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Duplex-Selective Ruthenium-based DNA Intercalators

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Abstract

We report the design and synthesis of small molecules that exhibit enhanced luminescence in the presence of duplex rather than single-stranded DNA. The local environment presented by a well-known [Ru(dipyrido[2,3-a:3',2'-c]phenazine)L₂]²⁺-based DNA intercalator was modified by functionalizing the bipyridine ligands with esters and carboxylic acids. By systematically varying the number and charge of the pendant groups, it was determined that decreasing the electrostatic interaction between the intercalator and the anionic DNA backbone reduced single-strand interactions and translated to better duplex specificity. In studying this class of complexes, a single Ru^{II} complex emerged that selectively luminesces in the presence of duplex DNA with little to no background from interacting with single stranded DNA. This complex shows promise as a new dye capable of selectively staining double versus single-stranded DNA in gel electrophoresis, which cannot be done with conventional SYBR dyes.

Keywords

DNA; Gels; Luminescence; Ruthenium

For many decades, gel electrophoresis has been the preferred method for the separation and characterization of nucleic acids.^[1] The imaging of nucleic acids within these gels is currently possible via small molecule staining agents, which provide a measureable colocalized response. Nucleic acid stains typically possess planar, conjugated π systems with excited-state photophysical properties, which allows them to be visualized using

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Supporting information for this article is available on the WWW.

fluorescence gel plate readers. The sensitivity of these methods relies on structures that luminesce in a nucleic acid environment but are quenched by the surrounding hydrogel matrix, thus reducing the overall background signal. Although it is possible to perform gel electrophoresis under denaturing conditions in order to remove the presence of base-pairing and other intramolecular interactions, running the gel under conditions that support hybridization to distinguish single-stranded DNA (ssDNA) from its duplex state could provide additional useful information regarding the structural state of the nucleic acids. Such information would be particularly helpful in applications that rely on differentiating the amount of double stranded DNA (dsDNA) from ssDNA, such as the DNA amplification step involved in polymerase chain reactions (PCR). To date, identifying molecules that exhibit specific interactions for dsDNA rather than ssDNA remains a challenge.^[2] Furthermore, although the interaction of metallointercalators with both duplex and single stranded DNA has been investigated, their behavior in the context of an application such as gel electrophoresis has not been studied.^[3]

To address this experimental limitation, we investigated synthetically modulating the electrostatic and steric character of a small molecule that is known to luminesce upon interaction with DNA and then determined how these modifications affected its specificity for duplex DNA. The choice of luminescent stain, a well-studied DNA intercalator $[\text{Ru}(\text{bpy})_2(\text{dppz})]^{2+}$ (dppz = dipyrido[3,2-a:2',3'-c]phenazine, bpy = 2,2'-bipyridine), was motivated by several factors. First, the dppz complexes exhibit luminescence through metal-to-ligand charge transfer in the hydrophobic interior of nucleic acids, yet are virtually spectroscopically silent in protic media, with a 10,000 $\times$ turn-on.^[4] Second, the overall charge of the complex can be synthetically tailored by varying the number of carboxylic acid groups appended to the ancillary ligand system that exist as carboxylates under physiological conditions.^[5] These modifications alter the steric and charge profiles across a series of complexes while maintaining their luminescent properties (Figure 1). This defines a window of attractive forces that exist between the planar π systems of the dppz ligand and nuclear bases and repulsive steric or electrostatic effects that could help to reduce the photoluminescence in the presence of ssDNA. Finally, while organic fluorophores are prone to rapid photobleaching, inorganic complexes are typically robust and can support prolonged experimentation as well as offer an extended shelf life.^[4d, 6] Taken together, ruthenium-based intercalators offer potential advantages for the development of tunable, duplex-selective nucleic acid stains.

The oligonucleotide sequences that were chosen for the investigation of selectivity are shown in Figure 2c. This sequence has been used extensively in the design of programmable assemblies of DNA-functionalized nanoparticles into well-ordered crystalline superlattices.^[7] This sequence is ideal because it does not form undesirable secondary structures and readily hybridizes to its complementary strand at the temperature and ionic strength specified in the current study. The luminescence of the parent complex, $[\text{Ru}(\text{dmb})_2(\text{dppz})]\text{Cl}_2$ (dmb = 4,4'-dimethyl-2,2'-bipyridine), was measured in phosphate-buffered saline and in solutions containing either the unhybridized or hybridized oligonucleotides (complex **1**, Figure 2a). For all fluorescence measurements, 3 μM of Ru^{II} complex and 10 μM of oligonucleotide were used. As is the case with similarly charged

complexes reported in literature,^[4a, 8] the luminescence of complex **1** is mostly quenched in buffer solution, but turned on in the presence of either ssDNA or dsDNA. To quantify the difference between luminescence signals produced in solutions containing ssDNA vs dsDNA, we used the following relationship to evaluate selectivity, which we term the *enhancement factor* (E.F.):

$$E.F. = \frac{I_{(dsDNA-Buffer)}}{I_{(ssDNA-Buffer)}}$$

where *I* refers to the integrated luminescence intensity of the Ru^{II} complex. In this manner, we observed that complex **1** emits approximately 1.5 times more photons in the presence of dsDNA than it does in the presence of ssDNA (Figure 2a).

Although the true physical nature of the interaction between these DNA intercalators and single-stranded oligonucleotides is not yet known,^[8–9] we hypothesize that electrostatic interactions allow the ssDNA to coil around the cationic complex and protect the photo-excited state of the complex from non-radiative deactivation. Thus, designing a ligand system that attenuates these electrostatic attractions should reduce the luminescence response of the complex to unhybridized oligonucleotide. Two routes to reducing the interaction between the intercalator complex and ssDNA were investigated: increasing steric hindrance by introducing bulky ester substituents and reducing the complex's overall positive charge by introducing pendant carboxylic acid groups. To evaluate the importance of sterics, [Ru(dppz)(dmb'-CO₂CH₃)(dmb)]Cl₂ (complex **2**), which possesses a methylcarboxypropyl group at the 4-position of one of the bipyridyl ligands, was synthesized. It was predicted that, in comparison to the parent complex **1**, the presence of the bulky ester group would reduce the interactions between the complex and ssDNA. However, no improvements in luminescent selectivity for this particular species (complex **2**, Figure 2b) were observed. On the other hand, the introduction of a negatively charged carboxylate group, to give [Ru(dppz)(dmb'-CO₂⁻)(dmb)]Cl (complex **3**, Figure 2b), increased the E.F. to 6.8, indicating that the local charge density reduced the interaction between the complex and ssDNA.^[5] Importantly, modification of complex charge does not impede the intercalation of the complex into the DNA duplex.

Next we considered complexes with two pendant groups to better elucidate the effects of steric hindrance and electrostatic repulsion on duplex specific luminescence (Figure 3). To determine the effect of steric interactions, we compared complex **2** to complex **4** ([Ru(dppz)(dmb'-CO₂CH₃)₂]Cl₂), which possess one and two pendant ester groups, respectively. Since complex **4** has a 3-fold higher E.F. than complex **2**, we concluded that increasing the steric bulkiness of the intercalator ancillary ligand promotes dsDNA selectivity. To further investigate the role of electrostatic repulsion, we replaced one of the ester-functionalized ligands with a carboxylic acid-functionalized ligand. This results in an intercalator, [Ru(dppz)(dmb'-CO₂CH₃)(dmb'-CO₂⁻)]Cl (complex **5**), that displays a significantly higher E.F. than both complex **4**, which possesses the same number of pendant groups but a different overall charge, and complex **3**, which has a different number of pendant groups but has the same charge. Based on the observations that two esters lead to greater enhancement

than a single ester (complex **4** > complex **2**) and that the combination of an ester group and a carboxylate results in greater enhancement than a single carboxylate (complex **5** > complex **3**), we hypothesized that increased electrostatic repulsion might result in the most dramatic enhancement in selectivity. Thus, we synthesized complex **6**, $[\text{Ru}(\text{dppz})(\text{dmb}'\text{-CO}_2^-)_2]$, which has two pendant chains, both of which terminate in carboxylates. This net neutral complex exhibited a remarkable E.F. of 58, which is two-fold higher than the second most selective compound (complex **5**). From this series of complexes, it is apparent that increasing the electrostatic repulsion between the ancillary ligands and the negatively charged backbone of DNA most significantly increases duplex specific luminescence, while steric hindrance plays a noticeable but smaller role.

To further expand our study and test the limits of this system, we synthetically modified one of the mono-functionalized ancillary ligands such that two functional groups were attached. A new class of ligand was synthesized in a manner analogous to the preparation of $\text{dmb}'\text{-CO}_2\text{R}$ by adjusting the stoichiometric parameters of the initial lithiation reaction (see Supporting Information). Combinations of mono- and bis-functionalized ancillary ligands were used to assemble four tris-heteroligated complexes, such that each complex possesses three functional groups (complexes **7–10**). In doing so, not only do we observe a similar trend in selectivity consistent with the structure–activity relationships we have discussed above (Figure 4), but we have also identified the limitations of this strategy of enhancing duplex specificity by reducing complex–DNA interaction. Continuing the investigation of the effect of pendant group steric hindrance, $[\text{Ru}(\text{dppz})(\text{dmb}''\text{-}\{\text{CO}_2\text{CH}_3\}_2)(\text{dmb}'\text{-CO}_2\text{CH}_3)]\text{Cl}_2$ (complex **7**), which is modified with three pendant ester functional groups, can be compared with those modified by two and one ester groups, complex **4** and complex **2**, respectively. The E.F. follows the trend: complex **7** > complex **4** > complex **2**, making it apparent that greater steric bulk leads to enhancement of duplex selectivity. Still, steric interactions of the ester-functionalized chains are less effective at increasing duplex selectivity than the introduction of acid-functionalized chains, since complex **7** is far less enhancing than either complex **5** or complex **6**. This is further demonstrated when one of the three pendant ligands becomes an acid group, as in $[\text{Ru}(\text{dppz})(\text{dmb}''\text{-}\{\text{CO}_2\text{CH}_3\}_2)(\text{dmb}'\text{-CO}_2^-)]\text{Cl}$ (complex **8**), which has an almost 3-fold higher E.F. than complex **7**.

In the remaining two complexes of the tris-heteroligated family, complexes **9** and **10**, we have identified the limitations of this approach for increasing duplex specificity. Further modification of the ligands appears to compromise the interaction of the complex with dsDNA. For example, $[\text{Ru}(\text{dppz})(\text{dmb}'\text{-CO}_2\text{CH}_3)(\text{dmb}''\text{-}\{\text{CO}_2^-\}_2)]$ (complex **9**), which possesses two pendant acid groups and one pendant ester group, has a lower E.F. than the sterically similar complex **8**. The extra pendant arm on complex **9** reduces the E.F. to far below that of the other net neutral molecule, complex **6**. The molecule with an overall negative charge, $\text{Na}[\text{Ru}(\text{dppz})(\text{dmb}''\text{-}\{\text{CO}_2^-\}_2)(\text{dmb}'\text{-CO}_2^-)]$ (complex **10**), does not exhibit significant luminescence in the presence of dsDNA. Presumably, this stems from an inability of the complex to bind to or intercalate into dsDNA due to the barrier presented by the steric hindrance and unfavorable electrostatic interaction introduced through the numerous negatively charged pendant groups.

In addition to studying the relative luminescence trends for each intercalator by solution based fluorescence spectroscopy, we also investigated these effects in the context of DNA staining in a gel electrophoresis assay. Solutions containing 40 μM of ssDNA or dsDNA were mixed with 25 μM of each complex in water and run in a 1% agarose gel (Figure 5). The relative selectivity exhibited by each complex for intercalating dsDNA within the agarose gel matched well with the trends observed in solution. Of particular interest is the photoluminescent staining response of the neutral complex **6**, which managed to selectively stain only duplex DNA within the window of fluorescence sensitivity of the gel scanners detectors. To probe the versatility of this complex, its interaction with a variety of sequences was determined (Table S1). Complex **6** exhibited selectivity for dsDNA across a range of GC content and sequence lengths.

The ability to selectively stain dsDNA by gel electrophoresis has important implications for DNA analysis and characterization. For example, the ability to visualize the degree of duplex formation in a given mixture of DNA can allow one to quickly assess the relative efficiency of enzymatic reactions such as the generation of shorter dsDNA cleavage products from linear dsDNA substrates after treatment with restriction enzymes. Furthermore, quantitative solution-based systems, which require selectivity of duplex versus ssDNA in solution, including those used in real time PCR reactions, could also benefit from such selectivity as these assays are designed to track the relative amounts of dsDNA generated from ssDNA starting materials such as primers and templates. SYBR green, the commercially available dye from Invitrogen used ubiquitously for PCR amplification, has been found to exhibit 20-fold greater luminescence when interacting with dsDNA vs. ssDNA at 10 dye molecules per DNA base pair whereas in our study, we have identified a complex that exhibits 58-fold enhancement in luminescence at only 0.017 dye molecules per DNA base pair.^[10] In principle, the methods developed here for improving duplex specific luminescence need not be limited to this class of intercalator complexes. This approach has been shown to be effective for controlling the interaction between a new class of ruthenium intercalators and DNA that can enable the development of highly specific, luminescent molecules useful for novel applications in biological assays.

Experimental Section

Ruthenium complex syntheses, characterization, and detailed procedures can be found in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Schematic representation of intercalator complexes synthesized for this study. Increasing contributions from both steric and electrostatic contributions were systematically studied through synthetic changes in the ancillary ligand side chains. X = PF_6^- as synthesized or Cl^- after salt exchange.

Figure 2.

Characteristic luminescence intensities of complexes at 3 μM with no or one pendant chain upon interaction with 10 μM of ssDNA and dsDNA in PBS (10 mM, pH 7.5). a) Emission profiles of complex **1** in the presence of ssDNA vs dsDNA and for b) complexes **2** and **3**, respectively. The temperature of the samples was maintained at 25 $^{\circ}\text{C}$, and the counter anion for all complexes was Cl^- . c) The 18 base DNA sequences used in these fluorescence assays.

Figure 3. Characteristic fluorescence intensities of complexes at 3 μM with two pendant chains upon interaction with 10 μM of dsDNA and ssDNA in PBS (10 mM, pH 7.5) at 25 $^\circ\text{C}$. The counter anion for all complexes was Cl^- .

Figure 4. Characteristic fluorescence intensities of complexes at 3 μM with three pendant chains upon interaction with 10 μM dsDNA and ssDNA in PBS (10 mM, pH 7.5) at 25 $^{\circ}\text{C}$. The counter anion for all complexes was Cl^- .

Figure 5.

Gel electrophoresis results showing the relative intercalative nature of each Ru complex when run in a mixture of ssDNA versus dsDNA samples. Samples included (from left to right) are complex **10**, complex **6**, complex **3**, complex **1**, and complex **7**. Degree of intercalation is indicated by the relative fluorescence intensities of the individual gel bands. Apparent from the differential staining of the DNA in each lane is that the neutral Ru^{II} complex can distinguish ssDNA from dsDNA, whereas the more positively charged Ru complexes cannot. Electrostatic repulsion between the negatively charged Ru complex and the negatively charged DNA backbone may significantly hinder intercalation, thus resulting in little to no fluorescence even in the presence of dsDNA. The gel to the right shows single and double stranded DNA samples post staining with the commercially available 1× SYBR Gold stain (Invitrogen) for comparison.