Scanning Force Microscopy of Small Ligand−Nucleic Acid Complexes: Tris(*o*-phenanthroline)ruthenium(II) as a Test for a New Assay

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Abstract: An understanding of DNA−ligand interactions at the molecular level is important for the design of new drugs and probes that can recognize specific DNA sequences and structural motifs. Interestingly, determining the mode-of-binding of a DNA ligand is not always straightforward due to uncertainties inherent in traditional assays. We have recently reported an exciting new assay utilizing scanning force microscopy (SFM) that can discern whether a ligand binds to DNA by intercalative or nonintercalative modes [Coury et al. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 12283−12286]. Visualization of individual DNA molecules by SFM and observation of ligand-induced lengthening provides direct evidence for intercalation. Metal complexes of polypyridyl ligands have been extensively studied as new probes of DNA structure and function because they exist as chiral molecules with the potential of enantioselective recognition of DNA. The binding mode of even the most widely studied of the members of this group, tris(*o*-phenanthroline)ruthenium(II) ($Ru(phen)_3^{2+}$), remains somewhat controversial due in large part to its low binding affinity. We report here the use of $Ru(phen)_3^{2+}$ as a test of our new assay toward the studies of weakly-binding ligands and to resolve the ambiguity surrounding the mode-of-binding of Δ and Λ-$Ru(phen)_3^{2+}$. Experiments reported here reveal that the experimental conditions of our assay do not preclude the binding of $Ru(phen)_3^{2+}$ to DNA and that NO lengthening occurs. Our findings are consistent with the view that $Ru(phen)_3^{2+}$ binds to duplex nucleic acids through nonintercalative modes.

DNA is a target of many clinically important chemotherapeutic agents. These agents bind to DNA within the grooves, ordinarily within the minor rather than the major groove, and/or by intercalating between base pairs. DNA ligands act through inhibition or disruption of such processes as replication, transcription, and translation. An understanding of DNA−ligand interactions at the molecular level is important for design of new drugs and probes that can recognize specific DNA sequences and structural motifs.

With conventional techniques, determining the mode-of-binding of a DNA ligand is not always straightforward. Detailed structural information is provided by X-ray diffraction and NMR spectroscopy. These three-dimensional techniques are often precluded by polymorphism in conformation, multiple modes-of-interaction, and low binding affinity and are limited to short DNA fragments. Other techniques such as hydrodynamics, sedimentation, and optical spectroscopy are indirect and inferential. These techniques average over population and time—a source simultaneously of strength and weakness.

We have recently reported an exciting new assay utilizing scanning force microscopy (SFM).[1] The premise of the assay is that intercalating ligands necessarily lengthen DNA, whereas groove-binding ligands do not. We use SFM to directly measure the length of individual DNA molecules of known molecular weight immobilized on a two-dimensional surface. From precise measurements of DNA length, affinity constants and exclusion numbers for several intercalators have been determined. Affinity constants obtained by SFM are in accordance with those obtained by other techniques, indicating the length of immobilized DNA "reports" its length in solution. Further evaluation of ligands with lower binding affinities is necessary to illustrate the generality of this new assay.

Metal complexes of polypyridyl ligands have been extensively studied as probes of DNA structure and function.[2−4] These complexes are especially interesting, because they exist as chiral molecules with the potential for enantioselective recognition of DNA. The most widely studied member of this group is tris(*o*-phenanthroline)ruthenium(II) ($Ru(phen)_3^{2+}$) (Figure 1). Chaires and co-workers have provided a brief review of what is known about $Ru(phen)_3^{2+}$ and its related polypyridyl analogs.[2]

Despite effort by a number of research groups, the binding mode of $Ru(phen)_3^{2+}$ remains ambiguous. Unwinding studies with closed circular DNA and absorption and fluorescence spectroscopies suggest that both enantiomers bind to DNA through intercalative modes.[5,6] NMR studies suggest that Δ-$Ru(phen)_3^{2+}$ prefers intercalation, while Λ-$Ru(phen)_3^{2+}$ prefers surface binding, possibly in the minor groove.[7] Energy minimization calculations and molecular modeling show that only partial insertion of one phenanthroline ring is feasible. Full insertion is blocked by the other two external phenanthroline ring systems.[8] Results of linear dichroism, equilibrium, and

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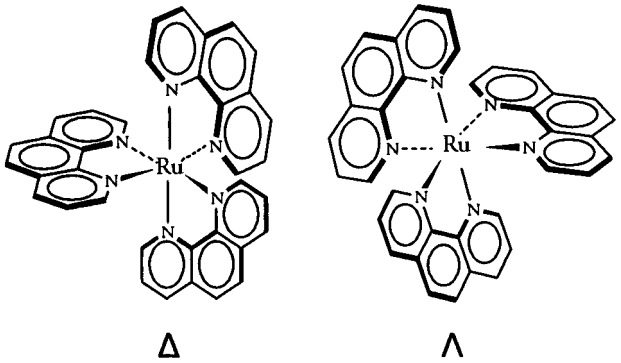

Figure 1. Enantiomers of tris(*o*-phenanthroline)ruthenium(II).

viscosity experiments suggest that neither isomer intercalates in DNA.[9,10] We use $Ru(phen)_3^{2+}$ to test our new SFM assay for study of weakly-binding ligands and resolve the ambiguity surrounding the mode-of-binding of Δ and Λ-$Ru(phen)_3^{2+}$.

Materials and Methods

Chemicals and Nucleic Acids. Reagents were used as received from Sigma-Aldrich. Nanopure water (18 MΩ-cm) was used throughout. Samples of $Ru(phen)_3^{2+}$ optical isomers were provided by J. B. Chaires. Enantiomeric purity (>95%) was measured by circular dichroism and absorption measurement. Concentrations of $Ru(phen)_3^{2+}$ stock solutions were determined from absorbance at 447 nm by using a molar absorptivity $\epsilon_{447} = 19\,000\ M^{-1}\ cm^{-1}$.[11] $Ru(tpy)(dppz)OH_2^{2+}$ (tpy = 2,2′,2″-terpyridine; dppz = dipyridophenazine) was provided by H. Holden Thorp. Concentrations of $Ru(tpy)(dppz)OH_2^{2+}$ stock solutions were determined from absorbance at 482 nm by using a molar absorptivity $\epsilon_{482} = 12\,000\ M^{-1}\ cm^{-1}$.[12] The DNA is 10.3 kb *p*BluBacHis b (*p*BBHb, In Vitrogen, V370−20) bacterially amplified, purified by CsCl gradient, linearized by Hind III (New England Biolabs), and resuspended in buffer (10 mM ammonium acetate, 5 mM $MgCl_2$ adjusted to pH 7.5−8.0 with sodium hydroxide). DNA−Ru-$(phen)_3^{2+}$ samples were prepared by appropriate dilution of DNA and $Ru(phen)_3^{2+}$ stock solutions in buffer. All operations involving potential ligands were carried out under subdued lighting conditions.

Scanning Force Microscopy. Images were obtained on a Nanoscope II or IIIa (Digital Instruments, Santa Barbara, CA). SFM substrates were freshly cleaved, Muscovite green mica disks (New York Mica Co., New York, NY) 3/8″ in diameter. Disks were placed on top of a droplet of DNA or DNA/ligand solution (0.1 μg DNA per mL) and allowed to stand for 10 min. Each DNA-laden disk was dipped sequentially in water, 50/50 ethanol/water, and twice in anhydrous ethanol. Excess liquid was "wicked" away with a Kimwipe, and the disk was blown dry with clean compressed chlorofluorocarbon gas (Tech Spray, Inc., Amarillo, TX) directed normal to the disk surface. Disks were stored overnight under anhydrous conditions prior to imaging. Samples were imaged under a minimum constant force in the repulsive-contact regime as indicated by force-distance curves obtained frequently during the imaging procedure. Total forces encountered were typically < 10 nN. Cantilevers with a force constant of 0.10 N/m and oxide-etched (i.e.,, sharpened) pyramidal Si_3N_4 probe tips of radius 20−40 nm (Sharpened Microlevers, Park Scientific Instruments, Sunnyvale, CA) were utilized. Images were obtained under a N_2 atmosphere to minimize the effects of humidity.[13] Contour lengths of DNA molecules were carefully measured using the cumulative X−Y distance measurement feature in the "TopView" mode within the microscope's off-line analysis software. DNA molecules with ambiguous topology were excluded from the analysis.

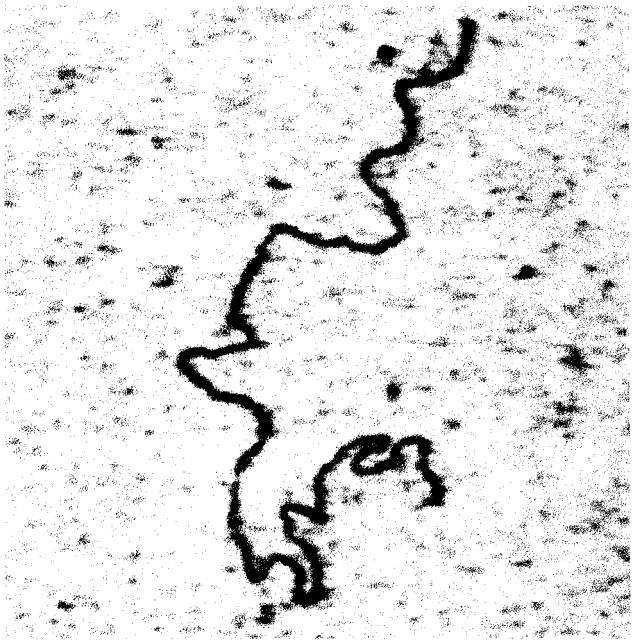

Figure 2. Representative SFM image of *p*BBHb on a mica substrate. Length = 3390 nm. Height of the DNA molecule is approximately 1.5 nm.

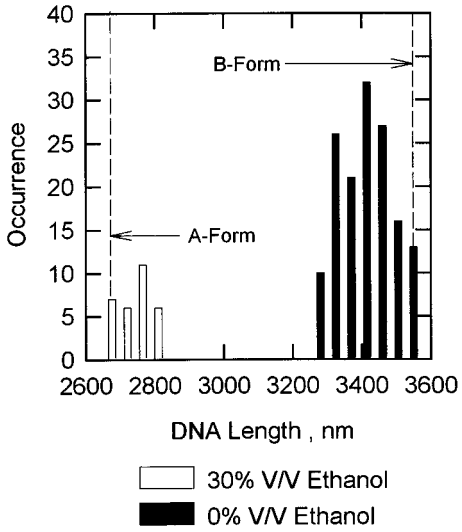

Figure 3. Histogram of measured molecular lengths for *p*BBHb following immobilization from buffer (10 mM ammonium acetate, 5 mM $MgCl_2$, pH 7.5) and 30% (v/v) ethanol in buffer.

Results

The length of immobilized DNA reports its length in solution. Figure 2 is a representative image of a *p*BBHb molecule without ligand immobilized on a mica substrate. The mean length of the plasmid was 3426 nm ($n = 145$, $\sigma = 76$ nm) illustrating that the unintercalated plasmid is predominantly in the B-form. Once immobilized on the surface, the plasmid length is fixed; no significant changes in mean length were observed over the course of several days. Addition of ethanol to B-DNA *in solution* converts the molecule to A-DNA. The mean length of plasmid immobilized from a 30% v/v ethanol solution was 2776 nm ($n = 32$, $\sigma = 55$ nm). A histogram of length data for both A-DNA and B-DNA is provided in Figure 3. By contrast, ethanol treatment following immobilization of DNA has no effect on its length.

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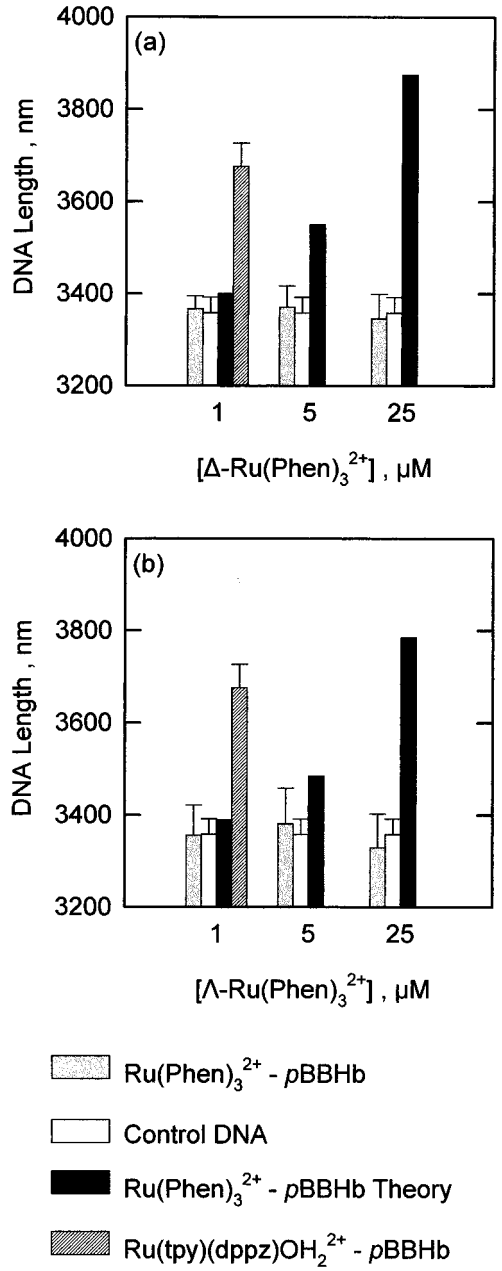

Figure 4. DNA length vs $Ru(phen)_3^{2+}$ concentration for the (a) Δ enantiomer and (b) Λ enantiomer. Error bars represent $\pm 1\sigma$. Over 100 high resolution SFM images of individual DNA complexes were obtained and examined. Bar descriptions from left to right: gray dotted pattern, white, black, and hatch pattern.

The lengths of DNA−$Ru(phen)_3^{2+}$ complexes were not statistically different from the lengths of unintercalated controls. Solutions of plasmid DNA with each enantiomer of $Ru(phen)_3^{2+}$ were prepared at a variety of ligand/bp ratios—i.e., 6.6 (1 μM $Ru(phen)_3^{2+}$), 33 (5 μM $Ru(phen)_3^{2+}$), and 165 (25 μM $Ru(phen)_3^{2+}$). Images of each complex were acquired in identical fashion with control images. Figure 4 displays the results of length measurements for each complex. Standard deviations in the lengths of DNA-$Ru(phen)_3^{2+}$ complexes were only 2−3% of the mean length of unintercalated DNA. The theoretical lengthening of DNA classically intercalated by $Ru(phen)_3^{2+}$ was calculated using reported binding affinities (K) of 4.9×10^4 and 2.8×10^4 M^{-1} and exclusion numbers (n) of 3.70 and 3.40, for the Δ and Λ enantiomers, respectively.[2] An exclusion number is the number of binding sites excluded by the binding of a single ligand. Lerman's model of classical intercalation,

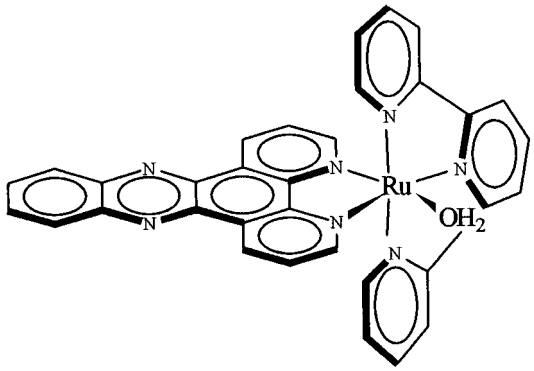

Figure 5. Structure of $Ru(tpy)(dppz)OH_2^{2+}$.

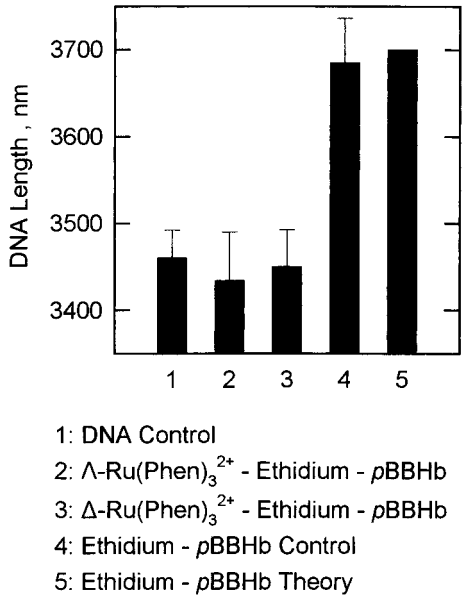

Figure 6. Results of competition binding experiments using 25 μM $Ru(phen)_3^{2+}$ and 2.5 μM ethidium (Et). Error bars represent $\pm 1\sigma$.

where the nucleic acid is lengthened by the van der Waals thickness of the intercalating moiety (3.4 Å), is assumed.[14,15]

Extension of the phenanthroline ring system, as in $Ru(tpy)(dppz)OH_2^{2+}$ (Figure 5), allows for classical intercalation and lengthening of the DNA.[16−19] A solution of plasmid DNA with $Ru(tpy)(dppz)OH_2^{2+}$ was prepared at a ligand/bp ratio of 6.6 (1 μM $Ru(tpy)(dppz)OH_2^{2+}$). $Ru(tpy)(dppz)OH_2^{2+}$ is unambiguously known to intercalate with a relatively high binding affinity (7×10^5 M^{-1}).[19] Micrographs reveal that the DNA length is approximately 200 nm longer than the control length.

Competitive binding experiments with ethidium exclude the possibility that our experimental conditions inhibit $Ru(phen)_3^{2+}$ binding in solution. Ethidium is a well-characterized bifunctional minor groove binder and intercalator[20] that lengthens the plasmid pBBHb to over 5200 nm at saturation.[1] Solutions were prepared by sequential addition of ligands to a DNA solution. Final concentrations were 2.5 μM ethidium (ethidium/bp = 16.5) and 25 μM in the enantiomer of $Ru(phen)_3^{2+}$ ($Ru(phen)_3^{2+}$/bp

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Table 1. Experimental Conditions and Binding Modes Reported in the Literature[a] for $Ru(phen)_3^{2+}$

date[b]	technique	ionic strength, mM[c]	Ru/bp	conclusion[d]	ref
2/1/83	circular dichroism	20	0.33	C_3 axis of $Ru(phen)_3^{2+}$ oriented differently relative to helix axis	29
8/26/83	absorbance spectroscopy	55	0.02[e]	Δ and Λ $Ru(phen)_3^{2+}$ intercalate	6
	fluorescence spectroscopy	55	0.033		
	electrophoresis	88			
	dialysis	55	≤1		
	circular dichroism	55	0.2		
10/14/83	circular dichroism	100	0.011	Δ $Ru(phen)_3^{2+}$ intercalates Λ $Ru(phen)_3^{2+}$ electrostatically bound	30
7/25/85	topoisomerase assay	10	0.044	racemic $Ru(phen)_3^{2+}$ intercalates	31
	thermal denaturation	30	≤0.02		
8/29/85	dialysis	55−205	0.02−0.2	two binding modes: intercalation and surface binding	3
	luminescence quenching	5	0.05		
	polarization	5	0.067		
	emission lifetime	5	0.05		
3/29/89	linear/circular dichroism	11	0.155	one mode: nonintercalative	9
10/19/89	^1H NMR	30	≤0.167	Δ $Ru(phen)_3^{2+}$ intercalates	7
	dialysis	100	≤0.03	Λ $Ru(phen)_3^{2+}$ surface binds	
	viscosity	5	0.05		
12/10/91	2D ^1H NMR	30	0.03(Δ) 0.095(Λ)	$Ru(phen)_3^{2+}$ binds in the minor groove	32
8/13/92	fluorescence titration	15	∼0.01−10[f]	one mode: nonintercalative	10
	dialysis	55			
	viscosity	55			
present	scanning force microscopy	25	6.7−167	nonintercalative	1

[a] These conditions were, to the best of our ability, reproduced directly from the manuscripts cited and not through personal communication with the authors. [b] This date reflects the receival date noted on the publication. [c] Buffer ionic strength, here, is defined as a molarity-scale ionic strength (vice molality-scale). These values do not reflect contributions to ionic strength made by the charged ligands but only of the buffer salts. [d] Unless otherwise noted, the conclusions are relative to both enantiomers of $Ru(phen)_3^{2+}$. [e] All ratios for these experiments are Ru/DNA vice Ru/bp. [f] Ru/DNA ratio estimated from experimental detail and Scatchard plots provided.

= 165). A 10-fold increase in concentration of $Ru(phen)_3^{2+}$ over ethidium was necessary due to the lower binding affinity of $Ru(phen)_3^{2+}$. Figure 6 presents the results of competitive binding experiments. Lengths of DNA−ethidium complexes (2.5 μM ethidium, ethidium/bp = 16.5) were approximately 240 nm longer than unintercalated DNA. The theoretical length at 2.5 μM ethidium was calculated assuming classical intercalation and using $K = 6.6 \times 10^4\ M^{-1}$ and $n = 2.00$ as determined by our SFM assay previously.[1] No lengthening was observed when $Ru(phen)_3^{2+}$ was present along with ethidium.

Discussion

SFM directly measures the length of individual DNA molecules of known molecular weight immobilized on a two-dimensional surface. Our sample preparation scheme uses small volumes of dilute DNA solutions to maximize the number of isolated molecules with topology amenable to unambiguous determination of length. The presence of Mg^{2+} (or other divalent cation) in the buffer is a prerequisite for acquisition of reproducible images.[21−23] The measured lengths of individual DNA molecules conform with expectations for A-DNA or B-DNA, depending on DNA solution conditions. The theoretical length of B-DNA is 3502 nm.[24] The B-DNA controls imaged and reported in this paper are within 4.1% of this length.[25] Similarly, the theoretical length of A-DNA is 2678 nm. Our measured lengths for A-DNA are within 3.6% of this value. It should be noted here that DNA immobilized from an aqueous solution and imaged under alcohol is in the B-form.[26] Thus, the length and conformation of immobilized nucleic acid molecules are commensurate with their length and conformation *in solution*.

The standard deviation in length measurements for controls was only 28 nm. Probe tip size impacts length measurement uncertainty by contributing to capillary forces (i.e., larger tip surface areas in contact with water layers produce greater capillary forces). In addition, all scanning probe images are convolutions of sample topography and tip geometry. Apparent widths of DNA images are always greater than what would be expected due to this convolution. For a hemispherical tip, the apparent widths can be shown to be $(8dR)^{1/2}$ where R is the radius of the tip and d is the actual diameter of the feature being measured.[27] If one were to assume a Watson−Crick diameter of 2.0 nm,[24] a tip of radius (20 nm) would provide an apparent width of 18 nm. The width of the DNA molecules in Figure 2 is 20 nm. As the apparent width increases, standard deviations in length measurements will increase. This is a result of the method by which the molecular lengths are determined. Line segments, drawn along the contour length of the molecule, are added cumulatively. As apparent widths of the molecules increase, bends in the molecule become obscured and can no longer be accurately measured thus contributing to greater deviation from molecule to molecule. The contribution to measured length from tip convolution at the ends of the DNA strand are minimal. Assuming a 20 nm tip radius and a DNA height of 2 nm, this contribution is only ∼18 nm or 0.5% of the control DNA lengths.

The premise of the assay is that intercalating ligands necessarily lengthen DNA, whereas groove-binding ligands do not. An inherent advantage of the assay is the high precision available for direct measurement of the length of single DNA

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molecules. For example, let us arbitrarily select 3σ of controls as the minimum observable lengthening (i.e., 85 nm). For *p*BBHb, this lengthening corresponds to the occupation of only 4.9% of the available sites by a simple, classical intercalator ($n = 2$, number of potential intercalating sites = 5150).

DNA samples, incubated at three relatively high $Ru(phen)_3^{2+}$ concentrations, do not reveal any lengthening when compared to control samples. Competition experiments with ethidium indicate that $Ru(phen)_3^{2+}$ is bound to the DNA *in solution* and inhibits the binding of ethidium. We thus conclude that neither enantiomer of $Ru(phen)_3^{2+}$ intercalates. SFM determined lengths of immobilized DNA accurately reports its length in solution. After immobilization, the length of the plasmid is fixed. The question as to whether the drug remains bound to the DNA after immobilization is an interesting one. Studies are currently underway using our previously reported marking strategy[28] to determine if noncovalently bound ligands remain with the DNA after sample preparation (i.e., rinsing and dehydration).

The ionic strength used in our experiment is comparable with those of others interested in $Ru(phen)_3^{2+}$−DNA binding (see Table 1). However, the $Ru(phen)_3^{2+}$/bp ratios used in our SFM experiments dramatically exceed those used by others for spectroscopy experiments which are limited to lower concentrations where Beer's Law is applicable.[33] Thus, a second advantage of the SFM assay is the ability to perform experiments over large ranges of ligand−DNA ratios.

Conclusions

$Ru(phen)_3^{2+}$ serves as a test to evaluate the usefulness of our new SFM assay toward ligands with low binding affinities. Our experimental conditions are quite similar to those of traditional assays and do not preclude the binding of $Ru(phen)_3^{2+}$ to DNA. No lengthening of the DNA occurs upon interaction with $Ru(phen)_3^{2+}$. Our findings are consistent with the view that $Ru(phen)_3^{2+}$ binds to duplex nucleic acids through nonintercalative modes.

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