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Dynamics of Supercoiled and Relaxed pTZ18U Plasmids Probed with a Long-Lifetime Metal-Ligand Complex

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$[Ru(bpy)_2(dppz)]^{2+}$ (bpy = 2,2'-bipyridine, dppz = dipyrido-[3,2-a:2',3'-c]phenazine) (RuBD), a long-lifetime metal-ligand complex, displays favorable photophysical properties. These include long lifetime, polarized emission, but no significant fluorescence from the complex that is not bound to DNA. To show the usefulness of this luminophore (RuBD) for probing the bending and torsional dynamics of nucleic acids, its intensity and anisotropy decays when intercalated into supercoiled and relaxed pTZ18U plasmids were examined using frequency-domain fluorometry with a blue light-emitting diode (LED) as the modulated light source. The mean lifetimes for the supercoiled plasmids ($<\tau>$ = 148 ns) were somewhat shorter than those for the relaxed plasmids ($<\tau>$ = 160 ns). This suggests that the relaxed plasmids were shielded more efficiently from water. The anisotropy decay data also showed somewhat shorter slow rotational correlation times for supercoiled plasmids (288 ns) than for the relaxed plasmids (355 ns). The presence of two rotational correlation times suggests that RuBD reveals both the bending and torsional motions of the plasmids. These results indicate that RuBD can be useful for studying both the bending and torsional dynamics of nucleic acids.

Keywords: Bending and torsional dynamics, Light-emitting diode, Long-lifetime metal-ligand complex, Supercoiled and relaxed pTZ18U

Introduction

The structure and dynamics of DNA are receiving more and more attention. They have been studied by various techniques including time-resolved fluorescence anisotropy (Schurr *et al*., 1992; Kielkopf *et al*., 2000; Lee and Choi, 2000; Okonogi *et al*., 2000). The interaction of transition metal-ligand complexes (MLCs) with DNA is an area of intense current interest. This is partly because of the potential of these compounds as novel probes of DNA structure and dynamics (Lakowicz *et al*., 1995; Malak *et al*., 1997; Lakowicz *et al*., 2000; Rajski *et al*., 2000; Kang and Lakowicz, 2001; Kang *et al*., 2002). Long-lifetime MLCs, which display decay times that range from 100 ns to more than 10 μs, have only recently become available (DeGraff *et al*., 1994; Terpetschnig *et al*., 1997; Lakowicz *et al*., 2000). They have favorable chemical, photochemical, and photophysical properties. They show large Stokes' shifts, polarized emission, as well as good water solubility and high chemical and photochemical stability (Terpetschnig *et al*., 1997; Lakowicz *et al*., 2000). In addition, the long lifetimes of the MLCs allow the use of gated detection, which can provide increased sensitivity (Haugen and Lytle, 1981).

Barton and co-workers (Friedman *et al*., 1990; Jenkin *et al*., 1992; Murphy and Barton, 1993) reported that the dipyrido[3,2-a:2',3'-c]phenazine (dppz) complexes of ruthenium appear to be a prime candidate for a spectroscopic probe for nucleic acids because of their "molecular light switch" properties for DNA. Since the luminescent enhancement upon DNA binding is $\geq 10^4$, compared to an enhancement of ~20 for ethidium bromide (EB), there is essentially no background with the dppz complexes of ruthenium. Barton and co-workers (Jenkin *et al*., 1992) also showed that the 2,2'-bipyridine (bpy) derivative of the ruthenium complex, $[Ru(bpy)_2(dppz)]^{2+}$ (RuBD), exhibited sensitivity to conformational differences in DNA because of the incomplete shielding of the dppz ligand from water in the presence of bpy, in contrast to the other phenanthroline derivative. In this laboratory, we introduced the use of fluorescence anisotropy decays of the dppz complexes of

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ruthenium to study the dynamics of nucleic acids, extending the measurable time scale of DNA dynamics to the submicrosecond range. Because these complexes display large fundamental anisotropies (r_0), we measured the anisotropy decays of calf thymus DNA (Lakowicz *et al*., 1995; Malak *et al*., 1997), as well as supercoiled and linear pTZ18U plasmids (Kang *et al*., 2002) using these ruthenium complexes.

In this study, we examined the intensity and anisotropy decays of RuBD intercalated into supercoiled and relaxed pTZ18U plasmids from *E. coli* HB101 in order to further show the usefulness of this luminophore (RuBD) for probing the bending and torsional dynamics of DNA. We used frequency-domain (FD) fluorometry with a high-intensity, blue light-emitting diode (LED) as the modulated light source. With this LED, we were able to directly modulate the excitation light up to 100 MHz without the need for an external modulator (like a Pockels cell) and to obtain very reliable time-resolved intensity and anisotropy decays of RuBD, which has a very low quantum yield ($Q = 0.008$) (Lakowicz *et al*., 2001).

Materials and Methods

Materials *E. coli* HB101, topoisomerase I, and bovine serum albumin (BSA) were purchased from Gibco BRL (Life Technologies, Grand Island, USA); LB-medium from Bio 101, Inc. (Vista, USA); agarose and ampicillin from Sigma (St. Louis, USA); pTZ18U plasmid from Bio-Rad (Hercules, USA); and a plasmid mega kit from Qiagen Inc. (Valencia, USA). RuBD was synthesized by the method described previously (Lakowicz *et al*., 1995). The chemical structure of RuBD is shown in Fig. 1. All of the other chemicals were of reagent grade. Water was deionized with a Milli-Q system. All of the measurements were carried out in a Topo-buffer (5 mM Tris-HCl, 10 mM $MgCl_2$, 50 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, and 30 μg/ml BSA, pH 7.4).

Absorption and steady-state fluorescence measurement The pTZ18U plasmids were purified with a Qiagen plasmid mega kit from 500 ml overnight cultures of *E. coli* HB101 in LB medium that contained ampicillin. The pTZ18U plasmids were relaxed with topoisomerase I in Topo-buffer for 2 h at 37°C. The completion of relaxing was monitored by electrophoresis in 1% agarose gel (Fig. 2). About 5-10 mM stock solution of RuBD was prepared in dimethylformamide. The plasmid concentration was 300 μM base pairs (bps), while the concentration of RuBD was 15 μM. The DNA and RuBD concentrations were determined using molar extinction coefficients of 13,300 $M^{-1}cm^{-1}$ (expressed as bp) at 260 nm and 13,000 $M^{-1}cm^{-1}$ at 440 nm, respectively. UV-visible absorption spectra were measured with a Hewlett-Packard 8453 diode array spectrophotometer. Steady-state intensity and anisotropy measurements were carried out using an Aminco · Bowman series 2 luminescence spectrometer (Spectronic Instruments, Inc., Rochester, USA). RuBD was nonfluorescent in Topo-buffer without pTZ18U plasmids in the absence and presence of topoisomerase I. This demonstrates the absence of any impurities, as well as any effect of topoisomerase I on the fluorescence intensity of RuBD (data not shown).

The intensity of the components of the fluorescence that were parallel (I_{VV}) and perpendicular (I_{VH}) to the direction of the vertically polarized excitation light was determined by measuring the emitted light through polarizers that were oriented vertically and horizontally. The steady-state anisotropy is given by:

$$r = \frac{I_{VV} - GI_{VH}}{I_{VV} + 2GI_{VH}} \quad (1)$$

where G is a grating correction factor for the monochromator's transmission efficiency for vertically and horizontally polarized light. This value is given by the ratio of the fluorescence intensities of the vertical (I_{HV}) to horizontal (I_{HH}) components when the exciting light is polarized in the horizontal direction.

FD intensity and anisotropy decay measurements Measurements were performed with the instruments that were described previously (Lakowicz and Maliwal, 1985) and modified with a data acquisition card from ISS, Inc. (Urbana, USA) (Feddersen *et al*., 1989). The excitation source was a blue LED LNG992CFBW (Panasonic, Japan) with luminous intensity of 1,500 mcd. An LED driver LDX-3412 (ILX Lightwave, Boseman, USA) provided

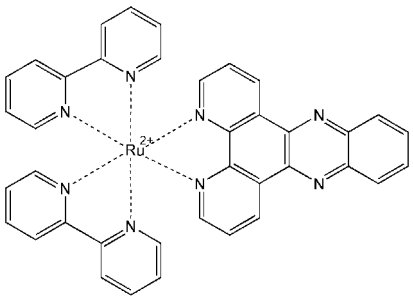

Fig. 1. Chemical structure of $[Ru(bpy)_2(dppz)]^{2+}$ (RuBD).

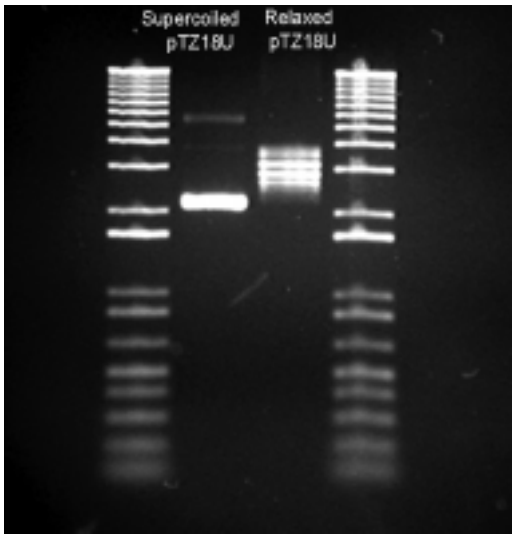

Fig. 2. Agarose gel electrophoresis pattern of supercoiled and relaxed pTZ18U plasmids. A 1kb DNA ladder was used.

30 mA of current at frequencies from 0.4 to 15 MHz. A 480 ± 20 nm interference filter and a 630 nm cut-off filter were used for isolating excitation and emission, respectively. Rhodamine B in water (τ = 1.68 ns) was utilized as a lifetime standard. All of the measurements were performed at 25°C.

The intensity decays were recovered from the FD data in terms of a multiexponential model:

$$I(t) = \sum_{i=1}^{n} \alpha_i e^{-t/\tau_i} \quad (2)$$

where the preexponential factor a_i is the amplitude of each component, $\Sigma a_i = 1.0$, τ_i is the decay time, and n is the number of exponential components. These values were determined by a nonlinear least squares analysis as described previously (Gratton *et al*., 1984; Lakowicz *et al*., 1984). Mean lifetimes were calculated by:

$$<\tau> = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} = \sum_i f_i \tau_i \quad (3)$$

where f_i is the fractional steady-state contribution of each component to the total emission, and Σf_i is normalized to unity. f_i is given by:

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \quad (4)$$

The best fits were obtained by minimizing χ_R^2 values:

$$\chi_R^2 = \frac{1}{\nu} \sum_\omega \left[\left(\frac{\varphi_\omega - \varphi_{c\omega}}{\delta\varphi} \right)^2 + \left(\frac{m_\omega - m_{c\omega}}{\delta m} \right)^2 \right] \quad (5)$$

where ν is the number of degrees of freedom, and φ_ω and m_ω are the experimental phase and modulation, respectively. The subscript c is used to indicate calculated values for assumed values of α_i and τ_i, and $\delta\varphi$ and δm are the experimental uncertainties.

The FD anisotropy decays were also analyzed in terms of the multiexponential model:

$$r(t) = \sum_i r_0 g_i e^{-t/\theta_i} \quad (6)$$

where g_i is the amplitude of the anisotropy component with a rotational correlation time θ_i, $\Sigma g_i = 1.0$, and r_0 is the anisotropy in the absence of rotational diffusion. The total anisotropy r_0 was a fitted parameter. The modulated anisotropy r_ω was calculated by:

$$r_\omega = \frac{\Lambda_\omega - 1}{\Lambda_\omega + 2} \quad (7)$$

where Λ_ω is the ratio of the amplitudes of the parallel and the perpendicular components of the modulated emission.

Results and Discussion

In this study, we demonstrated the usefulness of RuBD, a long-lifetime MLC, for probing the bending and torsional dynamics of supercoiled and relaxed pTZ18U plasmids. Fig. 3 shows the emission spectra of RuBD that is intercalated into the supercoiled and relaxed forms of the plasmids. For both forms, there was no difference in the emission spectra under our experimental conditions. RuBD showed an emission peak at about 620 nm. Steady-state anisotropy measurements also showed no difference in the anisotropy values between 4 and 45°C for both plasmid forms (Fig. 4).

The FD intensity decays of RuBD intercalated into the supercoiled and relaxed pTZ18U plasmids are shown in Fig.

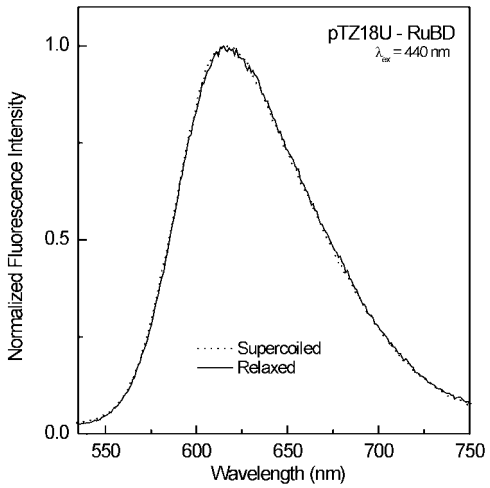

Fig. 3. Emission spectra of RuBD intercalated into supercoiled and relaxed pTZ18U plasmids.

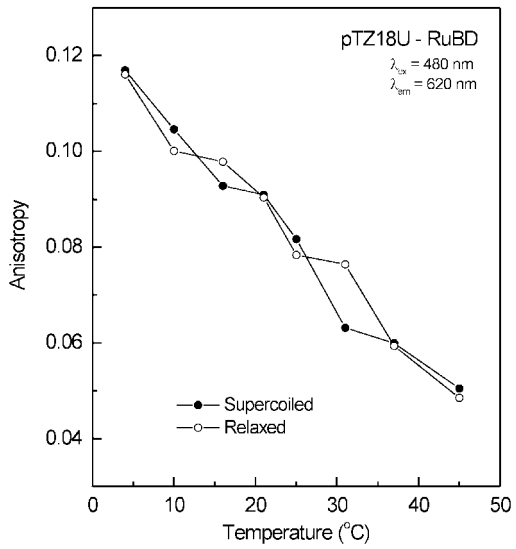

Fig. 4. Temperature-dependent steady-state anisotropy of RuBD intercalated into supercoiled and relaxed pTZ18U plasmids.

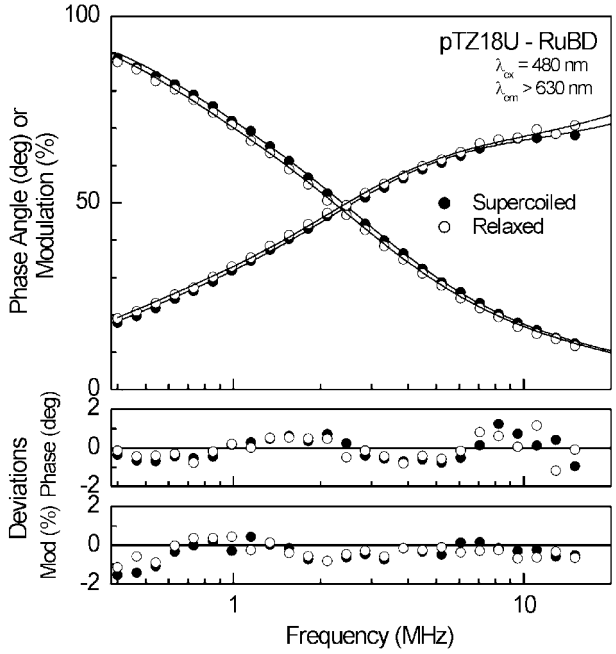

Fig. 5. Intensity decays of RuBD intercalated into supercoiled and relaxed pTZ18U plasmids. The symbols represent the measured phase and modulation values. The solid lines show the best multiexponential fits to the data. The middle and lower panels show plots of the residuals between the experimental data and the fitted curve.

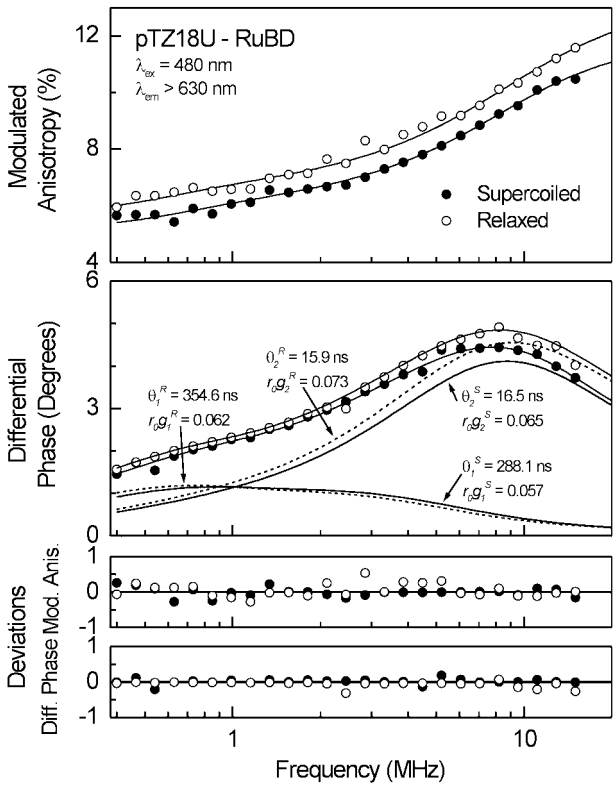

Fig. 6. Anisotropy decays of RuBD intercalated into supercoiled and relaxed pTZ18U plasmids. The symbols in the first and second panels represent the calculated modulated anisotropy and the measured phase shift values, respectively. The solid lines show the best multiexponential fits to the data. The differential phase data in the second panel are based on the rotational correlation times and amplitudes that are shown in Table 2. The superscripts *S* and *R* denote supercoiled and relaxed pTZ18U plasmids, respectively. The lower two panels show plots of the residuals between the experimental data and fitted curve.

5. The best fits of the intensity decay data were obtained using the triple exponential model. The results are summarized in Table 1. The mean lifetime values for the supercoiled and relaxed plasmids were 148 and 160 ns, respectively. The mean lifetime for the supercoiled plasmids was somewhat shorter than that for the relaxed plasmids. Because the interaction of water with the nitrogen atoms on the dppz quenches the luminescence, this result suggests that the RuBD MLC was more efficiently shielded from water in the relaxed than in the supercoiled plasmids. The observation of shorter lifetimes for the supercoiled plasmids agrees with our previous study that used supercoiled and linear pTZ18U plasmids (Kang *et al.*, 2002). The lifetime values of RuBD (Table 1) are larger than reported previously (Lakowicz *et al.*, 1995; Malak *et al.*, 1997; Kang *et al.*, 2002). The two earlier values (Lakowicz *et al.*, 1995; Malak *et al.*, 1997) were measured by time-correlated single photon counting, which weights the shorter decay times more heavily than does the FD measurements. In

Table 1. Multiexponential intensity decay analyses of $[Ru(bpy)_2(dppz)]^{2+}$ (RuBD) intercalated into supercoiled and relaxed pTZ18U plasmids.

Plasmid Type	τ_i (ns)	α_i	f_i [a]	$\langle\tau\rangle$ [a]	χ_R^2 [b]
Supercoiled	272.6	0.09	0.40	148.0	2.2
	70.9	0.49	0.55		
	7.7	0.42	0.05		
Relaxed	291.2	0.10	0.41	159.5	1.5
	74.3	0.51	0.54		
	9.7	0.39	0.05		

[a]Fractional intensities f_i and mean lifetimes $\langle\tau\rangle$ were calculated using Eqs. (4) and (3), respectively.
[b]The χ_R^2 values were calculated by Eq. (5), and the standard errors of phase angle and modulation were set at 0.2° and 0.005, respectively.

Table 2. Multiexponential anisotropy decay analyses of $[Ru(bpy)_2(dppz)]^{2+}$ (RuBD) intercalated into supercoiled and relaxed pTZ18U plasmids.

Plasmid Type	θ_i	$r_0*g(i)$	$\Sigma(r_0*g(i))$	$\chi_R^{2\ a}$
Supercoiled	288.1	0.057	0.122	3.2
	16.5	0.065		
Relaxed	354.6	0.062	0.135	4.5
	15.9	0.073		

[a]The χ_R^2 values were calculated by Eq. (5), and the standard errors of phase angle and modulation were set at 0.2° and 0.005, respectively.

general, resolution of a triple exponential decay is difficult (Lakowicz, 1999), and the individual decay times are significantly uncertain.

In addition to the intensity decay measurements, the FD anisotropy decays of RuBD intercalated into two forms of the pTZ18U plasmids were also measured (Fig. 6). The anisotropy decay data were best fit using the two ≈ exponential model. The results are summarized in Table 2. The obtained rotational correlation times were 288 and 17 ns for the supercoiled, and 355 and 16 ns for the relaxed plasmids. The slow and fast rotational correlation times appear to be consistent with the bending and torsional motions of the plasmids, respectively. Although the torsional motions showed no significant difference between the two plasmid forms, we observed somewhat shorter slow rotational correlation times for the supercoiled plasmids than the relaxed plasmids. This reflected a higher degree of bending motions in supercoiled than in relaxed plasmids. This result also agreed with our observations with the supercoiled and linear pTZ18U plasmids (Kang *et al*., 2002). The resolution of the differential phase values (Fig. 6, second panel) clearly showed that RuBD is a good probe for measuring the bending and torsional dynamics of the supercoiled and relaxed pTZ18U plasmids. Fig. 6 also shows the modulated anisotropy values for RuBD that was intercalated into both plasmid forms (Fig. 6, first panel). We observed slightly lower modulated anisotropy values for supercoiled plasmids. This is inconsistent with our steady-state anisotropy data, which showed no difference between the two forms (Fig. 4). Additionally, in our previous study (Kang *et al*., 2002), we observed slightly higher modulated anisotropy values for the supercoiled plasmids. Further experimentation is required to understand this discrepancy.

An important point of the present study is our use of a semiconductor light source for the FD intensity and anisotropy decay measurements. A variety of LEDs (including the high intensity UV, blue, and green LEDs) have been developed as an inexpensive and convenient light source. LEDs are easily modulated up to hundreds of MHz without the need for a Pockels cell (Sipior *et al*., 1996). We believe that LEDs will become an ideal light source for measuring the microsecond dynamics of biological macromolecules.

The use of RuBD, a long-lifetime MLC, allowed us to determine the bending motions of the plasmids, which were not measurable using the fluorescence anisotropy decay of EB ($<\tau> \approx 20$ ns). However, as in the previous study (Kang *et al*., 2002), it has to be pointed out that the lifetime of RuBD is far too short to measure the slower bending motions or end-over-end tumbling motions of the plasmids. The bending motions of DNA occur in about 100 ns to more than 100 μs (Schurr *et al*., 1992), which means that the time window of this report is near the lower end of bending dynamics of DNA. The time scale of end-over-end tumbling motions of plasmids spans from about 100 μs to a few milliseconds, depending on the size of the plasmid (Langowski and Giesen, 1989; Langowski *et al*., 1992; Chirico and Baldini, 1996; Fishman and Patterson, 1996). Additionally, somewhat high probe concentration was used because of the very low quantum yield ($Q = 0.008$) of RuBD (Lakowicz *et al*., 2001). This may partially explain the small difference in the bending and torsional dynamics between the supercoiled and relaxed plasmid forms, because the presence of the intercalating agents unwinds the supercoils of closed circular DNA (Waring, 1970). The use of long-lifetime MLCs to measure DNA dynamics is presently in its infancy. Additional MLCs with longer lifetime, higher quantum yield, as well as long-wavelength absorption are yet to be developed.

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