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Comparison of Mass Spectrometry and Other Techniques for Probing Interactions Between Metal Complexes and DNA

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Electrospray ionization mass spectrometry (ESI-MS) was used to study the binding interactions of two series of ruthenium complexes, $[Ru(phen)_2L]^{2+}$ and $[RuL'_2(dpqC)]^{2+}$, to a double stranded DNA hexadecamer, and derive orders of relative binding affinity. These were shown to be in good agreement with orders of relative binding affinity derived from absorption and circular dichroism (CD) spectroscopic examination of the same systems and from DNA melting curves. However, the extent of luminescence enhancement caused by the addition of DNA to solutions of the ruthenium complexes showed little correlation with orders of binding affinity derived from ESI-MS or any of the other techniques. Overall the results provide support for the validity of using ESI-MS to investigate non-covalent interactions between metal complexes and DNA.

Introduction

Interest in the non-covalent binding of metal complexes to nucleic acids now spans a period of more than 20 years, and has been reviewed on several occasions.[1,2] During this time, much of the attention has focused on the interactions of mononuclear octahedral complexes of inert transition metals such as ruthenium and rhodium, with calf thymus DNA (CT DNA). These studies have been conducted using a wide variety of physical, spectroscopic, and biochemical techniques, and provided considerable information about the effect of changing the ligand environment on the nature and strength of the binding interactions. Determining how metal complexes bind non-covalently to DNA remains one of the most important questions in these studies, and has been investigated using many techniques. For example, fluorescence spectroscopy,[3–5] viscosity measurements,[6] molecular modeling,[7] circular dichroism spectroscopy,[8,9] and isothermal titration calorimetry[10] have all been used to study the binding interactions between CT DNA and the enantiomers of $[Ru(phen)_3]^{2+}$ and $[Ru(phen)_2(dppz)]^{2+}$ (dppz = dipyrido[3,2-*a*:2′,3′-c]phenazine). NMR spectroscopy has been the most widely used spectroscopic technique for providing information about which base pairs in short DNA sequences are involved in non-covalent binding to metal complexes.[11–18]

DNA offers a variety of binding sites and binding modes for non-covalent interactions with small molecules. The three most important binding modes are: (i) electrostatic interactions, (ii) groove binding and (iii) intercalation. Electrostatic interactions occur between cationic metal ions or organic molecules, and the polyanionic phosphate backbone of DNA,

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whereas groove binding involves direct interactions between atoms or functional groups present in a metal complex or organic molecule, and those present on the edge of the base pairs in either the major or minor groove of DNA. Intercalation is a common mode of non-covalent binding for small molecules that have polycyclic planar aromatic or heterocyclic ring systems, often in addition to a formal positive charge. These properties allow intercalators to insert and stack in between base pairs in the hydrophobic interior of helical double stranded DNA (dsDNA). Many molecules interact with DNA using a combination of the above binding modes that is dependent upon the specific structural characteristics of the compound under investigation.

Interest in how metal complexes bind non-covalently to DNA has been driven not only by a desire to understand the fundamentals of these interactions but also by the variety of potential applications that may result from these studies, including use as synthetic restriction enzymes,[19] DNA repair agents,[20] selective probes of DNA structure,[21] and artificial regulators of gene expression.[22] The development of metal complexes into diagnostic or therapeutic agents requires techniques that can rapidly and accurately provide information about the effects of structural alterations on DNA selectivity and affinity. We recently showed that electrospray ionization mass spectrometry (ESI-MS) offers several features, including a minimal sample requirement, speed of use, and ease of analysis.[23,24] In addition, ESI-MS was shown to provide detailed information about the number, relative amounts, and stoichiometry of non-covalent complexes formed in solutions containing dsDNA 16mers and ruthenium compounds with the general formula $[Ru(phen)_2L]^{2+}$, where L was either phenanthroline or a related bidentate heterocyclic ligand.

Because mass spectrometry is a gas-phase technique, its applicability for analyzing solution systems is contingent upon being able to reproducibly generate a mixture of ions that accurately reflects overall solution composition. Therefore, the first aim of this work was to compare the order of relative binding affinities determined previously by ESI-MS for the series $[Ru(phen)_2L_2]^{2+}$,[24] with those obtained by several other techniques. These were absorption spectroscopy, which was used to determine overall binding constants for the Ru/DNA interactions as well as obtain DNA melting curves, circular dichroism spectroscopy, and luminescence spectroscopy. A second aim of this study was to investigate the binding of the complexes $[RuL'_2(dpqC)]^{2+}$ (dpqC = dipyrido[3,2-*a*:2′,3′-c](6,7,8,9-tetrahydro)phenazine) to one of the dsDNA 16mers used in our previous study using ESI-MS and subsequently compare trends in binding affinity revealed by this method to those obtained by the other spectroscopic techniques. These complexes differ in the identity of the ancillary ligands (L′), which were phen, bpy, and various methylated derivatives. This system was also chosen for examination because it was anticipated that their interactions with DNA would be dominated by the intercalating dpqC ligand, and show relatively minor variations due to changes in the number of methyl groups present on the ancillary ligands. The interactions of these complexes with DNA would therefore provide a more rigorous test of the ability of ESI-MS to provide information about the relative binding affinities of metal complexes toward DNA in solution.

Experimental Section

Materials. $[Ru(phen)_3]Cl_2$ and $[Ru(bpy)_3]Cl_2$ were obtained from Aldrich, whereas all other ruthenium compounds were prepared by literature methods.[13,15,25] The structures of the ruthenium compounds used, which were present as racemic mixtures in all experiments described below are shown in Figure 1. Single-stranded oligonucleotides were obtained from Geneworks, South Australia, and purified using procedures previously reported.[26] The concentrations of oligonucleotides were estimated by measurement of UV absorbance at 260 nm using values of ϵ_{260} for adenine, guanine, cytosine, and thymine of 15 200, 12 010, 7050, and 8400 $M^{-1}cm^{-1}$, respectively.[27] Stock solutions of individual ruthenium compounds were prepared by dissolving the appropriate amount of compound in 0.1 M ammonium acetate (NH_4OAc), pH 8.5.

Stock solutions containing the double-stranded DNA (dsDNA) molecule D2, shown below, were prepared by heating equimolar quantities of the two single strands (0.4−2.0 mM) in 0.1 M NH_4OAc, pH 8.5, to 20 °C higher than the melting temperature for ∼15 min and then annealing by cooling slowly overnight. Complexes of dsDNA with individual ruthenium compounds were prepared by annealing samples of DNA and then adding the required amount of ruthenium compound. The same procedure was used for competition experiments, except that quantities of two ruthenium compounds were added after the annealing process to give a final Ru/Ru:dsDNA ratio of 3:3:1.

D2 d(CCTCGGCCGGCCGACC/GGTCGGCCGGCCGAGG).

All mass spectra were obtained using a Waters extended-mass-range Q-ToF Ultima mass spectrometer equipped with a Z-spray probe and a quadrupole mass analyzer with an *m*/*z* range of 32 000. All samples were diluted with 0.1 M NH_4OAc, pH 8.5, giving a final concentration of dsDNA of 10 μM. These were injected using a Harvard model 22 syringe pump (Natick, MA, USA) at a flow rate of 10 μL min^{-1}. Negative ion ESI spectra were obtained using a capillary tip potential of 2500 V, a cone voltage of 100 V, and desolvation temperature of 100 °C. The transport and aperture were both set to 5, while the RF lens 1 energy was 70 V. Spectra were typically acquired over the range *m*/*z* 500−3000, with between 50

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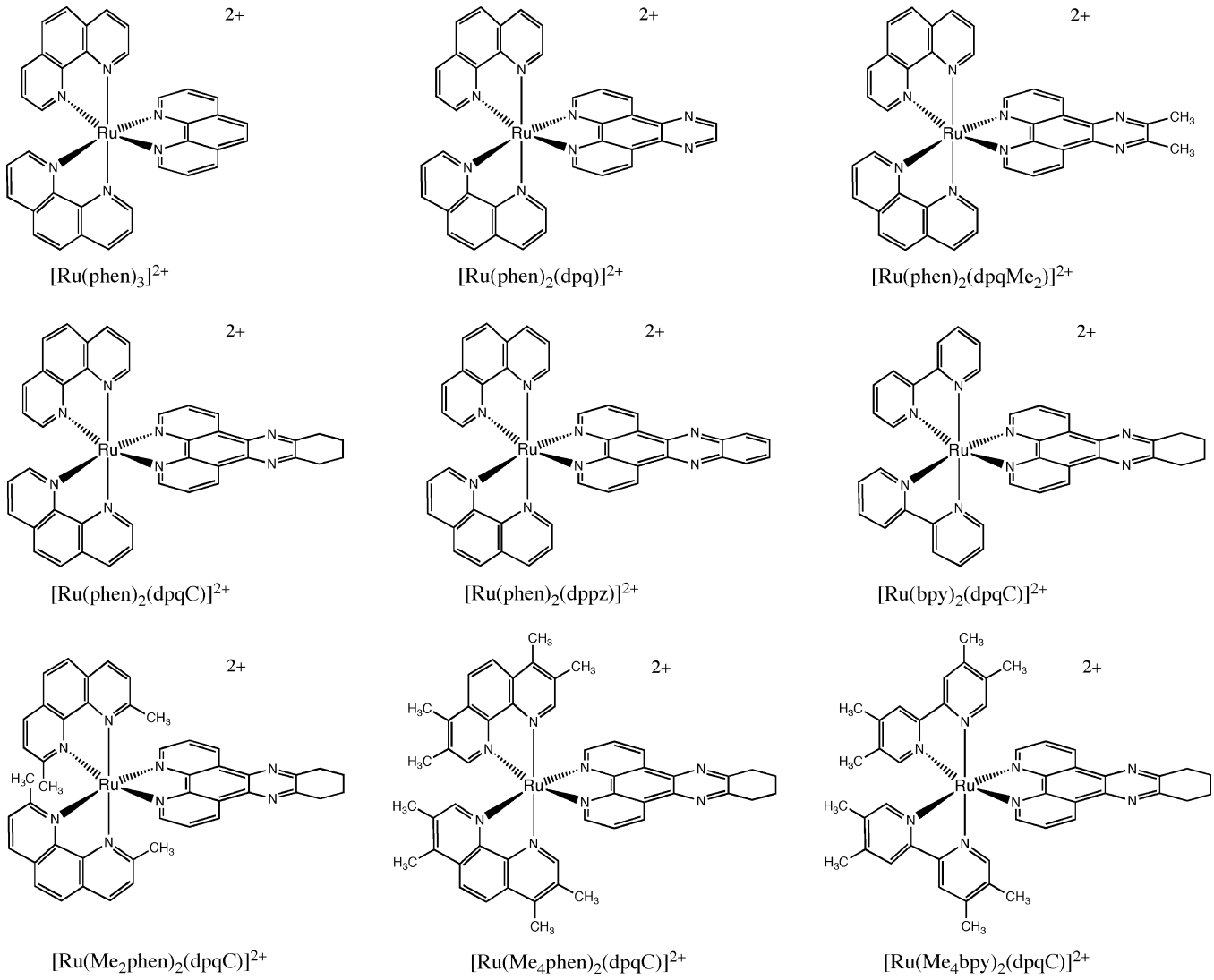

Figure 1. Structures of the ruthenium compounds used in this study.

and 100 scans averaged to give the final spectrum. All data were calibrated against a standard CsI solution (750 μM) over the same *m*/*z* range.

Absorption titrations were performed using a Shimadzu UV 1700 PharmaSpec spectrophotometer and 1 cm path length cells. A spectrum (260−600 nm) was first obtained using 2.5 mL of 10 μM ruthenium complex dissolved in 0.1 M NH_4OAc, pH 8.5. Aliquots of 1.25 mM D2 (also dissolved in in 0.1 M NH_4OAc, pH 8.5) were then added to the metal solution, which was mixed and allowed to stand at room temperature for 10 min prior to spectral analysis. This process was repeated until there were no further changes in the appearance of the absorption spectrum.

Reaction mixtures for melting temperature analysis were prepared by mixing 10 μL of 1 mM dsDNA (dissolved in in 0.1 M NH_4OAc, pH 8.5) and 100 μL of 100 μM ruthenium complex (dissolved in in 0.1 M NH_4OAc, pH 8.5) and making the final volume up to 1 mL using 0.1 M NH_4OAc, pH 8.5. This resulted in final solutions with dsDNA concentrations of 1 μM and Ru/dsDNA ratios of 3:1. The mixtures were left to stand at room temperature for 30 min before being analyzed using a Varian Cary 500 UV−vis/NIR spectrophotometer and its *Thermal-UV* software. Solution absorbances were measured at a wavelength of 260 nm. The start and end temperatures were 25 and 80 °C, respectively, while the ramping rate was 1 °C min^{-1}, the data interval 0.3 °C, and the filter size set to 101.

Circular dichroism (CD) spectra were obtained using a Jasco J-810 spectropolarimeter and stock solutions consisting of 0.5 mM ruthenium complex, 20 μM dsDNA and 0.1 M NH_4OAc, pH 8.5. Initially 300 μL of solution containing 20 μM D2 in 0.1 M NH_4OAc, pH 8.5 was placed into a cuvette and the CD spectrum obtained. After this, the required volumes of stock solution containing 0.500 mM ruthenium complex and 20 μM D2 were added to give final Ru/D2 ratios of 1:1, 3:1, 6:1, and 10:1. Each solution was allowed to stand at room temperature for 10 min prior to acquisition of another CD spectrum.

Luminescence spectra were obtained using a Varian Cary Eclipse spectrophotometer and 1 cm path-length cells. A spectrum (500−800 nm) was initially obtained using 2.75 mL of a 10 μM ruthenium complex dissolved in 0.1 M NH_4OAc, pH 8.5, and an excitation wavelength that corresponded to the peak of the metal-to-ligand charge transfer band in its absorption spectrum. Aliquots of 0.25 mM D2 (also dissolved in 0.1 M NH_4OAc, pH 8.5) were then added to the ruthenium solution, which was mixed and allowed to equilibrate for 10 min prior to

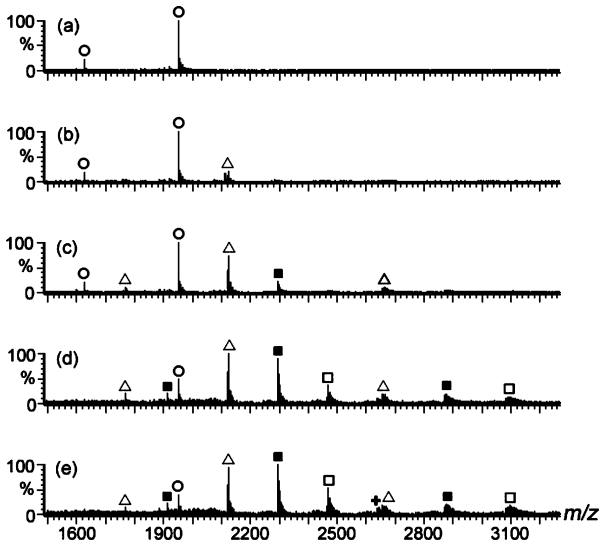

Figure 2. Negative ion ESI mass spectra of solutions containing different $[Ru(Me_4phen)_2(dpqC)]^{2+}$/D2 ratios: (a) free D2, (b) metal/D2 = 1:1, (c) metal/D2 = 3:1, (d) metal/D2 = 6:1, (e) metal/D2 = 9:1; ○ dsDNA, Δ dsDNA + $[Ru(Me_4phen)_2(dpqC)]^{2+}$, ■ dsDNA + 2 $[Ru(Me_4phen)_2(dpqC)]^{2+}$, □ dsDNA + 3 $[Ru(Me_4phen)_2(dpqC)]^{2+}$, + dsDNA + 4 $[Ru(Me_4phen)_2(dpqC)]^{2+}$.

further spectral analysis. This process was repeated until there were no further changes in the appearance of the fluorescence spectrum.

Results

Mass Spectrometry. ESI mass spectra were obtained of reaction mixtures containing $[RuL'_2(dpqC)]^{2+}$/D2 ratios from 1:1 up to a maximum of 9:1. Under the experimental conditions used, the most abundant ions from either free DNA or Ru/DNA complexes were 5- ions, with 6- and 4- ions of much lower abundance. For example, the negative ion ESI mass spectrum of a solution containing D2 alone (part a of Figure 2) contains ions of high abundance at *m*/*z* 1952.2 assigned to $[D2-5H]^{5-}$, and ions of low abundance at *m*/*z* 1626.7 due to $[D2-6H]^{6-}$. These ions are formed by loss of five and six protons respectively from a neutral D2 molecule. The spectra described here differ from those we have reported previously, as the latter generally contained 6- ions of greater abundance than either 5- or 7- ions.[23,24] This difference results from variations in ionization conditions between the mass spectrometer used in the current study and that used in our previous work. Despite these variations, spectra obtained using different instruments were consistent with the same overall solution composition for a given reaction mixture.

Addition of ruthenium complexes to solutions containing D2 resulted in the appearance of additional ions in ESI mass spectra at higher *m*/*z* values than those assigned to free D2. For example, Figure 2 shows the negative ion ESI mass spectra of solutions containing different ratios of $[Ru(Me_4phen)_2(dpqC)]^{2+}$ and D2. The spectrum of a solution containing a 1:1 ratio of Ru/D2 (part b of Figure 2) shows that the most abundant ions present were still those attributable to unbound DNA. However, ions of low abundance were also present at *m*/*z* 2124.0, which could be assigned to $[D2+Ru(Me_4phen)_2(dpqC)-7H]^{5-}$. When the Ru/D2 ratio was increased to 3:1, the mass spectrum (part c of Figure 2) showed that the abundance of these ions had increased. Ions attributable to different charge states of the same complex were also present along with ions at *m*/*z* 2295.6 that are assigned to $[D2 + 2Ru(Me_4phen)_2(dpqC)-9H]^{5-}$. When the metal/D2 ratio was increased further to 6:1, the most abundant ions present in the spectrum (part d of Figure 2) were from non-covalent complexes containing one and two ruthenium molecules bound to DNA. The abundance of ions assigned to unbound DNA had now decreased significantly, whereas new ions assigned to non-covalent complexes containing three ruthenium molecules bound to DNA were also present at *m*/*z* 2467.4 in low abundance. Increasing the metal/D2 ratio to 9:1 resulted in only minor changes to the appearance of the mass spectrum (part e of Figure 2), indicating that the binding of the ruthenium complexes to DNA was approaching the saturation point. Similar trends were seen in mass spectra of reaction mixtures containing the other ruthenium compounds, with ions assigned to more heavily substituted DNA molecules being observed at higher metal/D2 ratios. In all cases, new ions were observed at *m*/*z* values that corresponded exactly to formulations consisting of an intact dsDNA molecule, plus between one and four intact ruthenium molecules. The observation that all new ions contained ruthenium molecules with intact coordination spheres strongly suggests that binding to DNA was non-covalent in nature in all cases.

One of the principal aims of this study was to determine whether ESI-MS would reveal variations in DNA binding affinity between ruthenium compounds containing ancillary ligands with different numbers of methyl substituents. This was tested by comparing the negative-ion ESI mass spectra of solutions containing the same ratio (3:1) of different ruthenium compounds and D2. The results of these experiments are shown in Figure 3. In the case of solutions containing $[Ru(phen)_2(dpqC)]^{2+}$ and $[Ru(bpy)_2(dpqC)]^{2+}$, both mass spectra (parts a and d respectively of Figure 3) contained 5- ions of high abundance assigned to non-covalent complexes containing one and two ruthenium molecules bound to DNA, as well as 5- ions of medium abundance assigned to non-covalent complexes containing three ruthenium molecules. However, the spectra of solutions containing $[Ru(Me_2phen)_2(dpqC)]^{2+}$, $[Ru(Me_4phen)_2(dpqC)]^{2+}$, or $[Ru(Me_4bpy)_2(dpqC)]^{2+}$, and D2 (parts b, c, and e of Figure 3) showed ions of only medium abundance from complexes containing two ruthenium molecules bound to DNA, whereas ions containing three ruthenium molecules were of very low abundance or not present. These results are consistent with the proposal that $[Ru(phen)_2(dpqC)]^{2+}$ and $[Ru(bpy)_2(dpqC)]^{2+}$ have binding affinities toward D2 that are very similar to each other and greater than that of the complexes containing methylated ancillary ligands. Further examination of the spectra of solutions containing ruthenium complexes with phenanthroline or substituted phenanthroline ancillary ligands (parts a−c of Figure 3) reveals that the abundances of ions at *m*/*z* 1626.7 and 1952.2 from free D2 increased, suggesting the following order of relative D2 binding

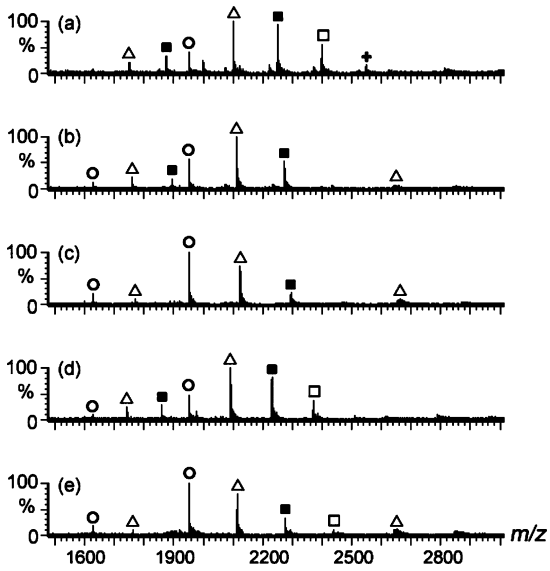

Figure 3. Negative ion ESI mass spectra of solutions containing a 3:1 ratio of ruthenium compound and duplex D2: (a) $[Ru(phen)_2(dpqC)]^{2+}$, (b) $[Ru(Me_2phen)_2(dpqC)]^{2+}$, (c) $[Ru(Me_4phen)_2(dpqC)]^{2+}$, (d) $[Ru(bpy)_2(dpqC)]^{2+}$, (e) $[Ru(Me_4bpy)_2(dpqC)]^{2+}$; ○ dsDNA, Δ dsDNA + $[Ru(L)_2(dpqC)]^{2+}$, ■ dsDNA + 2 $[Ru(L)_2(dpqC)]^{2+}$, □ dsDNA + 3 $[Ru(L)_2(dpqC)]^{2+}$, + dsDNA + 4 $[Ru(L)_2(dpqC)]^{2+}$.

affinities: $[Ru(Me_4phen)_2(dpqC)]^{2+}$ < $[Ru(Me_2phen)_2(dpqC)]^{2+}$ < $[Ru(phen)_2(dpqC)]^{2+}$. This order is supported by comparing the abundances of ions assigned to non-covalent complexes containing one and two ruthenium molecules. In the case of the solution containing $[Ru(phen)_2(dpqC)]^{2+}$ (part a of Figure 3), the ratio of the abundances of ions at *m*/*z* 2101.0 (due to $[D2+Ru(phen)_2(dpqC)-7H]^{5-}$) and 2250.3 (due to $[D2 + 2Ru(phen)_2(dpqC)-9H]^{5-}$) was approximately 1:1. However, for the $[Ru(Me_2phen)_2(dpqC)]^{2+}$ system (part b of Figure 3), the abundance of the corresponding ions assigned to non-covalent complexes containing two ruthenium molecules bound to D2 (at *m*/*z* 2273.1) was significantly less than that of ions at *m*/*z* 2112.8, assigned to non-covalent complexes containing one ruthenium molecule bound to D2. This is consistent with the conclusion that $[Ru(Me_2phen)_2(dpqC)]^{2+}$ has a lower affinity toward D2 than $[Ru(phen)_2(dpqC)]^{2+}$. When the ruthenium complex examined was $[Ru(Me_4phen)_2(dpqC)]^{2+}$, the mass spectrum obtained (part c of Figure 3) showed that the abundance of ions assigned to non-covalent DNA complexes containing two ruthenium molecule (at *m*/*z* 2295.6) was even lower relative to that of the corresponding ions containing just one bound ruthenium molecule (at *m*/*z* 2124.0), suggesting that this ruthenium compound has the lowest DNA affinity of the three in this subseries.

Further evidence of small, but discernible differences in DNA binding affinity between the different ruthenium compounds was provided by obtaining negative ion ESI mass spectra of competition mixtures containing a 3:3:1 ratio of two different compounds and D2. In general, the quality of these spectra was lower than that of solutions containing a single type of ruthenium compound and D2, owing to the greater heterogeneity of the reaction mixtures. However, the spectra obtained were always of sufficient quality to enable

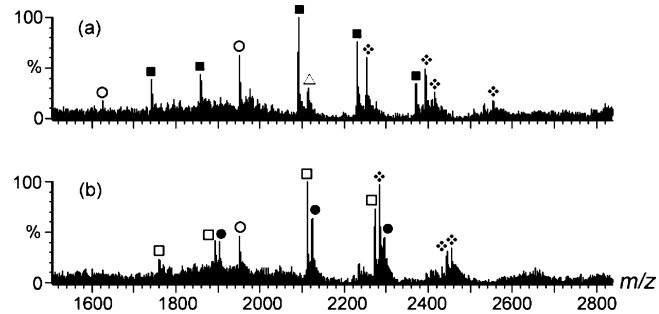

Figure 4. Negative-ion ESI mass spectra of solutions containing a 3:3:1 ratio of two ruthenium compounds and D2: (a) solution containing $[Ru(bpy)_2(dpqC)]^{2+}$ and $[Ru(Me_4bpy)_2(dpqC)]^{2+}$; (b) solution containing $[Ru(Me_2phen)_2(dpqC)]^{2+}$ and $[Ru(Me_4phen)_2(dpqC)]^{2+}$. ○ dsDNA; ■ dsDNA + *x* $[Ru(bpy)_2(dpqC)]^{2+}$, *x* = 1−3; Δ dsDNA + $[Ru(Me_4bpy)_2(dpqC)]^{2+}$; □ dsDNA + *x* $[Ru(Me_2phen)_2(dpqC)]^{2+}$, *x* = 1−2; ● dsDNA + *x* $[Ru(Me_4phen)_2(dpqC)]^{2+}$, *x* = 1−2; ❖ dsDNA + one or more of both ruthenium molecules.

a conclusion to be drawn as to which exhibited the greater DNA binding affinity. Representative spectra illustrating the general trends observed are shown in Figure 4. For example, part a of Figure 4 shows the spectrum of the solution containing $[Ru(bpy)_2(dpqC)]^{2+}$, $[Ru(Me_4bpy)_2(dpqC)]^{2+}$, and D2. The majority of ions of medium and high abundance in this spectrum are from non-covalent complexes containing different numbers of $[Ru(bpy)_2(dpqC)]^{2+}$ molecules bound to D2. In contrast, there is only one ion of low abundance (at *m*/*z* 2113.7) that can be assigned to non-covalent complexes containing one $[Ru(Me_4bpy)_2(dpqC)]^{2+}$ molecule bound to D2. These observations support the conclusion that the affinity of $[Ru(bpy)_2(dpqC)]^{2+}$ toward D2 is significantly greater than that of $[Ru(Me_4bpy)_2(dpqC)]^{2+}$.

The spectrum of a solution containing $[Ru(Me_2phen)_2(dpqC)]^{2+}$, $[Ru(Me_4phen)_2(dpqC)]^{2+}$, and D2 (part b of Figure 4) shows ions from non-covalent complexes containing one or more of both types of ruthenium molecules bound to DNA, as well as other ions containing a single type of ruthenium molecule. The presence of the former ions in the spectra of every competition mixture examined suggests that differences in DNA binding affinities between these ruthenium compounds are not great. This is consistent with our original hypothesis that the interactions of each ruthenium compound with DNA are dominated by the intercalating dpqC ligand, with only small variations being caused by changes to the ancillary ligands. Part b of Figure 4 contains four ions assignable to non-covalent complexes containing $[Ru(Me_2phen)_2(dpqC)]^{2+}$ molecules bound to D2, with two of these ions, at *m*/*z* 2112.5 and 2272.7, of high and medium abundance, respectively. In contrast, there are only three ions that can be assigned to non-covalent complexes containing $[Ru(Me_4phen)_2(dpqC)]^{2+}$ molecules bound to D2, with the most abundant of these (at *m*/*z* 2123.7) only of medium abundance. These results suggest first of all that the affinity of $[Ru(Me_2phen)_2(dpqC)]^{2+}$ for D2 is greater than that of $[Ru(Me_4phen)_2(dpqC)]^{2+}$, and second that the difference in affinity between these two compounds is not as great as that between $[Ru(bpy)_2(dpqC)]^{2+}$ and $[Ru(Me_4bpy)_2(dpqC)]^{2+}$ evident in part a of Figure 4.

The results of the above experiments show that ESI-MS can reveal reductions in DNA binding affinity caused by increasing the number of methyl groups in the ancillary ligands of the complexes $[RuL'_2(dpqC)]^{2+}$. Through comparison of the spectra shown in Figure 3, and analysis of mass spectra of competition mixtures, the binding affinities of the ruthenium compounds toward D2 were determined to increase in the following order: $[Ru(Me_4bpy)_2(dpqC)]^{2+} \sim [Ru(Me_4phen)_2(dpqC)]^{2+} < [Ru(Me_2phen)_2(dpqC)]^{2+} < [Ru(bpy)_2(dpqC)]^{2+} \sim [Ru(phen)_2(dpqC)]^{2+}$. In the following sections, this binding order, and that determined previously for the series $[Ru(phen)_2L]^{2+}$, will be compared to those derived using four other techniques.

Absorption Spectrophotometry. Monitoring the effect of adding increasing amounts of DNA on the absorption spectrum of a metal complex is one of the most widely used methods for determining overall binding constants. Addition of DNA generally has been reported to result in the absorption bands assigned to the metal complex exhibiting hypochromism and shifting to lower energy.[3,28–30] While the observed bathochromic shifts are generally small, the magnitude of such shifts has been used as a qualitative indicator of the strength of intercalative interactions.[28] A better indication of relative binding strengths may be obtained from binding constants determined by analysis of the observed hypochromism of the absorption bands. This has been accomplished by a variety of graphical procedures, including the use of eq 1:[28,30]

$$[DNA]/(\varepsilon_A - \varepsilon_F) = [DNA]/(\varepsilon_B - \varepsilon_F) + 1/K_b(\varepsilon_B - \varepsilon_F) \quad (1)$$

where ε_A, ε_F, and ε_B correspond to A_{obsd}/[Ru], the extinction coefficient for the free ruthenium complex, and the extinction coefficient for the ruthenium complex when fully bound to DNA, respectively.

Addition of increasing amounts of D2 to all of the ruthenium compounds under investigation resulted in significant changes in the appearance of the absorption spectrum of the metal complex. For example, Figure 5 illustrates the spectra obtained for $[Ru(phen)_2(dppz)]^{2+}$. With every metal complex examined, the spectra obtained consistently passed through several isosbestic points, indicating that equilibrium was achieved between the different non-covalent complexes formed throughout the course of the titrations. For $[Ru(Me_2phen)_2(dpqC)]^{2+}$, $[Ru(Me_4phen)_2(dpqC)]^{2+}$ and $[Ru(Me_4bpy)_2(dpqC)]^{2+}$ small (2−8 nm) bathochromic shifts

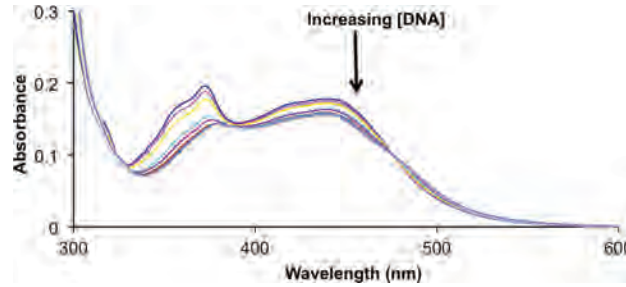

Figure 5. Visible absorption spectra of $[Ru(phen)_2(dppz)]^{2+}$ (10 μM) in the presence of increasing (0−40 μL) volumes of D2 (1.25 mM).

were observed for the metal-to-ligand charge transfer (MLCT) band, whereas for $[Ru(bpy)_2(dpqC)]^{2+}$ a small hypsochromic shift was noted. In the case of $[Ru(phen)_2(dppz)]^{2+}$, the MLCT band displayed only a very small bathochromic shift (<1 nm), whereas the intraligand band at higher energy exhibited a much larger red shift (8 nm). For each of the remaining compounds, it was impossible to determine whether a similar bathochromic shift occurred. This was because the spectra of these complexes contained two closely overlapping MLCT bands, which coalesced upon the addition of saturating amounts of DNA to give a single discernible peak with λ_{max} in between that of the original two absorption bands. In view of these observations, it was decided to base our conclusions regarding the relative binding affinities of the different metal compounds solely on the binding constants presented in Table 1, which were determined using eq 1 and the changes in absorbance observed at the maxima of the MLCT band of the metal complex. Figure 6 shows a representative plot obtained using this equation and data for $[Ru(phen)_2(dppz)]^{2+}$.

Examination of Table 1 shows that the binding constants obtained from absorption titrations for the complexes $[Ru(phen)_2L]^{2+}$ follow the order $[Ru(phen)_3]^{2+} < [Ru(phen)_2(dpq)]^{2+} < [Ru(phen)_2(dpqC)]^{2+} < [Ru(phen)_2(dppz)]^{2+} < [Ru(phen)_2(dpqMe_2)]^{2+}$. This sequence is very similar to that determined previously by ESI-MS, namely $[Ru(phen)_3]^{2+} < [Ru(phen)_2(dpq)]^{2+} < [Ru(phen)_2(dpqC)]^{2+} < [Ru(phen)_2(dpqMe_2)]^{2+} < [Ru(phen)_2(dppz)]^{2+}$,[24] with the one difference being reversal of the positions occupied by the last two complexes. Another interesting observation is that whereas the binding constants for $[Ru(phen)_3]^{2+}$, $[Ru(phen)_2(dpq)]^{2+}$, and $[Ru(phen)_2(dpqC)]^{2+}$ are significantly different to each other, the value for the latter complex is only a little less than that for $[Ru(phen)_2(dppz)]^{2+}$. This appears to be because the value obtained here for $[Ru(phen)_2(dppz)]^{2+}$ (6.4×10^4 M^{-1}) is significantly lower than expected, as literature binding constants for its interaction with CT DNA have typically been reported as $>10^6$ M^{-1},[21,30c,31] and even as high as $\sim 10^8$ M^{-1}.[32] There are several possible reasons for this discrepancy, including in most previous studies the use of a different experimental technique (luminescence titration),[21,31,32] different methods of data analysis (e.g the McGhee and von Hippel approach),[31,32]

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Table 1. DNA Binding Properties Of Ruthenium Compounds

compound	10^{-4} K_B[a,b]	ΔT_m [c]	CD $\Delta\lambda_{max}$ + $\Delta\epsilon$[d]	luminescence enhancement + $\Delta\lambda$[e]
$[Ru(phen)_3]^{2+}$	0.3 ± 0.1	1.0 ± 0.5	−6 (12)	1.3 (+2)
$[Ru(phen)_2(dpq)]^{2+}$	1.4 ± 0.2	2.4 ± 0.5	−8 (21)	4.4 (+2)
$[Ru(phen)_2(dpqC)]^{2+}$	6.1 ± 0.6	5.3 ± 0.5	−7 (25)	2.2 (0)
$[Ru(phen)_2(dppz)]^{2+}$	6.4 ± 0.3	9.3 ± 0.5	−8 (32)	47.0 (+1)
$[Ru(phen)_2(dpqMe_2)]^{2+}$	7.1 ± 0.2	5.8 ± 0.5	−8 (24)	2.2 (−2)
$[Ru(Me_2phen)_2(dpqC)]^{2+}$	1.9 ± 0.1	1.6 ± 0.5	−2 (14)	2.4 (+2)
$[Ru(Me_4phen)_2(dpqC)]^{2+}$	1.9 ± 0.1	3.5 ± 0.5	+1 (21)	4.5 (−14)
$[Ru(bpy)_2(dpqC)]^{2+}$	3.3 ± 0.5	2.7 ± 0.5	+7 (12)	2.1 (−1)
$[Ru(Me_4bpy)_2(dpqC)]^{2+}$	2.3 ± 1.5	2.2 ± 0.5	+10 (9)	4.2 (−17)

[a] Averaged value from at least two absorption titrations. The units for the binding constants are M(base pair)$^{-1}$. [b] The errors for the binding constants are the standard deviations obtained from multiple determinations of the binding constants. [c] Units for ΔT_m are °C. T_m for free D2 = 64 °C. [d] $\Delta\lambda_{max}$ is the shift in nanometers of the positive DNA CD band at 268 nm. $\Delta\epsilon$ (the value in parentheses) is the difference between the maximum ellipticity (in degrees) observed for the positive CD band in the spectrum of a 10:1 reaction mixture and the ellipticity observed at the same wavelength in the spectrum of free D2. [e] Luminescence enhancement is the ratio of luminescence in the presence of saturating amounts of DNA compared to the luminescence in the absence of DNA. $\Delta\lambda$ (the value in parentheses) is the shift in nanometers in the position of the emission band.

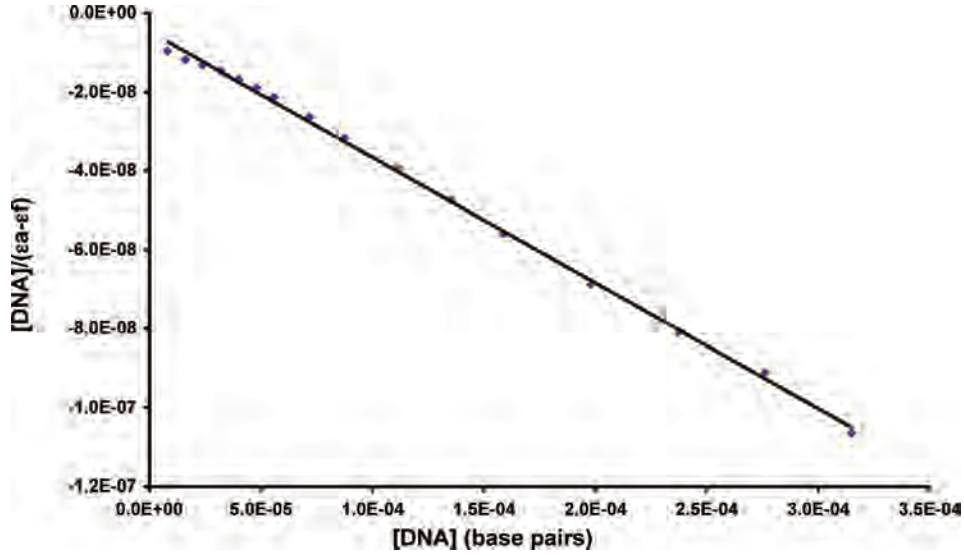

Figure 6. Binding isotherm derived using absorption spectrophotometric titration data for $[Ru(phen)_2(dppz)]^{2+}$ and eq 1.

and different DNA (calf thymus),[21,30c,32] to that used in the current work. In addition, we chose to perform our absorption titration experiments using a concentration of salt (0.1 M NH_4OAc) that was identical to that used in our ESI-MS experiments, but that provides a significantly higher ionic strength than that used in most literature studies. For example, two of the above literature studies used metal-complex and DNA solutions prepared in buffers consisting of 5 mM tris and 50 mM NaCl (pH 7.0 or 7.1),[21,30c] whereas the others used either a mixture of 1 or 10 μM phosphate, 10 mM NaCl, 1 mM sodium cacodylate, pH 7.0,[32] or 5 mM phosphate, pH 7.0.[31] Previous work by Haq et al. has demonstrated the sensitivity of metal-complex/DNA binding constants to ionic strength.[10] These workers showed that binding constants determined by fluorescence titration for the enantiomers of $[Ru(phen)_2(dppz)]^{2+}$ and CT DNA decreased from approximately 10^6 M^{-1}, to less than 10^5 M^{-1} in the case of the Λ enantiomer, when the concentration of Na^+ in the surrounding solution was increased from 0.06 to 0.2 M. This suggests that the relatively high salt concentrations present in our solutions may be the reason why the binding constant for $[Ru(phen)_2(dpqC)]^{2+}$ was only a little less than that for $[Ru(phen)_2(dppz)]^{2+}$. Further evidence for this is provided by a study that reported the binding constant for $[Ru(bpy)_2(dpqC)]^{2+}$ (8.5×10^4 M^{-1}) to be significantly less than that for $[Ru(bpy)_2(dppz)]^{2+}$ ($>10^6$ M^{-1}).[33] This study used solutions of calf thymus DNA and metal complexes prepared in a buffer consisting of 20 mM sodium phosphate, 10 mM NaCl, pH 7.85, which is again very different to the conditions we have employed.

In contrast to the significant differences between our binding constant for $[Ru(phen)_2(dppz)]^{2+}$ and values reported in the literature, our value for $[Ru(phen)_3]^{2+}$ (3×10^3 M^{-1}) is comparable to values determined previously by other workers (5.5×10^3 to 7.9×10^3 M^{-1}),[28,30c,34] despite variations in experimental conditions. This observation, and the overall good agreement found here between the orders of binding affinity determined by ESI-MS and the absorption titration method, suggests that the effect of ionic strength may have been most profound with the complex expected to be the most tightly binding, namely $[Ru(phen)_2(dppz)]^{2+}$. To investigate this further, we used the absorption titration technique to determine the binding constant for another metal complex known to have very high affinity toward DNA. The complex chosen for examination was $[Ru(bpy)_2(dppz)]^{2+}$, and

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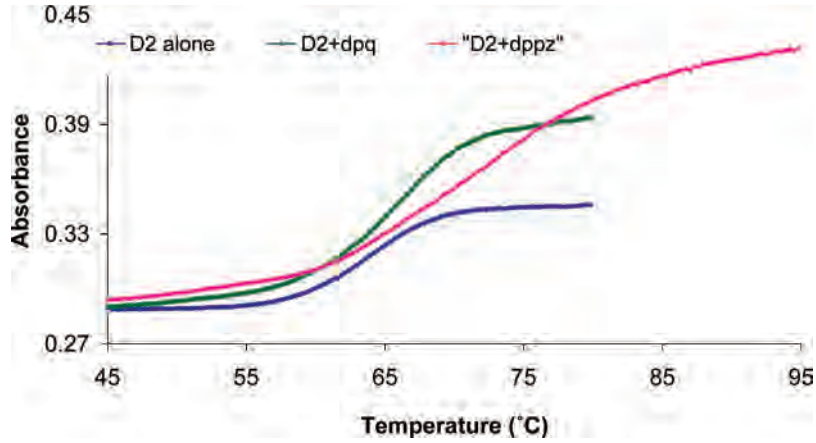

Figure 7. DNA melting curves for a solution containing D2 alone in 0.1 M ammonium acetate, and solutions containing D2 and 3 equivs of $[Ru(phen)_2(dpq)]^{2+}$ or $[Ru(phen)_2(dppz)]^{2+}$ in 0.1 M ammonium acetate.

it was found to have a binding constant of 7.3×10^4 M^{-1}, again significantly lower than values reported for this compound in the literature.[21,33]

These conditions do not appear, however, to have significantly affected binding constants for the other complexes examined, as there was generally a good degree of correlation between the order of binding affinity determined for the series $[RuL'_2(dpqC)]^{2+}$ by ESI-MS, and that determined by absorption titration. Both techniques showed that the complexes with the highest binding affinity were $[Ru(phen)_2(dpqC)]^{2+}$ and $[Ru(bpy)_2(dpqC)]^{2+}$. Furthermore, replacement of the phen ligands in $[Ru(phen)_2(dpqC)]^{2+}$ by Me_2phen, or the bpy ligands in $[Ru(bpy)_2(dpqC)]^{2+}$ by Me_4bpy, was found to result in decreases in the binding constant that mirrored lower DNA affinities revealed by ESI-MS. One surprising result, however, was that the binding constants for $[Ru(Me_4phen)_2(dpqC)]^{2+}$ and $[Ru(Me_2phen)_2(dpqC)]^{2+}$ were found to be identical within experimental error. In contrast, ESI-MS suggested that the complex with the more heavily methylated ligands did not bind DNA as avidly, as might be expected in view of the additional steric crowding present. Despite this, the overall good correlation found between orders of binding affinity based on binding constants determined using the absorption titration technique, and ESI-MS, support the conclusion that the latter method provides a good assessment of relative DNA binding affinity for these types of metal complexes.

DNA Melting Curves. It has been well documented that the binding of minor groove binding agents and intercalators to DNA increases the stability of the double helix, and as a consequence raises its melting temperature T_m.[29a,b,30b,c,34] Figure 7 shows the DNA melting curves for a solution containing D2 alone, and for solutions containing a 3:1 ratio of either $[Ru(phen)_2(dpq)]^{2+}$ or $[Ru(phen)_2(dppz)]^{2+}$, and D2. For these complexes and every other complex examined, well-defined sigmoidal shaped curves were obtained from which T_m, the temperature at which 50% of the double-stranded DNA molecules have undergone strand separation, was determined. Table 1 presents values of ΔT_m, the difference between the melting temperature of DNA in the presence and absence of the ruthenium compound. Each of the compounds investigated resulted in an increase in T_m, which is indicative of net stabilization of the double helical structure of DNA. For the series $[Ru(phen)_2L]^{2+}$, the order of relative binding affinities based on ΔT_m was found to be $[Ru(phen)_3]^{2+} < [Ru(phen)_2(dpq)]^{2+} < [Ru(phen)_2(dpqC)]^{2+} < [Ru(phen)_2(dpqMe_2)]^{2+} < [Ru(phen)_2(dppz)]^{2+}$. This sequence is identical to that derived from ESI-MS previously,[24] providing further support for the validity of the latter technique for investigating these types of systems.

For the $[RuL'_2(dpqC)]^{2+}$ series, the complex shown to have the greatest ability to increase T_m, and therefore the greatest affinity toward D2, was $[Ru(phen)_2(dpqC)]^{2+}$. Replacement of both phen ligands in $[Ru(phen)_2(dpqC)]^{2+}$ by Me_2phen resulted in a much smaller value of ΔT_m, as did replacing the bpy ligands in $[Ru(bpy)_2(dpqC)]^{2+}$ by Me_4bpy ligands. Each of the above results is consistent with trends in binding affinity revealed by ESI-MS and is not unexpected given that an increase in steric crowding of the ancillary ligands might be expected to generally reduce the ability of the intercalating dpqC ligand to insert into the double helix. A surprising observation, however, was that ΔT_m for $[Ru(Me_4phen)_2(dpqC)]^{2+}$ was greater than that for $[Ru(Me_2phen)_2(dpqC)]^{2+}$. This result indicates that the former complex has a greater stabilizing effect on the DNA structure, which is normally an indication of greater DNA binding affinity. However, this conclusion does not correlate with the relative DNA binding affinities of the two complexes, as judged by ESI-MS, and is as yet unexplained. It it worth noting, however, that the binding constants determined by the absorption titration method for $[Ru(Me_2phen)_2(dpqC)]^{2+}$ and $[Ru(Me_4phen)_2(dpqC)]^{2+}$ were identical, within experimental error. Furthermore, in the following sections additional surprising results will be described, which were obtained with $[Ru(Me_4phen)_2(dpqC)]^{2+}$ in CD and luminescence experiments.

Circular Dichroism (CD) Spectroscopy. This technique has been widely used to examine the non-covalent DNA binding interactions of metal complexes, owing to its ability to provide binding constants as well as information about nucleic acid conformation and the enantioselectivity of

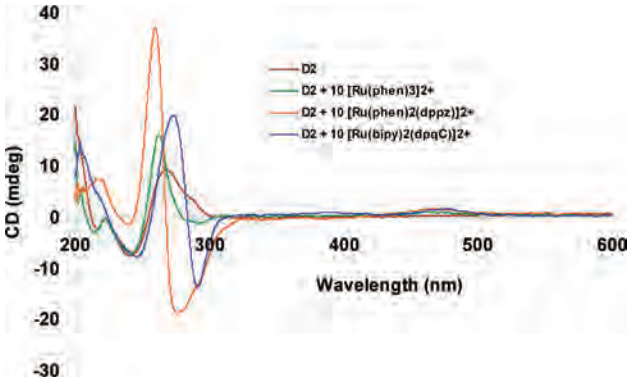

Figure 8. Circular dichroism spectra recorded over the wavelength range 200−600 nm for solutions containing a 10:1 ratio of $[Ru(phen)_3]^{2+}$, $[Ru(phen)_2(dppz)]^{2+}$, or $[Ru(bpy)_2(dpqC)]^{2+}$, and D2. The concentrations of ruthenium complex and D2 in each instance were 200 and 20 μM, respectively.

binding.[3,8,29a,c,30e,35–41] Most such studies have involved either CT DNA or DNA molecules with a homogeneous composition such as poly(dA-dT)$_2$ and poly(dG-dC)$_2$. In contrast, we have chosen to use CD to examine the interactions of metal complexes with a double stranded 16mer DNA molecule (D2) of defined length and base composition. CD spectra were obtained using solutions with metal/D2 ratios of 1:1, 3:1, 6:1, and 10:1. Figure 8 shows representative spectra of three solutions containing a 10:1 ratio of ruthenium complex and D2, recorded over the wavelength range 200−600 nm, as well as the CD spectrum of D2. For each complex, dramatic changes to the CD spectrum of the DNA molecule were observed between 200 and 320 nm. There was also some evidence for the appearance of new CD bands at longer wavelengths. These bands arise as a result of differing effects of the chiral DNA molecule on the CD bands of the enantiomers of the metal complex. It has been shown previously that the amplitude of these bands in the CD spectra of solutions containing racemic $[Ru(phen)_3]^{2+}$ and DNA is highly dependent on the identity of the DNA molecule used.[9] For example, whereas the CD spectrum of a solution containing racemic $[Ru(phen)_3]^{2+}$ and CT DNA contained signals of small but measureable amplitude in the DNA and metal regions of the spectrum, the signals in both of these regions were considerably smaller in magnitude when poly(dA-dT) was used instead.

The signals observed in the metal region of the CD spectra of our solutions were also very small in amplitude. This may be due to several factors, including small differences in the amounts of the two enantiomers bound to DNA for each metal complex.[9] Alternatively, the small CD signals may have been caused by different perturbations to the CD signals of enantiomers of a given ruthenium complex caused by their interactions with DNA.[9] Regardless of the cause, it is clear that the CD signals in the metal region of the spectrum cannot be used to provide an accurate measure of DNA binding affinity under the current experimental conditions. Consequently, we have focused our attention on the larger changes observed in the DNA region of the spectrum. Figure 9 shows representative spectra that illustrate the variety of spectroscopic changes that were observed in the wavelength range 200−320 nm. In all cases, significant changes to the CD spectra were detected when the metal/D2 ratio was increased. These changes are due to perturbation of the CD signals of DNA caused by interaction with the metal complexes and/or to induction of circular dichroism into the electronic absorption bands of the achiral metal complexes caused by the chiral DNA molecule. Therefore, changes in the CD spectra in the region between 200 and 320 nm provide further strong evidence for the formation of non-covalent complexes containing increasingly larger numbers of metal complexes bound to DNA as the metal/D2 ratio was increased.

The CD spectrum of free D2 was characterized by a positive band centered at 268 nm and a negative band at 241 nm, with zero ellipticity being observed at 255 nm. These values are consistent with those reported for B-form DNA.[35] Addition of increasing amounts of ruthenium complex resulted in several changes in the CD spectrum, including in all cases the positive CD signal shifting in position and growing in intensity. The magnitudes of these changes, which are reported in Table 1, show some interesting variations with the identity of the metal complex. For the series $[Ru(phen)_2L]^{2+}$, the wavelength of maximum ellipticity for the positive CD signal always shifted to higher energy by between 6 and 8 nm. This is clearly shown by the spectra in parts a and b of Figure 9. Replacing the phen ligands in $[Ru(phen)_2(dpqC)]^{2+}$ by Me_2phen resulted in a smaller shift to higher energy of only 2 nm, whereas introduction of further methyl groups onto the ancillary ligands to give $[Ru(Me_4phen)_2(dpqC)]^{2+}$ resulted in a shift to lower energy of 1 nm (part c of Figure 9). This variation highlights the sensitivity of CD spectra of these systems to interactions between the ancillary ligands of the ruthenium complex and the DNA molecule. Further evidence of this is provided by comparison of parts b and d of Figure 9, which show CD spectra of solutions containing $[Ru(phen)_2(dpqC)]^{2+}$ and $[Ru(bpy)_2(dpqC)]^{2+}$, respectively. Whereas the positive CD signal in the spectrum of the former compound shifts to higher energy by 7 nm, for the latter compound a shift to lower energy of 7 nm was measured. Introduction of four methyl groups onto each of the bpy ligands in $[Ru(bpy)_2(dpqC)]^{2+}$ to give $[Ru(Me_4bpy)_2(dpqC)]^{2+}$ resulted in a further shift of the positive CD band toward a lower energy of 3 nm.

As the ratio of Ru/DNA was increased, the width of the positive CD signal decreased significantly for most complexes, in addition to displaying the changes in wavelength and ellipticity noted above. This occurred as a result of decreases in ellipticity at wavelengths greater than λ_{max} for

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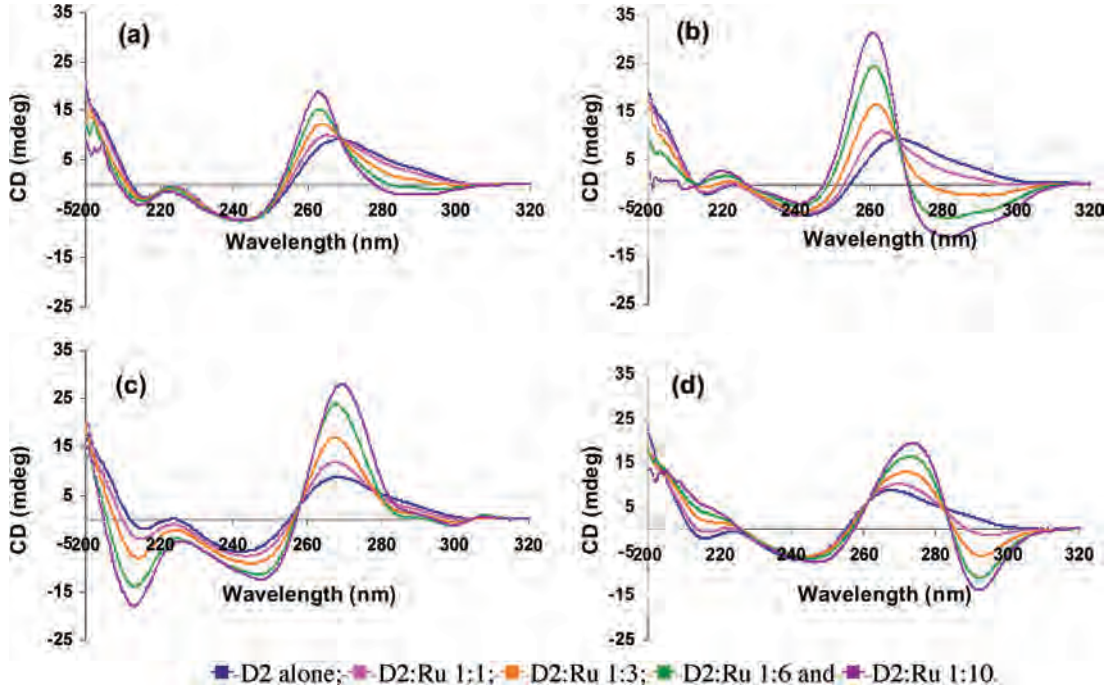

Figure 9. Circular dichroism spectra recorded over the wavelength range 200−320 nm for solutions containing different ratios of ruthenium complexes and D2: (a) $[Ru(phen)_3]^{2+}$, (b) $[Ru(phen)_2(dpqC)]^{2+}$, (c) $[Ru(Me_4phen)_2(dpqC)]^{2+}$, and (d) $[Ru(bpy)_2(dpqC)]^{2+}$; the concentration of D2 in each solution was 20 μM.

this signal that also showed a dependence on the identity of the ruthenium complex present. For example, part a of Figure 9 shows that the ellipticity was approximately zero between 280 and 300 nm for the solution containing $[Ru(phen)_3]^{2+}$ at the highest Ru/DNA ratio examined (10:1). In contrast, parts b and d of Figure 9 show that a significant negative CD band developed in this region as the Ru/DNA ratio was increased. This band was also prominent in the spectra of each of the other metal complexes examined, with the exception of $[Ru(Me_4phen)_2(dpqC)]^{2+}$ (part c of Figure 9) and $[Ru(Me_4bpy)_2(dpqC)]^{2+}$, which both showed little change in ellipticity as the amount of metal in solution was increased.

Table 1 reveals that there is no obvious parallel between the magnitude of the shift in position of the positive CD signal for the metal complexes, and their relative DNA binding affinities, as judged by ESI-MS. However, for the series $[Ru(phen)_2L]^{2+}$ the change in ellipticity for the positive CD band was found to increase according to the following sequence: $[Ru(phen)_3]^{2+} < [Ru(phen)_2(dpq)]^{2+} < [Ru(phen)_2(dpqMe_2)]^{2+} < [Ru(phen)_2(dpqC)]^{2+} < [Ru(phen)_2(dppz)]^{2+}$. This sequence is almost identical to the order of binding affinity derived from ESI mass spectra, providing additional support for the validity of ESI-MS as a technique for studying the non-covalent binding of metal complexes to DNA. For the $[RuL'_2(dpqC)]^{2+}$ series, the increase in ellipticity was found to be greatest when L = phen. Replacing the phen ligands in $[Ru(phen)_2(dpqC)]^{2+}$ by Me_2phen, and the bpy ligands in $[RuL_2(bpy)]^{2+}$ by Me_4bpy, resulted in decreases in ellipticity that indicated the affinity of the metal complexes for D2 had decreased. Each of the above observations parallels trends in DNA binding affinity determined from ESI-MS results. Surprisingly, replacing the Me_2phen ligands in $[Ru(Me_2phen)_2(dpqC)]^{2+}$ by Me_4phen was found to produce a significant increase in ellipticity, suggesting an increase in affinity for D2. Whereas this result is contrary to that obtained by ESI-MS, where a decrease in binding affinity was observed, it does correlate with trends in both binding constant and ΔT_m noted above for these complexes.

Luminescence Spectroscopy. Investigations of the effect of adding DNA on the luminescence of metal complexes have been widely used to provide information about binding constants as well as the number and type of DNA binding modes.[3,6,21,25,28,29b,30a–c,31–33,42] One of the most dramatic observations made in these studies has been the tremendous increase in luminescence intensity displayed by complexes such as $[Ru(phen)_2(dppz)]^{2+}$ and $[Ru(bpy)_2(dppz)]^{2+}$ when DNA is added.[21,30c,42] These complexes display negligible luminescence in aqueous solution, but luminesce strongly upon addition of B-form DNA, an effect that has been dubbed the "light switch effect".[21] Each of the complexes examined as part of the current study showed enhancements in luminescence upon addition of DNA as well as shifts in the position of the wavelength of maximum luminescence. Table 1 presents these parameters, whereas Figure 10 illustrates the changes in luminescence observed for $[Ru(phen)_2(dppz)]^{2+}$ and $[Ru(Me_4phen)_2(dpqC)]^{2+}$. In most cases, the position of the emission band did not shift appreciably upon addition of DNA. However, in the case of both $[Ru(Me_4phen)_2(dpqC)]^{2+}$ and $[Ru(Me_4bpy)_2(dpqC)]^{2+}$ significant shifts to lower energy were detected.

A luminescence enhancement of only 1.3 was measured for $[Ru(phen)_3]^{2+}$, which was the smallest value found and in good agreement with the very low binding affinity exhibited by this complex in experiments involving ESI-

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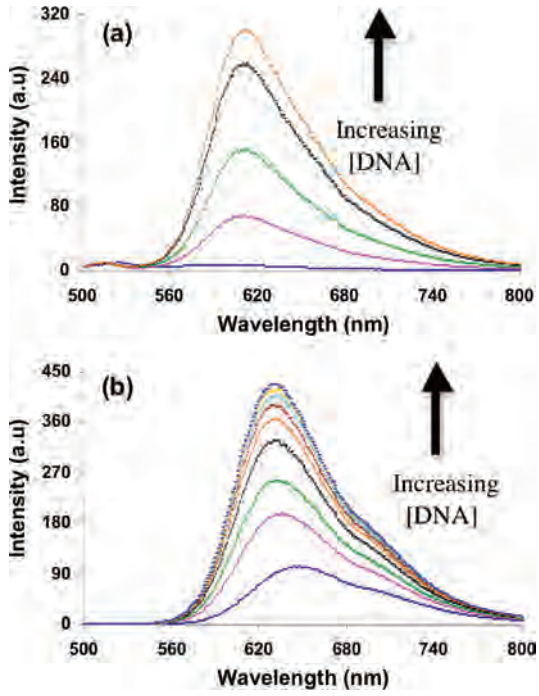

Figure 10. Luminescence spectra of solutions containing ruthenium complexes and increasing amounts of D2; (a) $[Ru(phen)_2(dppz)]^{2+}$, (b) $[Ru(Me_4phen)_2(dpqC)]^{2+}$.

MS and other techniques. However, for the majority of the other complexes in both series, luminescence enhancements were found to vary over a very narrow range and show no correlation with the overall orders of binding affinity determined by the other methods. The one exception to this was $[Ru(phen)_2(dppz)]^{2+}$, which showed the largest luminescence enhancement, consistent with its place at the top of the binding affinity order determined by ESI-MS. Despite this, $[Ru(phen)_2(dppz)]^{2+}$ only showed a maximum luminescence enhancement of 47.0, which is considerably less than values that have been reported previously for this complex with B-form CT DNA.[30c,42] This may be attributed to the presence of trace amounts of luminescent impurities in our solution, which can only be removed by additional chromatographic purification steps, and result in an extremely small but detectable amount of fluorescence.[32] It is important to note that the only complex where such impurities have been previously reported to be a problem in luminescence studies is $[Ru(phen)_2(dppz)]^{2+}$.[32] Furthermore, luminescence enhancements for most of the other complexes examined here are similar to values reported in the literature for the same or closely related complexes. For example, the enhancement found here for $[Ru(phen)_3]^{2+}$ is comparable to the value of 1.9 reported in the literature for this complex with CT DNA.[18] In addition, the complexes $[Ru(bpy)_2(dpq)]^{2+}$ and $[Ru(bpy)_2(dpqC)]^{2+}$, which are closely related to six of the other compounds in the present study, were reported to display luminescence enhancements of between 2 and 5,[33] in good agreement with the overall spread of values found for most complexes here. The overall conclusion to be taken from these studies, therefore, is that luminescence enhancements are not a reliable predictor of relative binding affinities under the conditions of our experiments.

Discussion

Over the past 25 years, a wide range of techniques have been used to analyze the non-covalent interactions of metal complexes with DNA. Whereas these studies have generated debate regarding the exact binding mechanisms used by some of these compounds, there has been good agreement between overall binding affinities determined for a small number of specific metal complexes using different spectroscopic techniques. The first aim of the current study was to compare the order of relative binding affinities determined previously by ESI-MS for the series $[Ru(phen)_2L]^{2+}$, with those obtained using some of the most widely applied techniques for investigating these systems. Our results showed that there was a strong correlation between orders of relative binding affinity determined by absorption titration, DNA melting curve, and circular dichroism methods, with that obtained previously by ESI-MS for the series $[Ru(phen)_2L]^{2+}$.[24] The only exception to this was the unexpectedly low binding constant obtained for $[Ru(phen)_2(dppz)]^{2+}$ (and subsequently $[Ru(bpy)_2(dppz)]^{2+}$) by the absorption titrimetric method, which suggested its affinity toward D2 was slightly less than that of $[Ru(phen)_2(dpqMe_2)]^{2+}$. This result is in contrast to ESI-MS results that suggest the opposite is true and is most likely due to the sensitivity of results obtained by the absorption titrimetric method to the nature and concentration of salt used in the experiments.

A second aim of the current study was to investigate the binding of the complexes $[RuL'_2(dpqC)]^{2+}$ to D2 using ESI-MS and compare the trends in relative DNA binding affinity revealed by this method with those determined by other spectroscopic techniques. In particular, we were interested in examining whether ESI-MS could distinguish between the relative binding affinities of these compounds toward D2 because they were expected to fall within a narrower range than for the $[Ru(phen)_2L]^{2+}$ series. The results of ESI-MS studies into the binding of the complexes $[RuL'_2(dpqC)]^{2+}$ to D2 revealed the following order of relative binding affinities: $[Ru(Me_4bpy)_2(dpqC)]^{2+} \sim [Ru(Me_4phen)_2(dpqC)]^{2+} < [Ru(Me_2phen)_2(dpqC)]^{2+} < [Ru(bpy)_2(dpqC)]^{2+} \sim [Ru(phen)_2(dpqC)]^{2+}$. It was not possible to distinguish between the relative binding affinities of $[Ru(Me_4bpy)_2(dpqC)]^{2+}$ and $[Ru(Me_4phen)_2(dpqC)]^{2+}$ on the one hand and $[Ru(bpy)_2(dpqC)]^{2+}$ and $[Ru(phen)_2(dpqC)]^{2+}$ on the other. However, ESI-MS did clearly show that replacement of the phen ligands in $[Ru(phen)_2(dpqC)]^{2+}$ by first of all Me_2phen ligands, and second Me_4phen ligands, did result in significant decreases in binding affinity in both instances. In addition, replacement of the bpy ligands in $[Ru(bpy)_2(dpqC)]^{2+}$ by Me_4bpy ligands also resulted in spectral changes consistent with a significant decrease in binding affinity.

None of the other spectroscopic methods used here produced an order of relative binding affinity for the series $[RuL'_2(dpqC)]^{2+}$ that exactly matched that obtained by ESI-MS. However, a number of trends are apparent when

comparing binding constants obtained by the absorption titration method, or the results of DNA melting curve measurements or CD spectroscopic studies, which match those present in the binding affinity series obtained by ESI-MS. For example, every one of the above four techniques showed that replacement of the bpy ligands in $[Ru(bpy)_2(dpqC)]^{2+}$ by Me_4bpy ligands resulted in a significant decrease in affinity toward D2. In addition, the binding constants determined by absorption spectrophotometry, and the results of DNA melting curve and CD spectroscopic studies, all indicated that $[Ru(phen)_2(dpqC)]^{2+}$ had the highest affinity of this series of complexes toward D2. This is also in agreement with the binding affinity series determined by ESI-MS, which placed $[Ru(phen)_2(dpqC)]^{2+}$ along with $[Ru(bpy)_2(dpqC)]^{2+}$ at the top of the series.

A surprising result, however, was that the results of CD spectroscopic studies, absorption titration experiments, and DNA melting curve measurements all suggested that the binding affinity of $[Ru(Me_4phen)_2(dpqC)]^{2+}$ was at least equal to, or greater than, that of $[Ru(Me_2phen)_2(dpqC)]^{2+}$. This result is opposite to that obtained by ESI-MS and to what might be expected in view of the greater steric demands imposed by the bulkier Me_4phen ligands. There are at least three possible explanations for this apparent anomaly. The first is that the ESI-MS technique does not accurately reflect slight differences in solution composition caused by the introduction of addition methyl groups onto the ancillary ligands. However, we believe this to be unlikely in view of the generally strong correlation between ESI-MS results and those obtained by the absorption titration, DNA melting curve, and circular dichroism techniques for the $[Ru(phen)_2L]^{2+}$ series. A second possible explanation is that unlike for most of the other compounds examined, there are significant differences in binding mechanisms displayed by the two enantiomers of $[Ru(Me_4phen)_2(dpqC)]^{2+}$, with the unexpected spectroscopic results observed being largely the result of intermolecular DNA interactions involving just one of the two isomers.

A third possible explanation is that intermolecular interactions involving the ancillary ligands of $[Ru(Me_4phen)_2(dpqC)]^{2+}$, and D2, have a disproportionately large effect on some of the spectroscopic measurements. Evidence supporting this explanation comes from the CD results. Addition of $[Ru(Me_2phen)_2(dpqC)]^{2+}$ to D2 resulted in a much smaller increase in ellipticity, and a smaller blue shift for the positive CD signal, than that caused by addition of $[Ru(phen)_2(dpqC)]^{2+}$ to D2. However, the introduction of even more methyl groups onto the ancillary ligands to give $[Ru(Me_4phen)_2(dpqC)]^{2+}$ reversed the above decrease in signal ellipticity, and produced a small red shift in the positive CD signal. The only other complexes of the nine examined here which showed red shifts in CD spectra upon addition to D2 were $[Ru(bpy)_2(dpqC)]^{2+}$ and $[Ru(Me_4bpy)_2(dpqC)]^{2+}$, highlighting the sensitivity of this technique to changes in intermolecular interactions caused by the ancillary ligands of these metal complexes. Further evidence is provided by the results of luminescence experiments, which showed that addition of DNA to either $[Ru(Me_4phen)_2(dpqC)]^{2+}$ or $[Ru(Me_4bpy)_2(dpqC)]^{2+}$ resulted in relatively large (14−17 nm) blue shifts for their emission bands. In contrast, each of the other complexes examined showed shifts to higher or lower energy of 1−2 nm at most.

The degree of luminescence enhancement caused by addition of DNA to the metal complexes proved to be the only qualitative indicator of relative binding affinity that did not show a good correlation with binding affinities determined by ESI-MS for either series of complexes. This was primarily a result of the very narrow range of luminescence enhancements displayed by most complexes and the sensitivity of luminescence spectra to different aspects of the metal complex/DNA interaction than the other techniques. Overall, it appears that luminescence enhancements, unlike each of the other parameters used here, are not a useful qualitative indicator of the relative DNA binding strength of metal complexes.

Attempts were also made to analyze the absorption spectroscopic data using a recently reported procedure[43] based on the intrinsic method,[35,44] which yields not only binding constants but also an average binding site size. Whereas this procedure afforded acceptable binding constants in some instances, on other occasions the resulting Scatchard plots showed significant deviations from nonlinearity possibly due to the presence of two or more binding modes at different metal/DNA ratios. Because the main purpose of this article was to demonstrate the validity of ESI-MS for investigating non-covalent binding of metal complexes to DNA, we therefore restricted our discussion of binding constants to those determined using eq 1, which has been shown on many occasions to provide a convenient estimate of overall binding strength.[28,30]

Conclusions

ESI-MS was used to determine an order of relative binding affinity for the complexes $[Ru(phen)_2L]^{2+}$ toward the ds DNA hexadecamer D2. This series matched very closely those obtained by absorption spectrophotometry, CD spectroscopy, and DNA melting curves. Although the degree of comparability between orders of binding affinity obtained in a similar study using the complexes $[RuL'_2(dpqC)]^{2+}$ was not as high, several trends in binding affinity were found in ESI-MS studies that matched those found using the other methods. Overall the results therefore support that ESI-MS can be used to provide an accurate picture of the non-covalent DNA binding interactions of metal complexes. The sensitivity and rapid analysis that is possible using ESI-MS means that this method can be used as a preliminary screen for relative orders of binding affinity of metal complexes for dsDNA. We are currently extending our studies to investigate the applicability of using ESI-MS for examining interactions of multinuclear metal complexes with DNA, including higher-order DNA structures.

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