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Highly Efficient Visible-Light-Induced Photocleavage of DNA by a Ruthenium-Substituted Fluorinated Porphyrin

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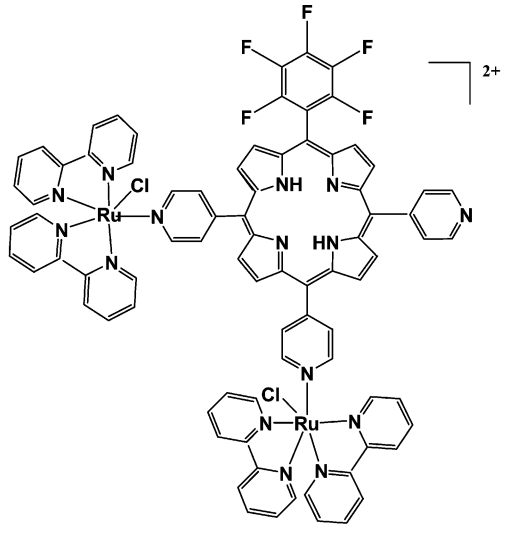
Keywords: Photodynamic therapy / Porphyrin / Ruthenium / DNA cleavage / DNA binding / Sensitizers / Charge-transfer

A new porphyrin, *meso*-5-(pentafluorophenyl)-10,15,20-tris(4-pyridyl)porphyrin, has been synthesized. Coordination of two $[Ru(bipy)_2Cl]^+$ moieties (where bipy = 2,2′-bipyridine) to the pyridyl nitrogen atoms in the 10,15-positions gives the target complex. Electronic transitions associated with the ruthenium–porphyrin include an intense Soret band and four less intense Q-bands in the visible region of the spectrum. An intense π–π^* transition in the UV region associated with the bipyridyl groups and a metal-to-ligand charge-transfer (MLCT) band appearing as a shoulder to the Soret band are also observed. Electrochemical properties associated with the complex include a redox couple in the cathodic region with $E_{1/2}$ = –0.84 V vs. Ag/AgCl attributed to the porphyrin and a redox couple in the anodic region at $E_{1/2}$ = 0.79 V vs. Ag/AgCl due to the $Ru^{III/II}$ couple. DNA titrations and ethidium bromide displacement experiments indicate the ruthenium porphyrin interacts with DNA potentially through a partial intercalation mechanism. Irradiation of aqueous solutions of the ruthenated complex and supercoiled DNA at a 100:1 base pair/complex ratio with visible light above 400 nm indicates that the complex causes double-strand breaks of the DNA.

Introduction

Porphyrins have been extensively studied as potential photosensitizers in photodynamic therapy (PDT).[1–3] Their planar aromatic structure coupled with their photophysical properties and synthetic versatility has made them attractive components for PDT.[4] PDT for cancer treatment is a non-invasive modality which utilizes light, a photosensitizer and molecular oxygen to create reactive oxygen species (ROS) capable of destroying malignant tumors. In addition to the advantages already mentioned for porphyrins as photosensitizers, they have been associated with a high affinity for tumor sites and an efficient formation of ROS.[5] Ruthenium-based cancer drugs have been of great interest over the past two decades in part due to their similarities to iron in biological systems, their numerous stable redox states under physiological conditions and the extensive library of synthetic complexes incorporating ruthenium.[6] Ruthenium–polypyridyl complexes have demonstrated the ability to create ROS when irradiated at the metal-to-ligand charge-transfer (MLCT) band, resulting in efficient photocleavage of supercoiled DNA.[7–10] An important issue in the development of photosensitizers for PDT is their ability to efficiently create the ROS, which can be linked to the photosensitizer's quantum efficiency. Fluorinated porphyrins have been studied due to their effective energy absorption and greater triplet quantum yields.[11]

This report describes the synthesis and characterization, by ^1H NMR spectroscopy, elemental analysis, UV/Vis spectroscopy and cyclic voltammetry of a new complex, which incorporates ruthenium(II)–polypyridyl moieties with a pentafluorophenyl-substituted porphyrin (**I**).



I

The ability of this complex to bind to calf thymus (CT) DNA and photocleave supercoiled DNA (pUC18) when irradiated with visible light is presented.

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Results and Discussion

Synthesis and Characterization

By using the appropriate stoichiometric ratio of aldehydes, 4-pyridinecarbaldehyde and pentafluorobenzaldehyde, to give a porphyrin composed of three *meso*-pyridyl and one *meso*-(pentafluorophenyl) substituent, led, in addition to 90% polymeric material, to the formation of six different porphyrins. Separation to obtain the target porphyrin proved challenging. With the appropriate choice of mobile phase the target porphyrin was obtained in low but not unreasonable yields for this procedure.[12] Coordination of $Ru(bipy)_2Cl_2$ to the pyridyl nitrogen atoms was accomplished by previously described procedures.[13] It has been noted that coordination of three $Ru(bipy)_2Cl_2$ moieties to the pyridyl nitrogen atoms of a porphyrin is extremely difficult under refluxing conditions in acetic acid.[14] However, coordination of two $Ru(bipy)_2Cl_2$ moieties under these conditions we found to be quite facile. Problems arise in separating the 10,15-bis(ruthenium–pyridyl)-substituted porphyrin from the 10,20-bis(ruthenium–pyridyl)-substituted porphyrin. Because the isomers' UV/Vis spectra, cyclic voltammograms, elemental analyses and ^1H NMR spectra are indistinguishable, the determination of whether we had all 10,15-bis(pyridyl)-substituted, all 10,20-bis(pyridyl)-substituted or a mixture of both proved challenging. By assuming that the isolated compound was a mixture, column chromatography on neutral alumina as stationary phase with acetonitrile as the eluent allowed for separation. Three bands appeared on the column. The first band, determined to be the mono(ruthenated) porphyrin, was discarded. The second band from the column represented only a trace amount of ruthenated porphyrin, whereas the third band was a tractable amount of compound. The last band off of the column was presumed to be the ruthenated 10,15-bis(pyridyl)porphyrin **I**, because it is expected to be more polar than the ruthenated 10,20-bis(pyridyl)porphyrin.

The electronic spectrum of complex **I** in acetonitrile at room temperature (Figure 1) shows very intense absorption bands in the visible region. The Soret band at 412 nm is only slightly shifted from the Soret band associated with the free-base porphyrin. Four less intense Q-bands in the 500–650 nm region are also associated with the porphyrin. Two less intense shoulders occurring just above and below the Soret band are attributed to $Ru(d\pi) \rightarrow bipy(\pi^*)$ metal-to-ligand charge-transfer (MLCT) transitions. The more intense absorption band at 295 nm is associated with the $bipy(\pi) \rightarrow bipy(\pi^*)$ charge-transfer transition.

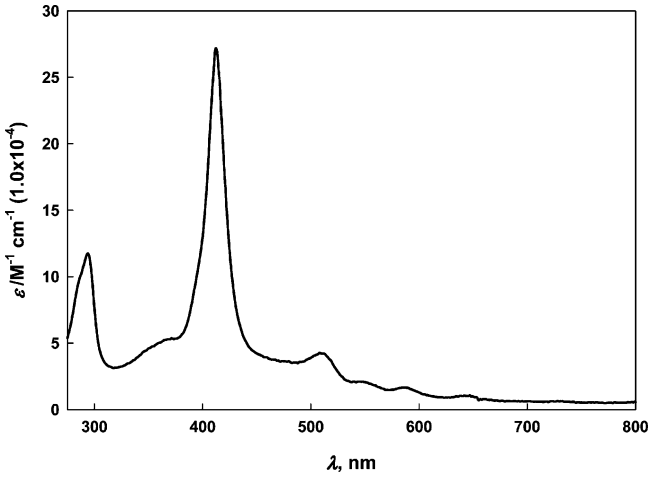

Figure 1. UV/Vis spectrum of complex **I** in acetonitrile at room temperature.

Electrochemistry

Solution-phase cyclic voltammetry in dry acetonitrile containing Bu_4NPF_6 as supporting electrolyte was performed by using a three-electrode system with a glassy-carbon working electrode. Figure 2 illustrates the results of this study where (A) is the cyclic voltammogram (CV) of the free-base porphyrin, (B) is the CV of the $Ru(bipy)_2Cl_2$ complex in the absence of the porphyrin and (C) is the CV of the ruthenated porphyrin complex **I**.

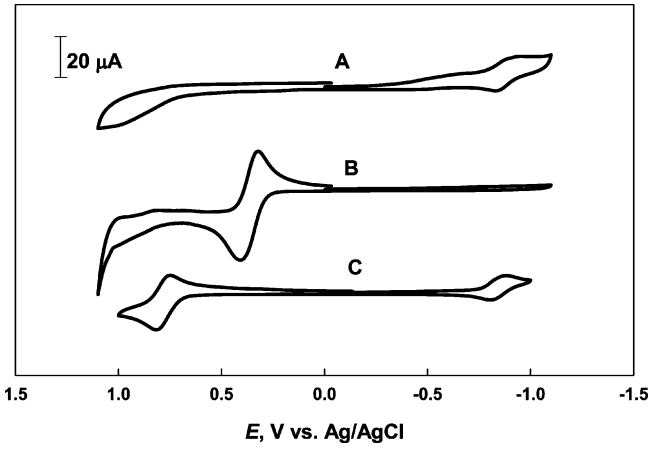

Figure 2. Cyclic voltammograms of complex **I** (C), the free-base porphyrin (A), and $Ru(bipy)_2Cl_2$ (B) in 0.1 M Bu_4NPF_6 at room temperature. v = 100 mV/s.

When the porphyrin solution is anodically cycled, a weak irreversible oxidation appears beyond 1.0 V vs. Ag/AgCl. This is due to oxidation of the porphyrin. In the cathodic direction one quasi-reversible redox wave with $E_{1/2}$ = –0.89 V vs. Ag/AgCl is observed and is associated with the one-electron reduction of the porphyrin to form the radical anion. The CV (B) (Figure 2) shows no redox activity in the cathodic direction indicating that reduction of 2,2′-bipyridine is more difficult than that of the porphyrin. In the anodic direction, a reversible redox couple with an $E_{1/2}$ value of 0.37 V is observed and can be attributed to the oxidation of the Ru^{II} center to Ru^{III}. The CV (C) (Figure 2) shows a reversible redox couple in the cathodic direction with an $E_{1/2}$ value of –0.84 V and a reversible redox couple in the anodic direction with an $E_{1/2}$ value of 0.79 V. The cathodic redox couple is very similar to that observed for the porphyrin alone and can therefore be attributed to the one-electron reduction of the porphyrin to the radical anion. The reversible redox couple in the anodic direction is attributed to the $Ru^{III/II}$ couple but has shifted by ca. 400 mV compared to the $Ru^{III/II}$ couple in CV (B). This

is expected when the chloride is replaced by a strong π-backbonding ligand like pyridyl, which stabilizes the Ru^{II} oxidation state making it more difficult to oxidize Ru^{II} to Ru^{III}. In addition, the coordinated Ru^{II} moieties of complex **I** are electronically independent of one another, because there is only one redox couple associated with both Ru^{II} metal centers. In addition, the ratio of the current associated with the Ru^{II} centers and the porphyrin is 2:1, consistent with coordination of two Ru^{II} complexes to one porphyrin unit.

DNA Binding Studies

A quantitative look at the ability of complex **I** to bind to CT-DNA was performed by using spectrophotometric titrations. Aqueous solutions of constant concentration of **I** were titrated with pH = 7.2 buffered solutions of CT-DNA. Figure 3 illustrates the results of this experiment. The Soret band as well as the band associated with the bipyridyl π–π* transition decrease in intensity upon addition of CT-DNA. The intrinsic binding constant K_b for the CT-DNA/complex **I** interaction was determined by a Scatchard plot according to Equation (1), where ε_a = absorbance/[complex **I**], ε_b and ε_f are the extinction coefficients for the fully bound and free form of complex **I**, respectively.[15] A linear fit of the plot of [DNA]/(ε_a – ε_f) vs. [DNA] gives a slope of 1/(ε_b – ε_f) and an intercept of 1/K_b(ε_b – ε_f). The binding constant determined by this method was 1.4×10^6 M^{-1}. This value is suggestive of intercalation but does not exclude the possibility of other binding interactions.[16]

$$[DNA]/(\varepsilon_a - \varepsilon_f) = [DNA]/(\varepsilon_b - \varepsilon_f) + 1/K_b(\varepsilon_b - \varepsilon_f) \quad (1)$$

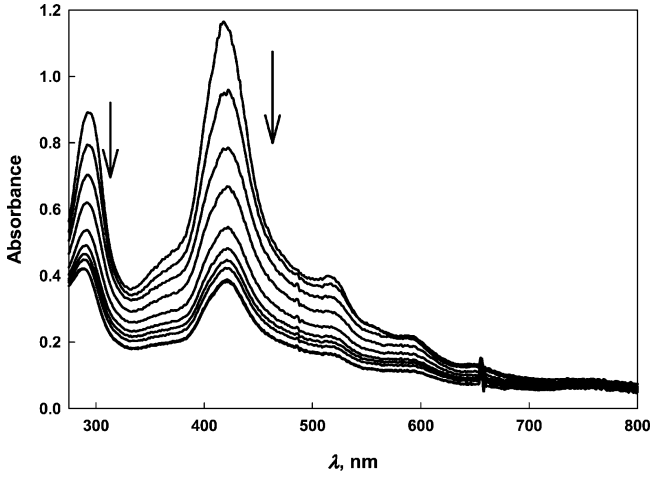

Figure 3. Absorption spectra of complex **I** in the presence of increasing amounts of CT-DNA, [complex **I**] = 1.0 μM, [DNA] = 0–30 μM.

For further proof of intercalation, an ethidium bromide (EthBr) competitive binding study was undertaken.[17] Figure 4 shows the electronic spectra of a buffered solution (43 μM, pH = 7.2) of EthBr (a). Spectrum (b) (Figure 4) represents a buffered solution, which is 45 μM in EthBr and 78 μM in CT-DNA. As the EthBr intercalates into the DNA, its absorption band decreases and shifts to lower energy. To this solution were added ca. 10 drops of a 10 μM solution of complex **I** [Figure 4 (c)]. Upon addition of complex **I**, the spectrum begins to revert back to its original spectrum prior to addition of DNA and complex **I**, again suggesting a competitive interaction for intercalation into DNA between EthBr, a known intercalator, and complex **I**. Due to the bulk of complex **I**, its interaction with DNA is most likely through a combination of groove binding and partial intercalation.[18] We have recently reported on the DNA binding of the dirutheneted 5,10-bis(pentafluorophenyl)-15,20-bis(4-pyridyl)porphyrin by similar methods.[19] The binding constant, determined to be 7.6×10^5 M^{-1}, was only slightly smaller than the K_b value for complex **I** (reported here) suggesting a similar binding mechanism.

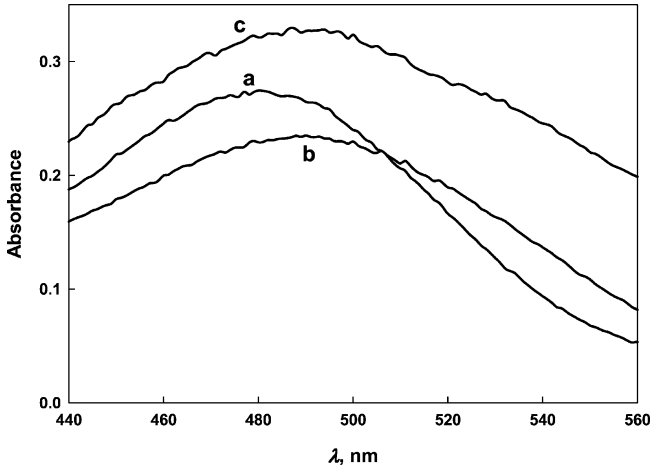

Figure 4. Absorption spectra: (a) EthBr, 4.5×10^{-5} M; (b) a + DNA 7.8×10^{-5}; (c) b + complex **I**.

Photocleavage Studies

When buffered, pH = 7.2, solutions of circular plasmid DNA (pUC18) containing complex **I**, in a ratio of 100:1 base pairs/metal complex were irradiated with a 100 W mercury arc lamp filtering out the UV light below 400 nm the supercoiled DNA was converted into the nicked form of DNA within 2 min of irradiation (Figure 5). Lane 10 (Figure 5) shows the supercoiled DNA in the absence of complex **I** prior to irradiation. Lanes 11–18 represent the irradiated supercoiled DNA taken at 2 min intervals. It is clear that within the irradiation time of the experiment the supercoiled DNA is intact in the absence of complex **I**. Lane 9 shows the supercoiled DNA and complex **I** at a 100:1 base pair/complex ratio before irradiation indicating that in the absence of light complex **I** does not react with DNA. After 2 min of irradiation with visible light (Lane 8, Figure 5) some of the supercoiled DNA is converted into the nicked form (**III**, Figure 5), a single-strand break, and some is converted into the relaxed or linear form (**II**, Figure 5). The linear form is most likely the result of multiple single-strand breaks. After 4 min of irradiation, all of the supercoiled DNA has been nicked; however, further irradiation (Lanes 1–7) shows a smear of the DNA on the gel, indicative of total DNA degradation.[20]

Compared to the reported DNA photocleavage experiments incorporating the diruthenated 5,10-bis(pentafluorophenyl)-15,20-bis(4-pyridyl)porphyrin, which requires a 5:1 base pair/complex ratio and longer irradiation times,[19] complex **I** is a more efficient photocleavage agent. The reasons for this unusual observation are currently being investigated.

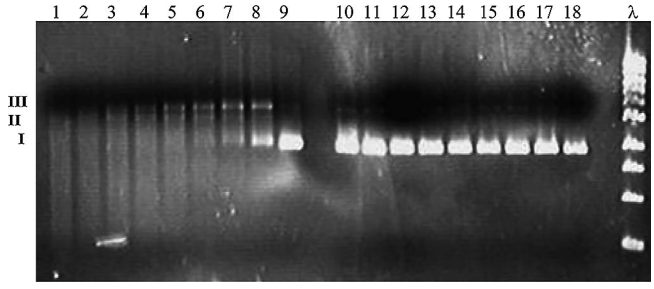

Figure 5. Gel electrophoresis of circular plasmid DNA (pUC18) in the presence of complex **I** at a 100:1 ratio of base pair/metal complex (Lanes 1–9) and without complex **I** (Lanes 10–18) and molecular-weight standard λ. Samples were irradiated with a 100 W mercury arc lamp equipped with a long-pass filter cutting off wavelengths below 400 nm. Samples were taken at 2 min intervals.

Conclusions

This report describes the synthesis of a new porphyrin combining pyridyl and pentafluorophenyl groups. Two of the pyridyl groups in the 10,15-positions are used to coordinate Ru^{II}–polypyridyl complexes. Investigation of the electronic transitions along with cyclic voltammetry experiments indicate a weak orbital interaction between the Ru^{II} metal centers and the porphyrin ring. Studies suggest a strong affinity for DNA and a partial intercalative mode of binding. Unprecedented double-strand cleavage of supercoiled DNA, when dilute solutions of the complex and DNA are briefly irradiated at energies lower than 400 nm, suggests a very unique interaction of this new photosensitizer with DNA. Further studies of this highly toxic light-induced photosensitizer with malignant tumor cells are currently underway.

Experimental Section

Materials: 4-Pyridinecarbaldehyde (ACROS), pentafluorobenzaldehyde (ACROS), propionic acid (ACROS), ammonium hydroxide (Fisher), methanol (Fisher), acetone (Fisher), acetonitrile (Fisher), diethyl ether (Fisher), dichloromethane (Fisher), ethyl acetate (Fisher), ethanol (Fisher), $RuCl_3$ trihydrate (Aldrich), 2,2′-bipyridine (Aldrich), silica gel (60–200 mesh) (Fisher), neutral alumina (Fisher), ammonium hexafluorophosphate (ACROS), glacial acetic acid (Fisher), tetrabutylammonium hexafluorophosphate (Bu_4NPF_6, used as supporting electrolyte for electrochemistry) (Aldrich), and ultra-dry (< 50 ppm H_2O) acetonitrile (for electrochemistry measurements) (Aldrich) were used without further purification. Pyrrole (Aldrich) was vacuum-distilled prior to use. *cis*-$Ru(bipy)_2Cl_2$ was synthesized as described previously.[21] The plasmid pUC18 was obtained from Bayou Biolabs. Electrophoresis-grade low-EEO agarose, tris(hydroxymethyl)aminomethane (Tris), boric acid and ethidium bromide (EthBr) were obtained from Fisher. The spectroscopic titrations were carried out at room temperature in a buffer (5 mM Tris-HCl, 0.1 M NaCl, pH = 7.2). Concentrations of the calf thymus DNA (Sigma) solutions used in the titrations were determined spectrophotometrically by using the extinction coefficient 6600 $M^{-1}cm^{-1}$ at 260 nm.[22] All aqueous solutions were prepared by using doubly distilled water. Elemental analyses were performed by Atlantic Microlab, Norcross, Ga.

***meso*-5-(Pentafluorophenyl)-10,15,20-tris(4-pyridyl)porphyrin:** A solution of 4-pyridinecarbaldehyde (4.3 mL, 45 mmol) and pentafluorobenzaldehyde (1.9 mL, 15 mmol) in propionic acid (100 mL) was first heated for 10 min. Freshly distilled pyrrole (4.2 mL, 60 mmol) was added to this solution, and the reaction mixture was heated to reflux for 2 h. The reaction mixture was cooled to room temperature. Then the mixture was divided into two fractions (50 mL each), and each fraction was neutralized with ice cooled methanol/ammonia (50:50). The slurry was filtered by vacuum filtration, and the powder was air-dried. The powder was dissolved in dichloromethane and chromatographed on silica gel with ethanol/ethyl acetate (50:50) as eluent. Three bands were separated. The third band off the column was collected to yield 120 mg (0.17 mmol, 1.11% yield). UV/Vis (CH_3CN): λ_{max} ($\varepsilon \times 10^{-4}$ $M^{-1}cm^{-1}$) = 414 (51.4), 509 (2.28), 542 (0.63), 585 (0.84), 640 (0.30) nm. ^1H NMR (300 MHz, $CDCl_3$, TMS): δ = 9.08 (6 H, 2,6-pyridyl), 8.9 (d, 8 H, pyrrole), 8.25 (6 H, 3,5-pyridyl), –2.92 (s, 2 H, internal pyrrole) ppm. $C_{41}H_{22}F_5N_7 \cdot H_2O$ (725.64): calcd. C 67.86, H 3.33, F 13.09, N 13.51; found C 67.71, H 3.19, F 12.73, N 13.31.

[*cis*-H_2MPFPTPyPRu_2(bipy)$_4Cl_2(PF_6)_2$]: A solution of porphyrin (0.050 g, 7.1×10^{-2} mmol) and *cis*-$Ru(bipy)_2Cl_2$ (0.077 g, 1.6×10^{-1} mmol) in glacial acetic acid (5 mL) was heated at reflux under nitrogen for 45 min. The glacial acetic acid was removed under reduced pressure, and methanol (2 mL) was added to the residue before being heated at reflux under nitrogen for 45 min. The reaction mixture was added dropwise to an aqueous solution of saturated ammonium hexafluorophosphate (100 mL) with stirring. The mixture was gravity-filtered, and the precipitate was vacuum-filtered for 10 min. The precipitate was chromatographed on a column of neutral alumina with acetonitrile as solvent. Of the three bands to come off the column, the third band was isolated, dissolved in a minimum of acetonitrile (ca. 3 mL) and flash-precipitated by addition to diethyl ether (50 mL). The product was gravity-filtered and vacuum-dried overnight to give 0.019 g (0.0097 mmol, 13% yield). UV/Vis (CH_3CN): λ_{max} ($\varepsilon \times 10^{-4}$ $M^{-1}cm^{-1}$) = 295 (11.6), sh 370 (5.31), 412 (27.2), 512 (4.23), 548 (2.10), 587 (1.67), 642 (1.07) nm. $C_{81}H_{54}Cl_2F_{17}N_{15}P_2Ru_2H_2O$ (1913.65): calcd. C 50.85, H 2.95, N 10.98; found C 50.46, H 2.99, N 10.59.

Measurements: ^1H NMR spectra were recorded with a Bruker 300 MHz spectrometer by using $CDCl_3$ as the solvent and TMS as the internal standard. UV/Vis spectra were recorded at room temperature by using a Shimadzu 1501 photodiode array spectrophotometer with 2 nm resolution. Samples were measured in dry acetonitrile using 1 cm quartz cuvettes.

Electrochemistry: Cyclic voltammograms were recorded by using a one-compartment, three-electrode cell (CH-Instruments), equipped with a platinum wire auxiliary electrode. The working electrode was a 2.0 mm diameter glassy-carbon disk (CH-Instruments), which was polished first by using 0.30 μm followed by 0.05 μm alumina polish (Buehler) and then sonicated for 10 s prior to use. Potentials were referenced to an Ag/AgCl electrode (CH-Instruments). The supporting electrolyte was 0.1 M tetrabutylammonium hexafluorophosphate (Bu_4NPF_6), and the measurements were carried out in extra-dry (< 50 ppm water) acetonitrile.

Photocleavage of Circular Plasmid DNA: Buffered solutions of pUC18 and pUC18/complex **I** at a 100:1 ratio of base pair/metal

complex were placed side by side in quartz cuvettes and irradiated with a 100 W mercury arc lamp (Oriel) equipped with a colored glass filter (Newport FSR-GG420) blocking wavelengths shorter than 400 nm. Samples were taken at 2 min intervals over a 16 min period and submitted to electrophoresis in 1 % agarose gel by applying 150 V in approximately 300 mL of Tris buffer solution for 1 h. The gels were stained with ethidium bromide and photographed by using UV illumination.

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