the absorption spectra above. While this binding affinity is somewhat smaller than that reported for dppz based ligands,[14] it is an order of magnitude greater than has been reported for Ru[phen$_3$]$^{2+}$ (50 mM NaCI, 5 mM Tris, pH 7.5).[16] These results clearly demonstrate the advantage of using **2** to push the absorption and emission wavelengths towards the NIR regions; suggesting that the lowest lying MLCT excited state is centred on the Ru-**2** part of **1**.

Thermal denaturation experiments further supported the interaction of **1** with DNA (see ESI†) where **1** was found to stabilise the double stranded DNA at both high and medium loading (P/D = 10 or 25, respectively, with a $\Delta T_m = 3.1$ °C at higher P/D loading). The affinity of **1** for stDNA was also confirmed by carrying out an ethidium bromide (EtBr) displacement assay (see ESI†);[17] which demonstrated that **1** effectively displaced EtBr from the DNA helix with an apparent binding constant (K_{app}) of ~10^5 M^{-1}. Furthermore, changes were evident in both the circular dichroism (CD) and the linear dichroism (LD) spectrum of **1** for the binding of the complex to DNA. For the former, structural changes in the signature CD of DNA were observed while in the LD, the evolutions of a positive signal occurred across the entire absorption of **1** in 10 mM phosphate buffer, at pH 7.4 (stDNA = 150 μM, see ESI†). The observation of a positive LD signal between 300 and 500 nm, as a function of decreasing P/D values, implies that these transitions are oriented parallel to the alignment of the helix axis in solution. Hence, these results support a binding mode in which the complex is edgewise

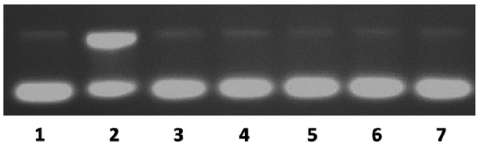

Fig. 5 Agarose gel electrophoresis of pBR322 DNA (1 mg ml^{-1}) after 30 min irradiation (2 J cm^{-2}) in 10 mM pH 7.4 phosphate buffer; lane 1: plasmid DNA control; lane 2: [Ru(bpy)$_3$]$^{2+}$ (P/D 5); lane 3 and 4: **1** (P/D 10, 5); lane 5: **1** in the dark (P/D 5); lanes 6 and 7: **1** + 10 mM NaN_3 (P/D 10, 5).

inserted into the grooves of DNA[18] but may also be partially-intercalating through the ancillary phen ligands as has recently been demonstrated for [Ru(TAP)$_2$(dppz)]$^{2+}$.[19] Given the structure of **1**, such a binding mode is not unexpected and further work is on-going in order to fully quantify this binding behaviour.

In order to further evaluate **1** as a potential biological imaging tool we also set out to evaluate any phototoxicity that might result from the photo excitation of **1** using Agarose gel electrophoresis of pBR322 plasmid DNA. As shown in Fig. 5 when incubated in the dark **1** showed no measurable DNA cleavage. Similarly, after 30 min of irradiation (using 400 nm cut off filter and 2 J cm^{-1}), under aerobic conditions at a P/D ratio of 5 and 10, **1** showed no photo cleavage effects, demonstrating its inability to cause DNA damage under light irradiation. Similarly, in the presence of NaN_3, a singlet oxygen scavenger, **1** also showed no photo-cleavage efficiency. As a control, the same pBR322 supercoiled DNA was treated with the known $^1O_2$ sensitizer [Ru(bpy)$_3$]$^{2+}$ which resulted in *ca.* 60% DNA photocleavage.

In summary, we have developed a novel ligand **2**, which was characterised fully using X-ray crystallography, and the corresponding Ru(II) polypyridyl complex **1**. The complex was shown to absorb at long wavelength and emit within an emission window of *ca.* 200 nm, between 680 and 860 nm in buffered aqueous solution upon binding to DNA; whereby the emission of **1** was '*switched on*'. We are currently further investigating the nature of the DNA interactions of **1**, as well as undertaking the synthesis of related structures, where various functional groups are being incorporated into **1**, with a view to achieving greater Φ_F values, longer absorption and emission wavelengths to evaluate their application as luminescent probes and *in vivo* imaging agents.

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Notes and references

‡Crystal data: $C_{20}H_{12}N_6$ (**2**), $M = 978.92$, monoclinic, $a = 15.066(3)$, $b = 7.0000(14)$, $c = 15.541(3)$ Å, $\beta = 107.45(3)°$, $V = 1563.6(5)$Å^3,

T = 115(2) K, space group $P2_1/n$, Z = 4, 10 976 reflections measured, 2528 unique (R_{int} = 0.1038) which were used in all calculations. Final wR_2 = 0.3629 (all data) and R_1 = 0.1357 ($I > 2\sigma$). CCDC 859051.

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