How to Make a Molecular Light Switch Sequence-Specific: Tethering of an Oligonucleotide to a Dipyridophenazine Complex of Ruthenium(II)

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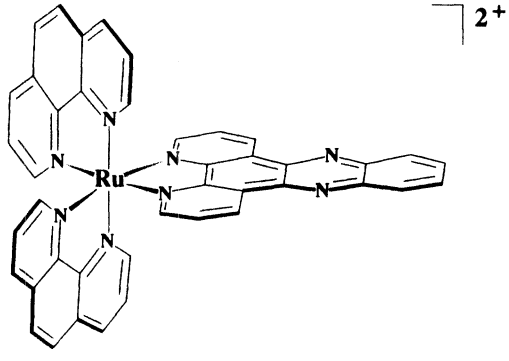
ABSTRACT

Considerable attention has been given recently to the design and development of nonradiative methods of recognizing DNA in a sequence-specific manner. Earlier, we reported that $Ru(bpy)_2dppz^{2+}$ (bpy = 2,2'-bipyridine, dppz = dipyrido[3,2:*a*-2',3':*c*]phenazine) shows no luminescence in aqueous solution, but upon intercalation into double-helical DNA, bright photoluminescence is observed (Friedman, A.E.; Chambron, J.-C.; Sauvage, J.P.; Turro, N.J.; Barton, J.K. *J. Am. Chem. Soc.*, **1990**, *112*, 4960). Based upon this observation, a sequence-specific molecular light switch has been designed in which a dppz complex of ruthenium(II) is tethered onto an oligonucleotide. An oligonucleotide modified at its 5' end has been constructed by coupling the sequence 5'-$H_2N(CH_2)_6$AGTGCCAAGCTTGCA-3' to $Ru(phen')_2dppz^{2+}$ (phen' = 5-amido-glutaric acid-1,10-phenanthroline). Like the parent complex $Ru(bpy)_2dppz^{2+}$, the single-stranded metal-oligonucleotide conjugate shows little detectable luminescence in aqueous solution. Addition of the complementary strand results in intense photoluminescence; time-resolved studies show that the emission is biphasic with excited state lifetimes of 500 (60%) and 110 (40%) ns. As expected, addition of a non-complementary strand produces no luminescence enhancement over that of the single-stranded metal-oligonucleotide. These results demonstrate that this oligonucleotide derivatized metal complex can be used to recognize and target specific sequences on DNA, a valuable feature which may lead to interesting and novel applications in hybridization technology.

The focus of our laboratory has been in the design and synthesis of transition metal complexes as probes of nucleic acid structure and conformation.[1] One complex of particular interest is (bis-1,10-phenanthroline)(dipyrido[3,2-*a*:2',3'-*c*]phenazine) ruthenium(II), $[Ru(phen)_2dppz]^{2+}$ (**1**). $[Ru(phen)_2dppz]^{2+}$ is a water-soluble complex



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that is coordinatively saturated, rigid, and inert to substitution. These properties are ideally suited for examining metal-DNA interactions in that the binding of the complex to DNA will be determined by the overall shape of the complex while the nature of this binding interaction can be studied using the spectroscopic properties of the complex imparted by the ruthenium center. Polypyridyl complexes of ruthenium (II) possess a well-characterized metal-to-ligand charge transfer (MLCT) transition[2] which is perturbed upon binding to DNA, providing a sensitive spectroscopic handle with which to characterize non-covalent binding interactions such as intercalation.

Recently, it was reported that dipyridophenazine (dppz) complexes of ruthenium(II) may serve as "molecular light switches" for DNA.[3] Excitation of the dppz complexes with visible light (440 nm) leads to localized charge transfer from the metal center.[4] In aqueous solutions, the emission resulting from this MLCT excited state is deactivated via a non-radiative energy transfer from the phenazine nitrogens to solvent water molecules. Both steady-state and time-resolved quenching studies in which water was used to quench the emission of the complex in anhydrous acetonitrile give a k_{H_2O}/k_{D_2O} of 2.2[5], which is consistent with the notion of quenching through vibrational deactivation via a hydrogen bonding pathway. When double-stranded DNA is added to aqueous solutions of the complex, the complex binds by intercalation, an interaction which protects the phenazine nitrogens from solvent water molecules, resulting in bright photoluminescence. Shown in Fig. 1 are the emission spectra of the complex in the absence and presence of calf thymus DNA. In the absence of DNA, there is no detectable emission. Upon addition of double-stranded DNA, the complex luminesces intensely.

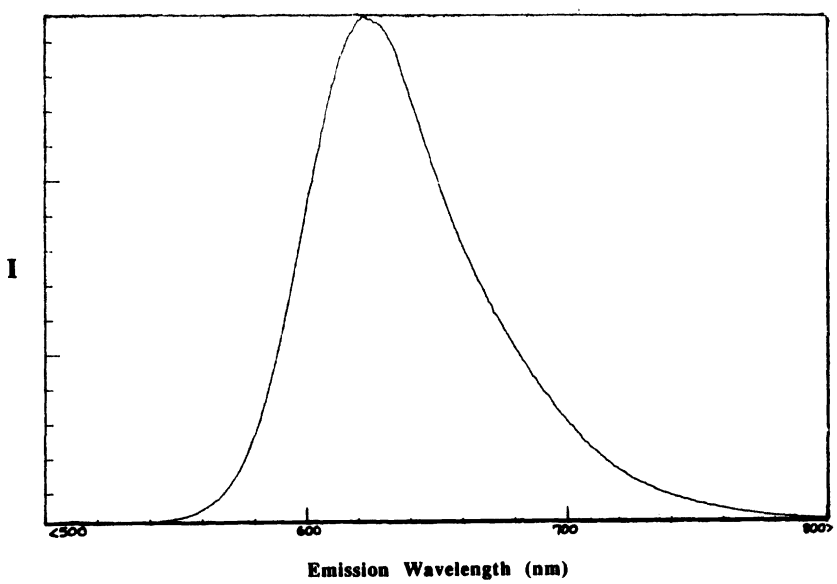


Figure 1. Emission spectra of $[Ru(phen)_2dppz]^{2+}$ in the absence and presence of calf thymus DNA.

The dppz ligand has an extended aromatic surface area which enables the complex to easily intercalate between adjacent base pairs of the DNA double helix, giving rise to binding constants on the order of 10^7 M^{-1}. This unwinding is consistent with intercalation. Figure 2 shows the results of an assay in which pBR322 DNA has been unwound with topoisomerase I in the presence of increasing amounts of $[Ru(bpy)_2dppz]^{2+}$ (bpy = 2,2'-bipyridyl). The agarose gel displaying the unwinding has been visualized using a solution of the ruthenium complex as a luminescent stain. The

emission enhancement of the complex upon binding to DNA is $\geq 10^4$, compared to an enhancement of ~20 for ethidium.[6] Due to the substantial emission enhancement of the ruthenium complex, no destaining step is required to eliminate background luminescence, which would be advantageous in instances requiring high contrast. The luminescence quantum yield for the ruthenium complex is, however, lower than for ethidium, so that experiments requiring high signal would benefit more from application of ethidium.

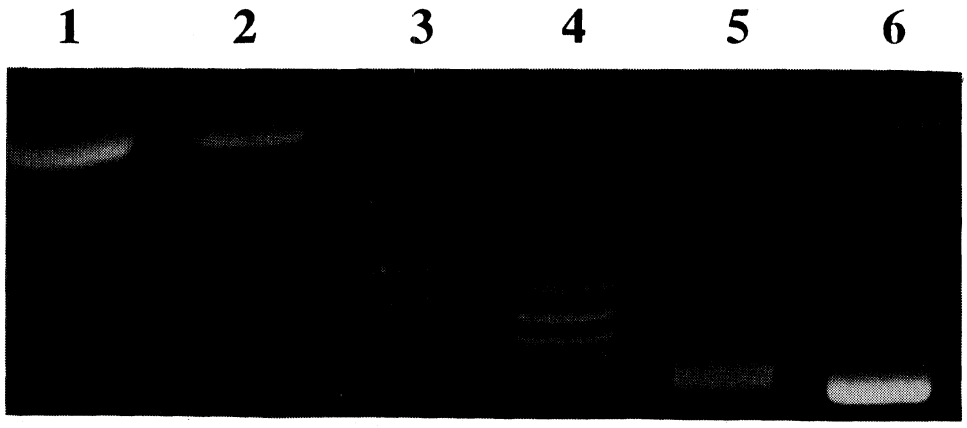


Figure 2. Agarose gel showing unwinding of pBR322 DNA using topoisomerase I in the presence of increasing amounts of ruthenium complex. Lanes 1-5 show the topoisomers present after incubation of DNA, enzyme, and increasing ruthenium concentrations from 3.9 to 8.3 μM. Lane 6 shows DNA incubated without enzyme or ruthenium complex. The gel has been stained with a solution of the ruthenium complex.

Shown in Table 1 are the changes in the steady-state and time-resolved emission characteristics observed upon binding to various DNAs by $[Ru(phen)_2dppz]^{2+}$.

Table 1. Emission Characteristics of $[Ru(phen)_2dppz]^{2+}$ upon Binding to Nucleic Acids of Varying Conformations.[a]

Nucleic Acid	τ [b](ns)	%[c]	λ_{max} (nm)	R.I.[d]
Calf Thymus DNA	770	40	617	0.94
	120	60		
Z-poly [d(GC)] •	270	60	608	0.60
poly [d(GC)]	70	40		
poly [r(AU)] •	490	20	620	0.10
poly [r(AU)]	80	80		
poly (dT) • poly (dA) •	530	60	621	1.45
poly (dT)	170	40		

[a]All measurements were conducted at 20°C using 10 μM ruthenium/100 μM nucleotides and excitation at 440 nm. [b]Error is estimated to be 10% for both steady-state and time-resolved measurements. [c]The percentage of each component of the biexponential emission decay was calculated from the magnitudes of the preexponential factors produced by the fitting program. [d]Relative emission intensities (R.I.) were determined as a ratio of emission relative to a 10 μM $[Ru(bpy)_3]^{2+}$ solution.

It is evident that the luminescent parameters for the metal complex vary depending upon helix conformation and base composition. What is particularly interesting to note is the correlation between the luminescent lifetimes and the relative luminescent intensities with how well the conformations of each of the different helices may shield the phenazine nitrogens from the solvent. This correlation is most clearly illustrated in the cases of poly [r(AU)] · poly [r(AU)] and the triple helix. In A-form nucleic acids such as poly [r(AU)] · poly [r(AU)], the base pairs are pushed back toward the periphery of the major groove, creating a major groove which is very deep and very narrow.[7] The shape of this cavity hinders the intercalation of tris(phenanthroline) complexes of ruthenium(II)[1d] and likely also affects the longer dppz ligand in the same manner. This relatively poor protection results in short excited-state lifetimes and correspondingly low luminescent intensities. In the triple helix, poly (dT) · poly (dA) · poly (dT), a different effect is observed. Intercalation into the major groove of the triplex by the ruthenium complex results in an interaction where the base triples adjacent to the intercalating ligand completely surround the phenazine nitrogens, resulting in greater water protection and therefore longer luminescent lifetimes and higher luminescent intensities.

Deconvolution of the time-resolved luminescence decay traces results in a biexponential decay in emission, indicating the presence of two different, distinguishable modes of binding for the complex to DNA. Previous photophysical studies done on polypyridyl complexes of ruthenium(II) were consistent with two possible modes of binding to DNA[8]; one is a surface bound mode in which the ancillary ligands of the metal complex rest against the minor groove of DNA and the other, an intercalative mode where one of the ligands inserts between adjacent base pairs in the double helix. Quenching studies using both cationic quenchers such as $[Ru(NH_3)_6]^{3+}$ (unpublished data) and anionic quenchers such as $[Fe(CN)_6]^{4-}$ have indicated that for the dppz complexes, both binding modes may be intercalative in nature.[5] Figure 3 shows

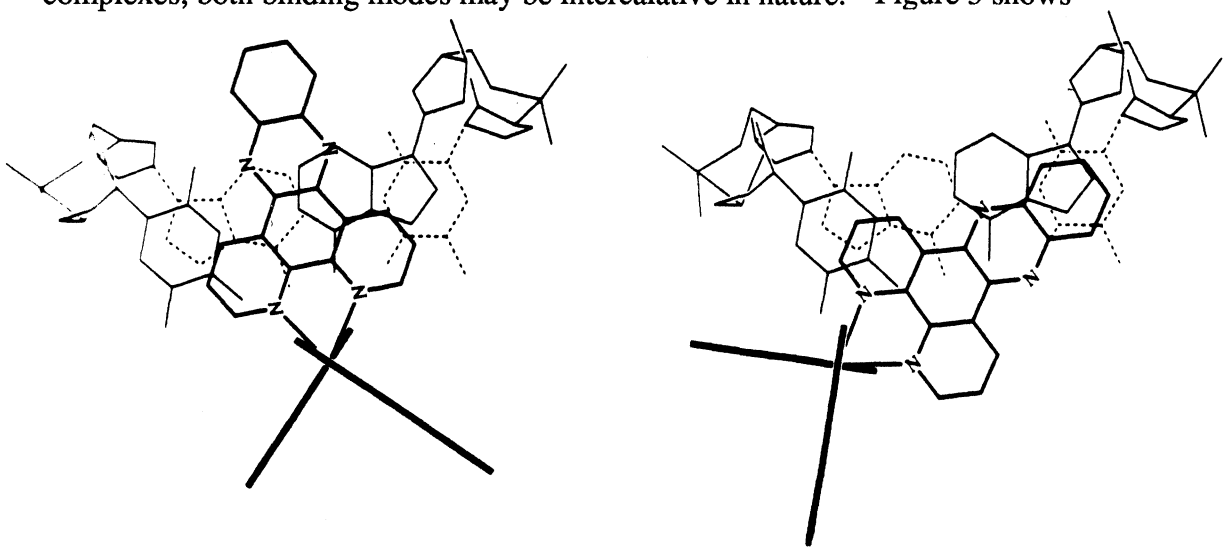
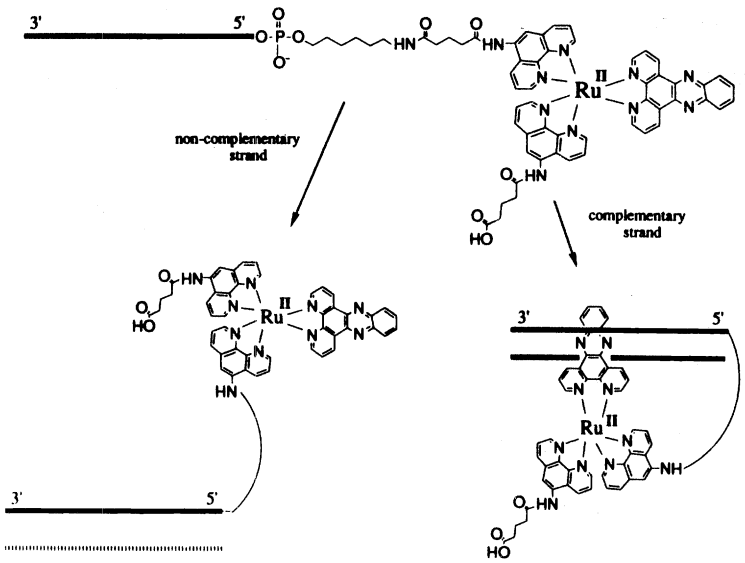


Figure 3. "Front-on" (left) and "side-on" (right) models of binding of $[Ru(phen)_2dppz]^{2+}$ to DNA. The view is looking down the helix axis with the major groove on the bottom. Note the difference in the overlap of the intercalated ligand with the base pairs above and below for the two models. In the side-on mode of binding, the phenazine nitrogens of the dppz are more exposed to the solvent than in the front-on mode, which may result in substantial differences in the luminescence.

a MACROMODEL generated illustration[9] displaying two possible intercalative binding modes for the complex which are consistent with the results obtained here and with photophysical studies done on various dppz derivatives in which the ligand has been substituted in either symmetric or asymmetric fashion.[3c] In the model on the left, the long axis of the dppz is perpendicular to the DNA dyad axis ("front-on intercalation") whereas in the right model, the long axis of the dppz is almost parallel to the DNA dyad axis ("side-on" intercalation). In the "front-on" model of intercalation, there are bases stacked both above and below the intercalating ligand which effectively shield it from water, thus accounting for the long component of the lifetime. In the "side-on" model of intercalation, one of the phenazine nitrogens of the intercalating ligand actually protrudes into the major groove. In this mode of binding, the intercalating ligand is much more accessible to water, resulting in a substantial decrease in the excited-state lifetime.

It has therefore been established that the ruthenium complex, $[Ru(phen)_2dppz]^{2+}$, may serve as a "molecular light switch" for double-helical DNA and that in addition, the light switch effect is a sensitive reporter of the helix environment. How could these novel optical properties be utilized in the detection of DNA? If we covalently attached an oligonucleotide to the ruthenium complex, we could potentially make this luminescent effect sequence-specific (see Scheme I). If the single-stranded metal complex were added to a non-complementary sequence, no duplex would form and complex intercalation could not occur, therefore resulting in no luminescence. If, however, the single-stranded metal complex were added to the complementary sequence, then duplex formation would create a platform for the metal complex to intercalate into, thus preserving the emission of the complex and signaling the presence of the desired sequence.

Scheme 1



An oligonucleotide derivatized at its 5'-terminus with a ruthenium complex was therefore prepared by coupling a 15-mer functionalized with a hexyl amine linker to $[Ru(phen')_2dppz]^{2+}$ (phen' = 5-amidoglutaric acid-1,10-phenanthroline). Figure 4 shows the emission spectra of the metalated oligonucleotide in the absence and the presence of different DNAs. By itself, the single-stranded metal complex shows little detectable luminescence. When annealed to its complementary strand, bright photoluminescence is observed. In contrast, in the presence of a non-complementary strand, there is little

emission enhancement. In effect, the oligonucleotide-derivatized metal complex functions as a sequence specific molecular light switch.

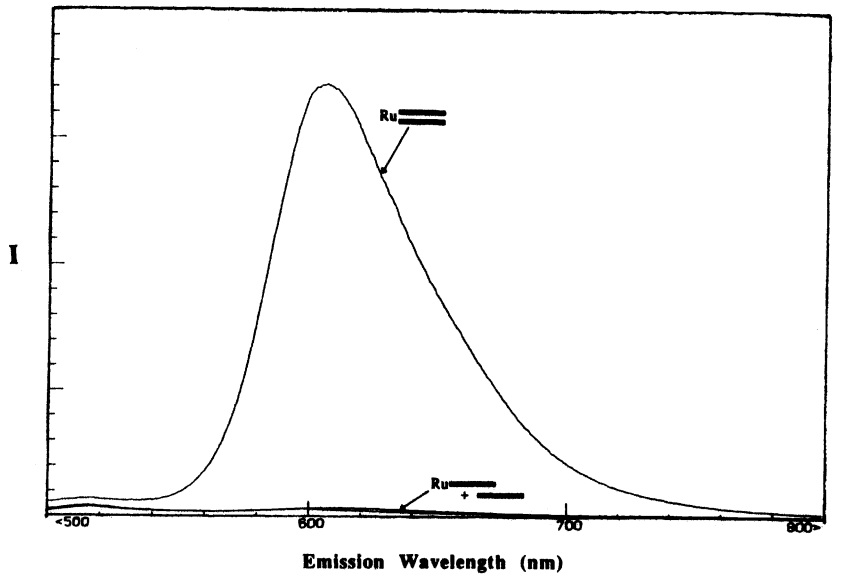


Figure 4. Emission spectra of the metalated oligonucleotide by itself (bottom emission trace), in the presence of a non-complementary strand (Ru– + —), and in the presence of complementary strand (Ru═).

We have explored the sensitivity of the emission of this metalated oligonucleotide to the presence of base mismatches at various positions along the duplex. Table 2

Table 2. Luminescence data for the metalated oligonucleotide annealed to a series of oligonucleotides containing base mismatches near or far from the metal intercalation site.

Sample	*Number of base mismatches*	*R.I.*
ACGTTCGAACCGTGA TGCAAGCTTGGCACT + Ru	0	1
ACGTTCGAACCGTGA-$(CH_2)_6$-NH_2—(Ru) TGCAAGCTTGGCACT	0	1.16
ACGTTCGAACCGTGA-$(CH_2)_6$-NH_2—(Ru) TGCAAGCTTGGCCCT	1	.68
ACGTTCGAACCGTGA-$(CH_2)_6$-NH_2—(Ru) TGCCAGCTTGGCACT	1	.37
ACGTTCGAACCGTGA-$(CH_2)_6$-NH_2—(Ru) TGCCAGCTTGACACT	2	.26
ACGTTCGAACCGTGA-$(CH_2)_6$-NH_2—(Ru) TACCCGCCTGACACT	5	0.11

All mismatch experiments were conducted at duplex concentrations of 5 μM. The relative luminescent intensity of the duplex with 5 base mismatches is comparable to that of the single-stranded metal complex by itself.

The luminescence generally correlates with the relative stability of the duplexes. The luminescence is decreased with a mismatch near the 5' terminus of the metalated strand, at a site where complex intercalation is likely, but is decreased to much greater extent when the mismatch is at the 3' terminus; the destabilizing effects of a base mismatch near the 5'end may be moderated by the stabilizing effect of complex intercalation but a similar compensation may not be available at the 3'-end. The mismatch results, therefore, are consistent with intramolecular intercalation by the dppz complex tethered to the 5' terminus.

These results taken together establish that an oligonucleotide functionalized with a dppz complex of ruthenium can be used to target single-stranded DNA in a sequence-specific fashion. This complex could be extremely valuable in the development of novel hybridization probes both for heterogeneous and homogenous assays.

APPENDIX

Experimental Methods

Buffers and Chemicals. All emission experiments were conducted at pH 7.0 in Milliport water containing 50 mM NaCl/5 mM Tris-OH buffer. $RuCl_3 \cdot 3H_2O$ was purchased from Johnson and Matthey/AESAR and used without further purification. Glutaric acid monoethylester chloride was purchased from Fluka. Dicyclohexyl-carbodiimide and 1-hydroxybenzotriazole were from Aldrich Chemicals.

Oligonucleotides. All oligonucleotides used in experiments were synthesized on an Applied Biosystems DNA Synthesizer Model 392 and were purified using reverse phase HPLC (Hewlett-Packard 1050 HPLC, Dynamax C_{18} or Vydac C_{18} column). The 5'-amino modifier (Aminolink 2) was purchased from Applied Biosystems, Inc.

Spectroscopic Measurements. For instrumentation, see Y. Jenkins; A.E. Friedman; N.J. Turro; and J.K. Barton, *Biochemistry*, **1992**, *31*, 10809.

Preparation of bis(5-amidoglutaric acid-1,10-phenanthroline)(dipyrido[3,2-a:2',3'-c]phenazine)ruthenium(II)(hexafluorophosphate), [Ru(phen')$_2$dppz](PF$_6$)$_2$. [Ru(phen')$_2$dppz](PF$_6$)$_2$ was prepared analogously to the method of Amouyal, E.; Homsi, A.; Chambron, J.-C; Sauvage, J.-P. *J. Chem. Soc. Dalton Trans.*, **1990**, 1841. Product was characterized using ^{1}H-NMR and FAB-MS.

Coupling Reaction. Dicyclohexylcarbodiimide and 1-hydroxybenzotriazole were added to a solution of [Ru(phen')$_2$dppz](PF$_6$)$_2$ (C_1 isomer) in 1:1 DMF/dioxane. Aminolinked oligonucleotide, 5'-$H_2N(CH_2)_6$AGTGCCAAGCTTGCA-3', was added to the activated metal complex solution as a suspension followed by the addition of LiOH. After shaking at 37°C and monitoring by reverse-phase HPLC, the reaction was quenched with water and the desired product isolated on a Hewlett Packard 1050 HPLC system (Vydac C_{18} column using a triethylamine-acetic acid/acetonitrile gradient). Product analysis was conducted by ultraviolet-visible (ε(440 nm) = 2.1 x 10^4 $M^{-1}cm^{-1}$), atomic absorption spectroscopy, colorimetry for phosphate (Lindberg, O.; Ernsten, L. *Meth. Biochemical Analysis*, **3**, D. Glick, Ed.; Interscience: New York, 1954), and enzymatic digestion for detection of DNA bases.

ACKNOWLEDGEMENTS

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