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Dynamics of Supercoiled and Linear pTZ18U Plasmids Observed with a Long-Lifetime Metal–Ligand Complex

JUNG SOOK KANG,[1] OMOEFE O. ABUGO,[2] JOSEPH R. LAKOWICZ[2]

[1] Department of Oral Biochemistry and Molecular Biology, College of Dentistry, Pusan National University, Pusan 602-739, Korea

[2] Center for Fluorescence Spectroscopy, Department of Biochemistry and Molecular Biology, University of Maryland at Baltimore, 725 West Lombard Street, Baltimore, Maryland 21201

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ABSTRACT: The metal–ligand complex, $[Ru(bpy)_2(dppz)]^{2+}$ (bpy = 2,2′-bipyridine, dppz = dipyrido[3,2-a:2′,3′-c]phenazine) (Ru-BD), was used as a spectroscopic probe for studying nucleic acid dynamics. The Ru-BD complex displays a long lifetime of over 100 ns and a molecular light switch property upon DNA binding due to shielding of its dppz ligand from water. To further show the usefulness of this luminophore (Ru-BD) for probing DNA dynamics, we examined its intensity and anisotropy decays when intercalated into supercoiled and linear pTZ18U plasmids using frequency-domain fluorometry with a light-emitting diode (LED) as the modulated light source. Compared to the supercoiled plasmids with an average intensity decay time of 120.8 ns at 25°C, we obtained somewhat longer lifetimes for the linear plasmids ($\langle\tau\rangle$ = 141.4 ns at 25°C), suggesting a more efficient shielding from water by the linear plasmids. The anisotropy decay data also showed longer rotational correlation times for the linear plasmids (495 and 35 ns at 25°C) as compared to the supercoiled plasmids (412 and 27 ns at 25°C). The slow and fast rotational correlation times appear to be consistent with the bending and torsional motions of the plasmids, respectively. The anisotropy values were quite similar, although the values of the supercoiled plasmids were slightly higher in both the steady-state and anisotropy decay measurements. These results indicate that Ru-BD can be applied in the study of both bending and torsional dynamics of nucleic acids.
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Keywords: supercoiled and linear plasmids; metal–ligand complex; bending and torsional dynamics

INTRODUCTION

The hydrodynamic properties of DNA in solution have been the object of many biophysical studies. The use of time-resolved fluorescence anisotropy for DNA dynamics originated with the measurement of the anisotropy decay of ethidium bromide (EB) bound to DNA.[1–4] Since these early reports, there has been considerable progress in the use of fluorescence to study DNA dynamics.[5–7] However, the short decay times of about 1–20 ns for most DNA-bound dyes have been a serious limitation because DNA is expected to display a wide range of relaxation times.[1,2] The depolarization due to the torsional motions around the long axis of DNA and that due to the bending motions about the short axis occur on very different time scales. In fact, most fluorescence studies of DNA dynamics

Correspondence to: J. R. Lakowicz (lakowicz@cfs.umbi.umd.edu).

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report only the torsional motions of DNA, which are detectable on the nanosecond time scale. The slower bending motions of DNA are often undetectable when nanosecond-lifetime probes are employed.

Long-lifetime metal–ligand complexes (MLCs) that display decay times ranging from 100 ns to more than 10 μs have only recently become available[8–10] and have been proven to have favorable chemical, photochemical, and photophysical properties. Because of their large Stokes' shift, the MLCs do not display significant radiative or nonradiative homo transfer.[9,10] For this reason we believe that anisotropy decays of the MLCs represent motions of the probe, not the loss of anisotropy due to energy transfer. In general, the MLCs display good water solubility and high chemical and photochemical stability.[9,10] In addition, the long lifetimes of the MLCs allow the use of gated detection, which can be employed to suppress interfering autofluorescence from biological samples and can thus provide increased sensitivity.[11] Finally, most MLCs display usefully large fundamental anisotropies (r_0), making them useful for microsecond hydrodynamics.[9,10]

In the use of long-lifetime MLCs for probing DNA structure, Barton and coworkers[12–14] reported the use of dipyrido[3,2-a:2′,3′-c]phenazine (dppz) complexes of ruthenium as spectroscopic probes. Ruthenium complexes containing dppz display a molecular light switch property for DNA. These probes have little or no emissions in aqueous media. However, when bound to DNA, they become highly fluorescent with greater than 10^4 luminescent enhancement. This results in very little interfering background emissions during measurements: essentially all the observed emission originates from the DNA-bound complex. Barton et al.[13] also showed that the 2,2′-bipyridine (bpy) derivative of the ruthenium complex, $[Ru(bpy)_2(dppz)]^{2+}$ (Ru-BD), 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 common phenanthroline derivative.

In our laboratory we introduced the use of fluorescence anisotropy decay of the dppz complexes of ruthenium to study the dynamics of nucleic acids. We examined the intensity and anisotropy decays of calf thymus DNA labeled with these ruthenium complexes and observed rotational correlation times up to several hundred nanoseconds.[15,16] To further show the usefulness of these ruthenium complexes for DNA dynamics, we studied the photophysical properties of Ru-BD intercalated into supercoiled and linear pTZ18U plasmids from *E. coli* HB101 in the present work. We observed a clear difference in the bending and torsional dynamics of supercoiled and linear pTZ18U plasmids using Ru-BD.

For this study we used a high-intensity, blue light-emitting diode (LED) as the excitation source with our frequency-domain (FD) fluorometer. 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. We previously had to use external modulators with our cw laser light sources in our measurements using MLCs in order to obtain modulation. However, for an MLC like Ru-BD with a low quantum yield ($Q = 0.008$),[17] it was very difficult to obtain reliable anisotropy decay measurements. For this reason time-correlated single-photon counting (TCSPC) was used in our previous studies with Ru-BD.[15,16] However, with a blue LED as the light source, we were able to obtain very reliable time-resolved intensity and anisotropy decays using the FD method.

MATERIALS AND METHODS

Materials

The *E. coli* HB101, *Sma*I, and REACT 4 buffer (20 m*M* Tris-HCl, 5 m*M* $MgCl_2$, 50 m*M* KCl, pH 7.4) were purchased from Gibco BRL (Life Technologies, Grand Island, NY). Luria–Bertani (LB) medium was procured from Bio 101, Inc. (Vista, CA), and agarose and ampicillin were from Sigma (St. Louis, MO). The pTZ18U plasmid [2860 base pair (bp)] was acquired from Bio-Rad (Hercules, CA), and the plasmid mega kit was from Qiagen Inc. (Valencia, CA). A racemic mixture of Ru-BD was synthesized as the PF_6^- salt by the method described previously,[15,16] and the chemical structure is shown in Figure 1. All other chemicals were reagent grade, and the water was deionized with a Milli-Q system. All measurements were carried out in REACT 4 buffer. The PF_6^- ion on the Ru-BD was expected to immediately exchange with the Cl^- ion in the buffer.

Absorption and Steady-State Fluorescence Measurement

The pTZ18U plasmids were purified with the Qiagen plasmid mega kit from 500-mL overnight cul-

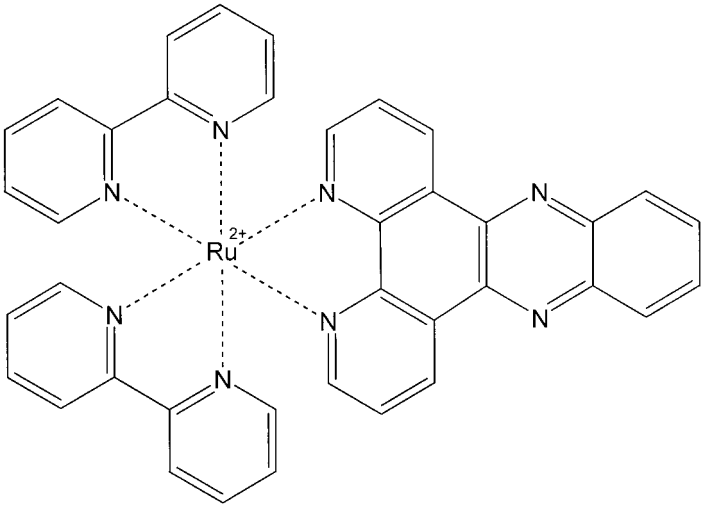

Figure 1. The chemical structure of $[Ru(bpy)_2(dppz)]^{2+}$ (RuBD).

tures of *E. coli* HB101 in LB medium containing ampicillin. Overnight *Sma*I digestion was performed in REACT 4 buffer at 37°C and confirmed by 1% agarose gel electrophoresis (Fig. 2). The DNA concentration was 300 μM bp while that of Ru-BD was 15 μM. The concentrations of DNA and Ru-BD 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. The 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 SLM AB2 spectrofluorometer (Spectronic Instruments, Inc., Rochester, NY). The Ru-BD was excited at 440 nm while the emission was observed at 620 nm. The excitation and emission slits were both 4 nm.

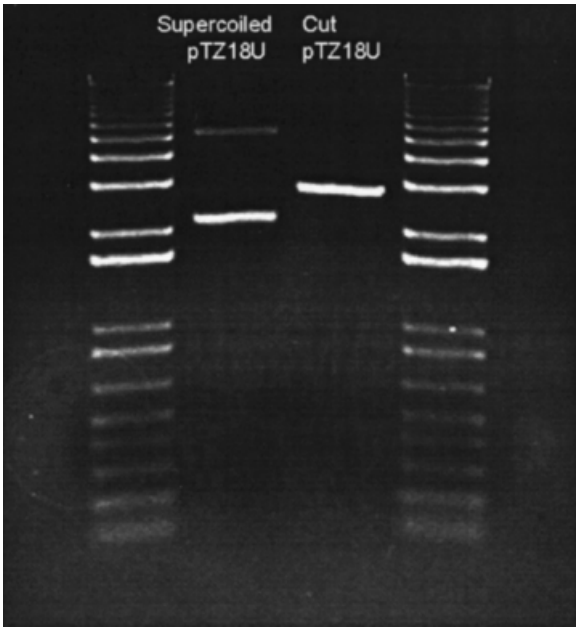

Figure 2. The agarose gel electrophoresis patterns of supercoiled and linear pTZ18U plasmids. A 1-kb DNA ladder was used.

FD Intensity and Anisotropy Decay Measurements

Measurements were performed using the instruments described previously[18] and modified with a data acquisition card from ISS, Inc. (Urbana, IL).[19] The excitation source was a blue LED (LNG992CFBW, Panasonic) with a luminous intensity of 1500 mcd. An LED driver (LDX-3412, ILX Lightwave, Boseman, MO) provided 30 mA of current at frequencies from 0.4 to 15 MHz. A 450 ± 20 nm interference filter and a 630 nm cutoff filter were used for isolating the excitation and emission, respectively. Rhodamine B in water (τ = 1.68 ns) was utilized as a lifetime standard.

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

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

where the preexponential factor α_i is the amplitude of each component, $\Sigma\alpha_i = 1.0$, τ_i is the

decay time, and n is the number of exponential components. These values were determined by nonlinear least squares analysis as described previously.[20,21] The mean lifetimes were calculated according to equation

$$\langle \tau \rangle = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} = \sum_i f_i \tau_i \quad (2)$$

where f_i is the fractional steady-state contribution of each component to the total emission and Σf_i is normalized to unity. The equation for f_i is

$$f_i = \frac{\alpha_i \tau_i}{\sum_i \alpha_i \tau_i} \quad (3)$$

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

$$r(t) = \sum_i r_0 g_i \exp(-t/\theta_i) \quad (4)$$

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 variable parameter. We used the nonassociated anisotropy decay model in which the individual decay times are not associated with the individual correlation times. This model seems appropriate because the DNA-bound probe seems to be in a single environment. The associated model would be appropriate if emissions were observed from both the bound and free probes.

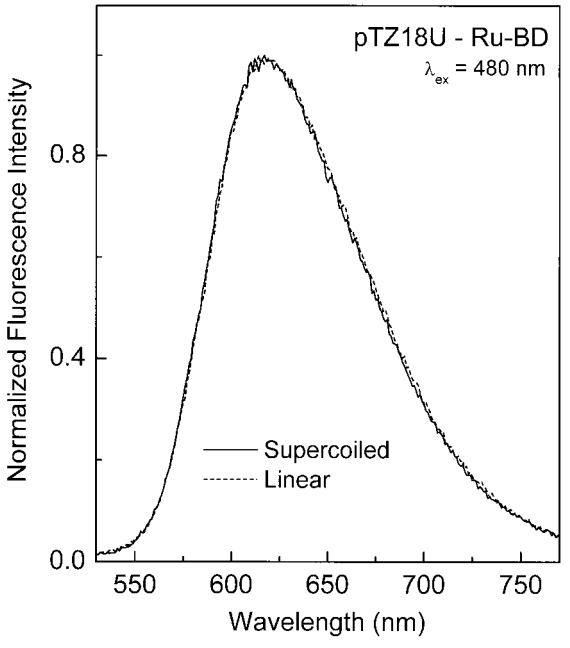

Figure 3. The emission spectra of Ru-BD intercalated into supercoiled and linear pTZ18U plasmids.

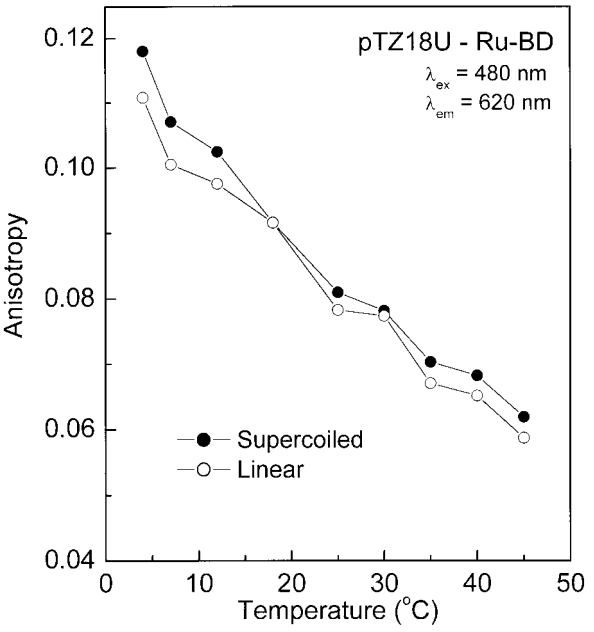

Figure 4. The temperature-dependent steady-state anisotropy of Ru-BD intercalated into supercoiled and linear pTZ18U plasmids.

RESULTS

In this study we characterized the photophysical properties of Ru-BD intercalated into supercoiled and *Sma*I-cut linear pTZ18U plasmids. Figure 3 shows the emission spectra of Ru-BD intercalated into the supercoiled and linear forms of the plasmids. There was no difference in the emission spectra for both forms under our experimental conditions. They both had an emission peak at about 620 nm. However, in the steady-state anisotropy measurements, we observed a slight but consistent difference in the anisotropy values between 5 and 45°C for both forms of the plasmids (Fig. 4). The supercoiled form had consistently higher anisotropy values, except at 18°C where they were similar. At 25°C these values were

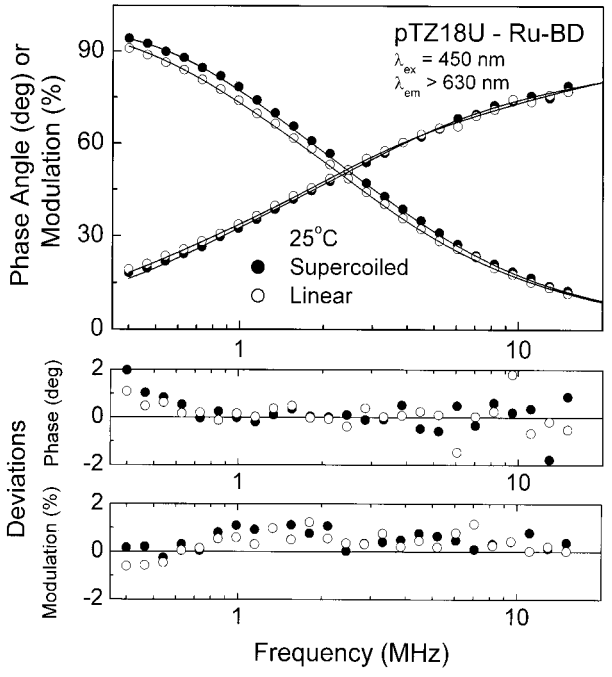

Figure 5. The intensity decays of Ru-BD intercalated into supercoiled and linear pTZ18U plasmids at 25°C.

0.081 and 0.078 for the supercoiled and linear plasmids, respectively.

The FD intensity decays of Ru-BD intercalated into supercoiled and linear plasmids are shown in Figure 5. The intensity decays were best fit by a triple exponential decay. Table I shows the intensity decay results for both plasmid forms at 4 and 25°C. At both temperatures the mean decay times for the linear plasmids were consistently longer than those for the supercoiled plasmids. The mean lifetime values obtained at 4 and 25°C were 164.5 and 141.4 ns, respectively, for the linear, and 133.5 and 120.8 ns, respectively, for the supercoiled plasmids. This result suggests that the Ru-BD MLC was more efficiently shielded from water in the linear plasmids than in the supercoiled plasmids, resulting in a longer lifetime. It seems that the linear pTZ adopts more favorable conformations for Ru-BD intercalation. As expected, we observed increased decay times for both plasmid forms at 4°C in comparison to 25°C, which is probably due to the reduced collisional motions at the lower temperature. These decay times (Table I) are larger than reported previously.[15,16] The earlier values were measured by TC-SPC, which, in our experience, weights the shorter decay times more heavily than does the FD measurements. Additionally, resolution of a triple exponential decay is difficult[22] and the individual decay times are subject to significant uncertainty.

In addition to the intensity decay measurements, we also measured the anisotropy decays of Ru-BD intercalated into the two forms of the pTZ18U plasmids at 4 and 25°C (Fig. 6) and the results are summarized in Table II. The best fits of the anisotropy decay data were obtained using the two correlation time model. The slow and fast rotational correlation times appear to be consis-

Table 1. Multiexponential Intensity Decay Analyses of $[Ru(bpy)_2(dppz)]^{2+}$ Bound to Supercoiled and Linear pTZ18U Plasmids at Different Temperatures

Plasmid	Temperature (°C)	τ_i (ns)	α_i	f_i	$\langle\tau\rangle^a$ (ns)	$\chi_R^{2\,b}$
Supercoiled pTZ18U	4	220.1	0.16	0.46	133.5	1.40
		62.7	0.62	0.50		
		12.2	0.22	0.04		
	25	170.4	0.27	0.57	120.8	1.99
		56.7	0.59	0.41		
		10.4	0.14	0.02		
Linear pTZ18U	4	279.5	0.11	0.42	164.5	1.80
		72.8	0.57	0.53		
		11.9	0.32	0.05		
	25	233.9	0.16	0.44	141.4	1.51
		75.6	0.58	0.51		
		17.4	0.26	0.05		

[a] Mean lifetimes were calculated using $\langle\tau\rangle = f_i\,\tau_i$, where f_i is the fractional steady-state contribution of each component to the total emission.

[b] The standard errors of the phase angle and modulation were set at 0.2° and 0.005, respectively.

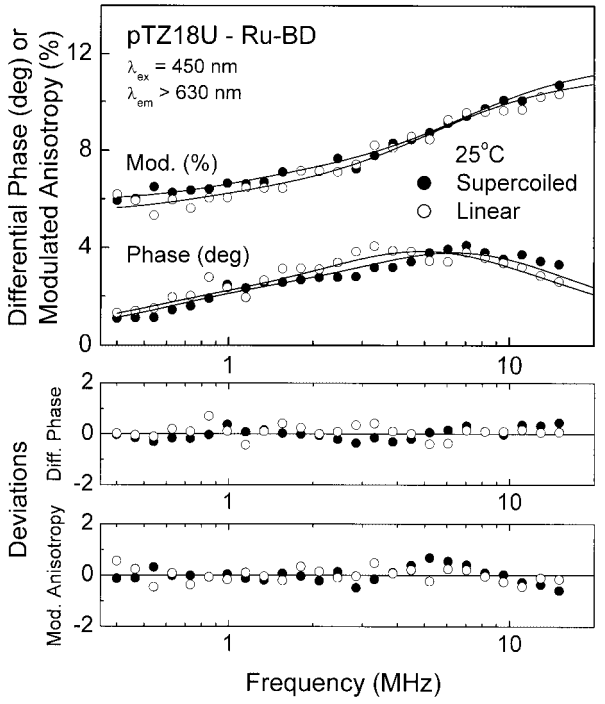

Figure 6. The anisotropy decays of Ru-BD intercalated into supercoiled and linear pTZ18U plasmids at 25°C.

tent with bending (flexure of the helix axis) and torsional (twisting of base pair) motions of plasmids, respectively. At both temperatures we observed longer rotational correlation times for the linear plasmids than for the supercoiled plasmids, reflecting a higher degree of internal flexibility in the supercoiled plasmids than in the cut ones. At 25°C the obtained rotational correlation times were 495 and 35 ns for the linear plasmids and 412 and 27 ns for the supercoiled plasmids. The observation of larger values of the slow rotational correlation times for linear plasmids agrees with the studies of Langowski et al.[23] They used dynamic light scattering to show that the internal diffusion coefficient (D_i) for linear plasmids is smaller than that for supercoiled plasmids. Because they interpreted the D_i as the relative motion of subsegments of the DNA within the polymer coil,[24,25] it is thus likely that the D_i largely reflects the bending motions. Although the slow rotational correlation times increased with decreasing temperature, the observed fast rotational correlation times remained almost constant for both plasmid forms, irrespective of temperature (Table II). Thus, it seems that only bending motions depend on temperature within the time resolution of our measurements. In the top panel of Figure 6 we also see the modulated anisotropy values for Ru-BD intercalated into both plasmid forms at 25°C. We expected higher modulated anisotropy values for the linear plasmids because of their corresponding longer rotational correlation times. However, we observed slightly lower modulated anisotropy values for linear plasmids at frequencies below 5 MHz. This is in good agreement with our observations with the steady-state anisotropy measurements that are also smaller for the linear plasmids (Fig. 4). This may be at least partly due to a significantly reduced torsion elastic constant for linear plasmids than supercoiled plasmids because of relaxation of the superhelical strain in the linear plasmids.[26]

DISCUSSION

We demonstrated the usefulness of Ru-BD, a long-lifetime MLC, for probing the bending and

Table II. Anisotropy Decay Analyses of $[Ru(bpy)_2(dppz)]^{2+}$ Bound to Supercoiled and Linear pTZ18U Plasmids at Different Temperatures

Plasmid	Temperature (°C)	θ_i (ns)	$g(i)*r_0$	$\Sigma(g(i)*r_0)$	$\chi_R^{2\,a}$
Supercoiled pTZ18U	4	514.4	0.054	0.128	1.86
		26.8	0.074		
	25	412.1	0.058	0.117	3.15
		27.1	0.059		
Linear pTZ18U	4	625.9	0.046	0.115	6.52
		36.2	0.069		
	25	494.9	0.047	0.112	2.59
		35.2	0.065		

[a] The standard errors of the phase angle and modulation were set at 0.2° and 0.005, respectively.

torsional dynamics of supercoiled and linear pTZ18U plasmids. The torsional motions of DNA were measured with the fluorescence anisotropy decay of EB with a mean decay time of about 20 ns. Direct observation of bending motions over short distances came from the studies using transient electric birefringence[27,28] and transient electric dichroism,[29,30] which are insensitive to torsional motions.[7] The important point of this report is that the use of a long-lifetime MLC enabled us to extend the measurable time scale of DNA dynamics to submicrosecond and to measure both the bending and torsional motions of the plasmids using fluorescence anisotropy decay.

However, it should be pointed out that the time window of the bending motions of the present study (about 500–600 ns) is near the lower end of the 10^{-7}–10^{-4} second bending motions of DNA.[7] The lifetime of Ru-BD is still too short to measure slower bending motions or end-over-end tumbling motions of the plasmids. In addition, the rotational correlation times of the two plasmid forms did not show a substantial difference. A possible explanation for this may be the probe concentration, which is about 1:20 for the Ru-BD to DNA (bp) molar ratio. Because of the very low quantum yield (Q = 0.008) of Ru-BD,[17] we had to use a somewhat high probe concentration. The unwinding of the supercoils of closed circular DNA by intercalating agents is well known.[31] Considering the apparent unwinding angle of 30 ± 11° for Ru-BD[12] and the presence of about 140 molecules of Ru-BD per pTZ18U molecule, the possibility of significant unwinding of supercoiled pTZ18U by Ru-BD cannot be ruled out. The use of MLCs to measure DNA dynamics is just beginning, and additional MLCs with longer lifetimes and higher quantum yields are yet to be developed.

Another interesting aspect of this study was our use of a blue LED as the source of excitation. By using this light source we were able to obtain approximately 3–5 mW of excitation power, depending on the current applied to the LED. The use of the LED also allowed us to have a simpler turnkey operation (i.e., less optical adjustments and easier electronic operations). Direct electronic modulation of up to 100 MHz was performed without the use of external modulators.[32] When compared to laser light sources that can cost from about $10,000 to several hundred thousand dollars, LEDs are very inexpensive (about $2–10 each) and the required electronics cost a few hundred dollars. From our experience we therefore believe that the low cost of LEDs together with their simpler mode of operation will allow laboratories to perform fluorescence measurements with less sophisticated fluorescence equipments. In general, the use of the new high intensity UV, blue, and green GaN LEDs will facilitate the use of time-resolved measurements for many laboratories and a wide range of applications.

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