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Absence of quenching by  $[\text{Fe}(\text{CN})_6]^{4-}$  is not proof of DNA intercalation†

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The quenching of the  $^3\text{MLCT}$  emission of three Ru(II) complexes of known DNA binding mode is compared. This work shows that relative binding constants dictate whether quenching is observed in the presence of DNA rather than protection of the probe by intercalation, as has been commonly stated.

The DNA binding of luminescent ruthenium complexes has been investigated extensively for numerous applications,<sup>1–5</sup> where the binding of probes to DNA is characterized by their binding constant,  $K_b$ , and binding mode.<sup>6,7</sup> For example,  $[\text{Ru}(\text{bpy})_3]^{2+}$  (**1**, bpy = 2,2'-bipyridine) is known to bind DNA electrostatically with a relatively small binding constant ( $K_b = 700 \text{ M}^{-1}$ ),<sup>8</sup> whereas  $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$  (**2**; dppz = dipyrido-[3,2-*a*:2',3'-*c*]phenazine) intercalates between the DNA bases and exhibits a significantly larger binding constant,  $3.2 \times 10^6 \text{ M}^{-1}$  (Fig. 1).<sup>9</sup> Shifts in the DNA melting temperature, changes in relative viscosity, and various optical techniques need to be taken together to determine the DNA binding mode of these and other probes.<sup>10</sup> Optical measurements include changes in the absorption spectrum and emission enhancement of a probe when bound to DNA, however, it is known that these methods cannot be used exclusively to prove intercalation, since surface aggregation often leads to similar results.<sup>11,12</sup> Another common optical method is the relative luminescence quenching of molecules

bound to DNA by ferrocyanide,  $[\text{Fe}(\text{CN})_6]^{4-}$ , which has often been used to assign the DNA binding mode as intercalation.<sup>13–17</sup>

$[\text{Fe}(\text{CN})_6]^{4-}$  remains free in the aqueous environment in the presence of polyanionic DNA owing to Coulombic repulsion between the two species. It has been postulated that ferrocyanide is able to quench the emission of probes that are electrostatically bound to the DNA backbone, but that its quenching efficiency is reduced significantly when the luminescent compound is intercalated between the DNA bases, since the latter are protected from quenchers in solution.<sup>13–17</sup> However, since binding mode and binding constant are often closely correlated, it has proven difficult to establish whether the magnitude of the binding constant of the probes bound to DNA, rather than binding mode, is a more important parameter to be considered in the relative quenching by  $[\text{Fe}(\text{CN})_6]^{4-}$ .

$[(\text{bpy})_2\text{Ru}(\text{tpphz})\text{Ru}(\text{bpy})_2]^{4+}$  (**3**; tpphz = tetrapyrrodo-[3,2-*a*:2',3'-*c*:3'',2''-*h*:2''',3'''-*j*]-phenazine) was recently shown to bind to DNA as strongly as an intercalator ( $K_b = 1.9 \times 10^5 \text{ M}^{-1}$ ), but in an electrostatic manner.<sup>18</sup> Therefore, comparison of the emission quenching by  $[\text{Fe}(\text{CN})_6]^{4-}$  for **1–3** bound to DNA is expected to provide insight into the role of binding mode and binding constant on the use of the ferrocyanide quenching method for the determination of DNA binding mode.

Complexes **1–3** were prepared as described previously.<sup>18,19</sup> The luminescence quenching of  $10 \mu\text{M}$  **1** in 5 mM Tris buffer (pH = 7.2, 50 mM NaCl) and in the presence of 400  $\mu\text{M}$  DNA by  $[\text{Fe}(\text{CN})_6]^{4-}$  has been previously reported to result in a Stern–Volmer quenching rate constant,  $k_q$ , of  $9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ , showing that the presence of DNA does not affect the quenching.<sup>20</sup> The  $^3\text{MLCT}$  (metal-to-ligand charge transfer) excited state of **1** is known to be reductively quenched by  $[\text{Fe}(\text{CN})_6]^{4-}$ .<sup>21</sup> Stern–Volmer constants,  $K_{\text{SV}}$ , of  $14\,000 \text{ M}^{-1}$  and  $7000 \text{ M}^{-1}$  were obtained from linear fits of plots of  $I_0/I$  vs.  $[\text{Q}]$  ( $\text{Q} = \text{quencher, ferrocyanide}$ ) for **1** in deoxygenated 5 mM Tris buffer (pH = 7.5) with 5 mM and 50 mM NaCl, respectively. The latter results in a quenching rate constant,  $k_q$ , of  $1.2 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$  ( $\tau_0 = 600 \text{ ns}$ ) in the absence of DNA, slightly greater than the previous report.<sup>20</sup> However, in that work the solutions were not deoxygenated, such that the oxygen concentration was decreasing with increasing  $[\text{Fe}(\text{CN})_6]^{4-}$ , since  $[\text{O}_2]$  is dependent on ionic strength in water and at the surface of polyelectrolytes.<sup>22,23</sup>

As expected from electrostatic screening, a lower quenching rate constant for **1** by  $[\text{Fe}(\text{CN})_6]^{4-}$  was previously measured in

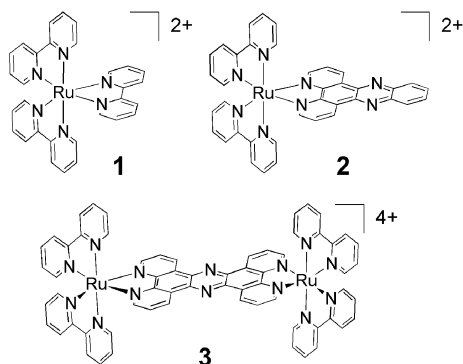
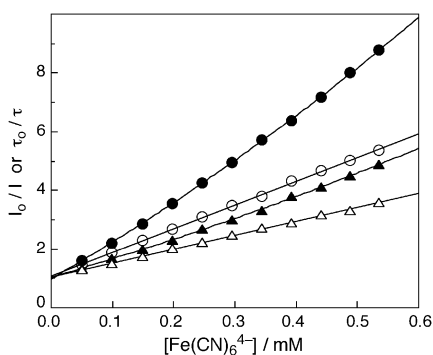


Fig. 1 Schematic representation of the molecular structures of **1–3**.

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† Electronic supplementary information (ESI) available: Experimental details, absorption changes with  $[\text{Fe}(\text{CN})_6]^{4-}$ , emission quenching plots and Stern–Volmer plots with DNA in 5 mM NaCl. See DOI: 10.1039/c0cc04973e



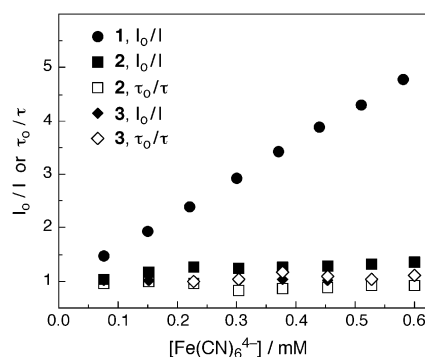
**Fig. 2** Stern–Volmer plots of 10  $\mu\text{M}$  **1** in 5 mM Tris buffer (pH = 7.5) showing quenching of lifetime (open symbols) and emission intensity (closed symbols) in 5 mM NaCl (circles) and 50 mM NaCl (triangles).

water containing 0.5 M NaCl ( $k_q = 3.5 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ ).<sup>21</sup> From this result and the data presented here, it is apparent that ionic strength plays a role in the magnitude of the quenching rate constant in deoxygenated solutions and it is possible that static quenching may be operative, especially at low ionic strengths. In order to explore the contributions from dynamic and static quenching, the Stern–Volmer plots obtained from  $\tau_0/\tau$  and  $I_0/I$  vs.  $[\text{Q}]$  are compared (Fig. 2). It is apparent from Fig. 2 that the dynamic quenching component ( $\tau_0/\tau$  vs.  $[\text{Q}]$ ) results in lower  $K_{\text{SV}}$  values (slopes) than those derived from the total emission intensity, which contains contributions from both dynamic and static quenching, at each NaCl concentration. The lifetime quenching data shown in Fig. 2 can be fitted with a line with intercept of 1.0, as expected for dynamic quenching behavior, with  $k_q = 1.4 \times 10^{10} \text{ M}^{-1} \text{ s}^{-1}$  and  $k_q = 7.9 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$  in 5 mM and 50 mM NaCl, respectively. The emission intensity quenching data,  $I_0/I$ , in Fig. 2 are not linear and such attempted fits result in poor correlation coefficients and intercepts of 0.69 and 0.91 for 5 mM and 50 mM NaCl, respectively, consistent with contributions from both dynamic and static quenching to the decrease in luminescence intensity.

In order to ascertain the contributions of dynamic and static quenching in this system, a combined equation was used to fit the  $I_0/I$  data in Fig. 2, eqn (1), where  $K_{\text{S}}$  and  $K_{\text{D}}$  represent the

$$\frac{I_0}{I} = (1 + K_{\text{S}}[\text{Q}])(1 + K_{\text{D}}[\text{Q}]) \quad (1)$$

static and dynamic Stern–Volmer constants, respectively.<sup>24</sup> Derivation of eqn (1) assumes that the quencher, Q, is in excess, and that there is no luminescence from pre-associated  $[\text{Ru}(\text{bpy})_3]^{2+}/[\text{Fe}(\text{CN})_6]^{4-}$ , with equilibrium constant of  $K_{\text{S}}$ . Under these conditions, only **1** free in solution at the time of excitation emits light and is quenched dynamically ( $K_{\text{D}} = \tau_0 k_q$ ). Fits of the 5 mM data in Fig. 2 to eqn (1) using the slope of the corresponding fits of  $\tau_0/\tau$  Stern–Volmer plot as  $K_{\text{D}}$  ( $8.3 \times 10^3 \text{ M}^{-1}$ ) result in  $K_{\text{S}} = 1.2 \times 10^3 \text{ M}^{-1}$ , indicating that even at low ionic strength the predominant quenching mechanism is dynamic. The static component is relatively smaller in 50 mM NaCl with  $K_{\text{S}} = 694 \text{ M}^{-1}$  and  $K_{\text{D}} = 4.9 \times 10^3 \text{ M}^{-1}$ . As expected, the magnitude of the association constant  $K_{\text{S}}$  is dependent on ionic strength, and the values measured here are consistent with those previously



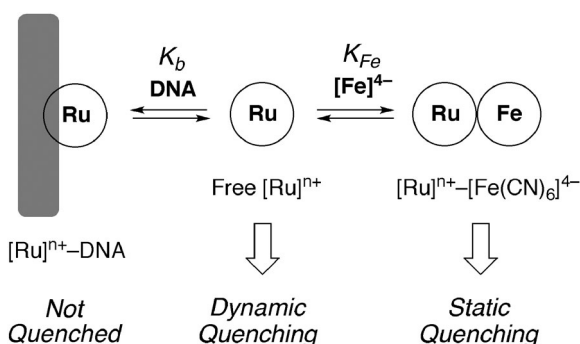
**Fig. 3** Stern–Volmer plots of 10  $\mu\text{M}$  **1**, **2**, and **3** showing selected changes in emission intensity ( $I_0/I$ ) and lifetime ( $\tau_0/\tau$ ) in the presence of 100  $\mu\text{M}$  DNA and 50 mM NaCl (5 mM Tris buffer, pH = 7.5).

reported for the  $[\text{Ru}(\text{bpy})_3]^{2+}/[\text{Fe}(\text{CN})_6]^{4-}$  system.<sup>25</sup> The low emission intensity of **2** and **3** in buffer alone precluded this type of analysis in the absence of DNA.

The emission intensity of 10  $\mu\text{M}$  **1** is unaffected by the addition of 100  $\mu\text{M}$  DNA in deaerated buffer (5 or 50 mM NaCl), whereas those of **2** and **3** increase significantly, as previously reported.<sup>1a,18</sup> Although a slight increase in the lifetime of **1** was previously reported in aerated solutions upon addition of DNA, it is likely that interaction of the complex with the DNA backbone and reduced  $\text{O}_2$  concentration surrounding the polyanion contributed to this observation, as was reported for anionic dendrimers.<sup>23</sup>

Fig. 3 shows the Stern–Volmer plots for 10  $\mu\text{M}$  **1–3** in the presence of 100  $\mu\text{M}$  DNA in 50 mM NaCl buffer (5 mM Tris, pH = 7.5). The quenching data for 5 mM NaCl are shown in Fig. S5, ESI.† As previously reported, the quenching of **1** in 50 mM NaCl is nearly independent of the presence of DNA, consistent with its weak ionic binding to the double helix.<sup>20</sup> The quenching of **1** by ferrocyanide is reduced from pure buffer to DNA solutions at low salt concentration (5 mM NaCl), which can be attributed to the contribution of the polyanion in electrostatic screening to the formation of the donor–acceptor complex. Indeed, the quenching of the luminescence of **1** is identical within the error limit at both 5 mM and 50 mM NaCl when 100  $\mu\text{M}$  DNA is present, indicative that the polyanionic backbone serves to increase the local ionic strength around the probe to a greater extent than the ions in the bulk solution.

In contrast to **1**, complexes **2** and **3** exhibit little luminescence intensity or lifetime quenching in the presence of DNA (Fig. 3). This behavior is expected for probes that bind to DNA *via* intercalation, and was previously published for the known intercalator **2**.<sup>26</sup> Complex **2** exhibits a biexponential emission decay when bound to DNA,<sup>26</sup> similar to the related complex  $[\text{Ru}(\text{phen})_2(\text{dppz})]^{2+}$  (phen = 1,10-phenanthroline).<sup>27,28</sup> The long lifetime component, for which  $\tau_0 = 149 \text{ ns}$ , was used in the  $\tau_0/\tau$  vs.  $[\text{Q}]$  plot in Fig. 3, however, it should be noted that the shorter component (47 ns) is also invariant as the ferrocyanide concentration is increased with 100  $\mu\text{M}$  DNA in solution. In the presence of 100  $\mu\text{M}$  DNA (5 mM Tris, pH = 7.5, 50 mM DNA) the luminescence of **3** can be fitted to a monoexponential decay with  $\tau_0 = 107 \text{ ns}$  in the absence of quencher. In contrast to **2**, **3** has been shown to bind electrostatically to DNA, albeit with a binding constant that is similar



**Fig. 4** Schematic representation of quenching processes of a cationic Ru(II) complex by ferrocyanide in the presence of DNA.

to that of the well known intercalator ethidium bromide, for which  $K_b = 1.7 \times 10^5 \text{ M}^{-1}$ .<sup>29</sup> Therefore, from the data in Fig. 3, it can be concluded that it is the relative binding constants among the three species in solution, cationic probe,  $[\text{Fe}(\text{CN})_6]^{4-}$ , and DNA, that determine the ability of the quencher to access the emissive complex, and not intercalation.

Fig. 4 illustrates the predominant equilibria for a cationic Ru complex in the presence of both the polyanionic DNA and  $[\text{Fe}(\text{CN})_6]^{4-}$ . It is clear from this representation that the competitive binding for  $[\text{Ru}]^{n+}$  by DNA and ferrocyanide is modulated by the relative magnitudes of the equilibrium constants,  $K_b$  and  $K_{\text{Fe}}$ , respectively, and the concentrations of the anionic species present in solution. It is expected that for the divalent complex **2**,  $K_{\text{Fe}}$  should be of similar order of magnitude to that for **1**,  $\sim 10^3 \text{ M}^{-1}$ . Using average radii of 13.5 Å for the tetravalent complex **3** and 4.5 Å for  $[\text{Fe}(\text{CN})_6]^{4-}$  results in values of  $K_b$  of  $\sim 2\text{--}3 \times 10^4 \text{ M}^{-1}$  from 5 to 50 mM NaCl using the Debye–Hückel formalism.<sup>25b,30</sup> Therefore, for **2** the value of  $K_b$  is  $\sim 3$  orders of magnitude larger than that of  $K_{\text{Fe}}$ , and this difference is a factor of  $\sim 10$  for **3**. Given these equilibrium constants, even at the highest  $[\text{Fe}(\text{CN})_6]^{4-}$  concentration in Fig. 3, it can be estimated that nearly all **2** and **3** is bound to DNA, consistent with the lack of emission intensity and lifetime quenching observed for these complexes. The absence of dynamic quenching of the DNA-bound species shows that the on/off rate of **3** with DNA must be significantly slower than the dynamic quenching rate constant,  $k_q \sim 10^{10} \text{ M}^{-1} \text{ s}^{-1}$ .

The present work shows that there is little emission quenching by  $[\text{Fe}(\text{CN})_6]^{4-}$  of **2** and **3**, both of which bind to DNA strongly. Although **2** is a known DNA intercalator, **3** binds electrostatically to the double helix. Therefore, this work shows that the absence of luminescence quenching by ferrocyanide cannot be used as proof of DNA intercalation.

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