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Dynamics of Supercoiled and Linear pBluescript II SK(+) Phagemids Probed with a Long-lifetime Metal-ligand Complex

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We extended the measurable time scale of DNA dynamics to microsecond using $[Ru(phen)_2(dppz)]^{2+}$ (phen = 1,10-phenanthroline, dppz = dipyrido[3,2-a:2',3'-c]phenazine) (RuPD), which displays a mean lifetime near 500 ns. To evaluate the usefulness of this luminophore (RuPD) for probing nucleic acid dynamics, its intensity and anisotropy decays when intercalated into supercoiled and linear pBluescript (pBS) II SK(+) phagemids were examined using frequency-domain fluorometry with a blue light-emitting diode (LED) as the modulated light source. The mean lifetime for the supercoiled phagemids ($<\tau>$ = 489.7 ns) was somewhat shorter than that for the linear phagemids ($<\tau>$ = 506.4 ns), suggesting a more efficient shielding from water by the linear phagemids. The anisotropy decay data also showed somewhat shorter slow rotational correlation times for supercoiled phagemids (997.2 ns) than for the linear phagemids (1175.6 ns). The slow and fast rotational correlation times appear to be consistent with the bending and torsional motions of the phagemids, respectively. These results indicate that RuPD can have applications in studies of both bending and torsional dynamics of nucleic acids.

Keywords: Bending and torsional dynamics, Light-emitting diode, Long-lifetime metal-ligand complex, Supercoiled and linear pBluescript II SK(+)

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Introduction

Time-resolved fluorescence anisotropy is a powerful technique for investigating macromolecular dynamics (Badea and Brand, 1979). A number of attempts have been made to use fluorescence anisotropy decay to study DNA dynamics. However, the short decay time of about 1 to 30 ns displayed by most DNA fluorophores have been a serious limitation to reflect a wide variety of DNA motions (Schurr *et al*., 1992). Information on the rotational motion is usually available over a time scale not exceeding three times the lifetime of the fluorophore, after which there is too little signal for accurate anisotropy measurements. The applications of fluorescence anisotropy decay are now more limited by probe lifetime than by instrumentation.

Long-lifetime metal-ligand complexes (MLCs), which display decay times ranging from 100 ns to more than 10 μs, have only recently become available (DeGraff and Demas, 1994; Terpetschnig *et al*., 1997; Lakowicz *et al*., 2000) and show a number of characteristics that make them versatile biophysical probes. Because of the large Stokes' shift, the MLCs do not display significant radiative or nonradiative homo energy transfer (Terpetschnig *et al*., 1997; Lakowicz *et al*., 2000). In general, the MLCs display good water solubility and high thermal, 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 be employed to suppress interfering autofluorescence from biological samples and can thus provide increased sensitivity (Haugen and Lytle, 1981). Finally, most MLCs display polarized emission, making them useful for microsecond hydrodynamics. A number of Os, Re, and Ru complexes have been synthesized and used to study microsecond dynamics of biological macromolecules (Terpetschnig *et al*., 1997; Lakowicz *et al*., 2000; Kang *et al*., 2002a,b,c).

Barton and co-workers (Friedman *et al*., 1990; Jenkins *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 prime candidates for a spectroscopic probe for nucleic acids because of their "molecular light switch" properties for DNA. Since the luminescent enhancement upon DNA binding is $\geq 10^4$, there is essentially no background with the dppz complexes of ruthenium. There are two common dppz complexes of ruthenium, the 2,2'-bipyridine (bpy) derivative $[Ru(bpy)_2(dppz)]^{2+}$ (RuBD) and the 1,10-phenanthroline (phen) derivative $[Ru(phen)_2(dppz)]^{2+}$ (RuPD). Because both complexes display large fundamental anisotropies (r_0), they were used to measure the anisotropy decays of calf thymus DNA (Lakowicz *et al*., 1995; Malak *et al*., 1997) and supercoiled, linear, and relaxed pTZ18U plasmids [2860 base pair (bp)] (Kang *et al*., 2002a,b). RuPD has some advantages over RuBD because of its longer lifetime and higher quantum yield (Jenkin *et al*., 1992; Murphy and Barton, 1993; Malak *et al*., 1997; Kang and Lakowicz, 2001). However, with one exception (Malak *et al*., 1997), all anisotropy decay measurements were performed using the bpy derivative RuBD (Lakowicz *et al*., 1995; Malak *et al*., 1997; Kang *et al*., 2002a,b).

To evaluate further the usefulness of RuPD for probing nucleic acid dynamics, we examined the intensity and anisotropy decays of RuPD intercalated into supercoiled and linear pBluescript (pBS) II SK(+) phagemids from *E. coli* XL1-Blue MRF'. 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 with simpler and low cost instrumentation.

Materials and Methods

Materials *Sma*I and REACT4 buffer (20 mM Tris-HCl, 5 mM $MgCl_2$, 50 mM KCl, pH 7.4) were purchased from Gibco BRL (Life Technologies, Grand Island, USA); LB-medium from Beckton, Dickinson and Company (Franklin Lakes, USA); agarose and ampicillin from Sigma (St. Louis, USA); pBS II SK(+) phagemid (2961 bp) and *E. coli* XL1-Blue MRF' from Stratagene (La Jolla, USA); and plasmid mega kit from Qiagen Inc. (Valencia, USA). RuPD was synthesized by the method described previously (Lakowicz *et al.*, 1995). All other chemicals were of the reagent grade, and water was deionized with a Milli-Q system. All measurements were carried out in REACT4 buffer.

Absorption and steady-state fluorescence measurements pBS II SK(+) phagemids were purified with Qiagen plasmid mega kit from 500 ml overnight cultures of *E. coli* XL1-Blue MRF' 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. 1). About 5-10 mM stock solution of RuPD was prepared in dimethylformamide. The phagemid concentration was 300 μM bp while the concentration of RuPD was 15 μM. The concentrations of DNA and RuPD were determined using molar extinction coefficients of 13,300 $M^{-1}cm^{-1}$ (expressed as bp) at 260 nm and 21,000 $M^{-1}cm^{-1}$ 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 a Cary Eclipse fluorescence spectrophotometer (Varian Inc., Palo Alto, USA).

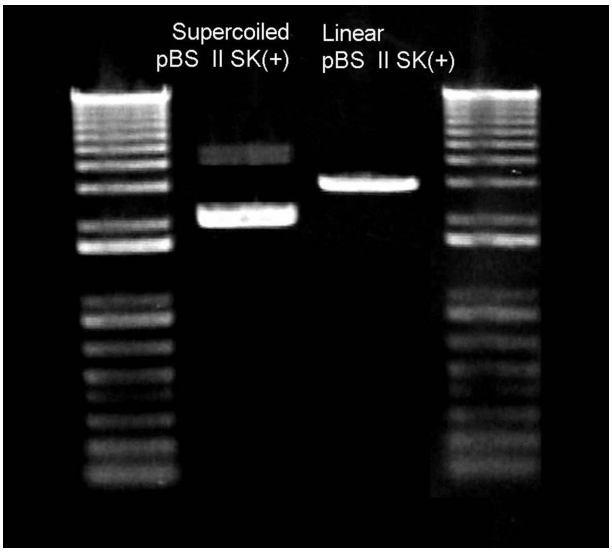

Fig. 1. Agarose gel electrophoresis pattern of supercoiled and linear pBluescript (pBS) II SK(+) phagemids. 1kb DNA ladder was used.

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 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 an ISS Koala instrument (ISS Inc., Champaign, USA) using a blue LED LNG992CFBW (Panasonic, Japan) as the excitation source. An LED driver LDX-3412 (ILX Lightwave, Boseman, USA) provided 30 mA of current at frequencies from 0.07 to 10 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 measurements were performed at 25°C.

The intensity decays were recovered from the FD data in terms

of a multiexponential model using nonlinear least squares analysis (Gratton *et al*., 1984; Lakowicz *et al*., 1984; Lakowicz and Gryczinski, 1991; Kang and Lakowicz, 2001; Kang and Kostov, 2002; Kang *et al*., 2002a,b,c):

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

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. Mean lifetimes were calculated by:

$$\langle\tau\rangle = \frac{\sum_i \alpha_i \tau_i^2}{\sum_i \alpha_i \tau_i} = \sum_i f_i \tau_i \tag{3}$$

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:

$$f_i = \frac{\alpha_i \tau_i}{\sum_j \alpha_j \tau_j} \tag{4}$$

The calculated (c) values of the phase $\phi_{c\omega}$ and modulation $m_{c\omega}$ at each modulation frequency ω are given by:

$$\phi_{c\omega} = \arctan(N_\omega/D_\omega) \tag{5}$$
$$m_{c\omega} = (N_\omega^2 + D_\omega^2)^{1/2} \tag{6}$$

where

$$N_\omega = \sum_i \frac{\alpha_i \omega \tau_i^2}{1+\omega^2\tau_i^2} \Big/ \sum_i \alpha_i \tau_i \tag{7}$$

$$D_\omega = \sum_i \frac{\alpha_i \tau_i}{1+\omega^2\tau_i^2} \Big/ \sum_i \alpha_i \tau_i \tag{8}$$

The values of α_i and τ_i were determined by minimizing χ_R^2 values:

$$\chi_R^2 = \frac{1}{\nu}\sum_\omega \left[\left(\frac{\phi_\omega - \phi_{c\omega}}{\delta\phi}\right)^2 + \left(\frac{m_\omega - m_{c\omega}}{\delta m}\right)^2\right] \tag{9}$$

where ν is the number of degrees of freedom, and ϕ_ω and m_ω are the measured phase and modulation, respectively. $\delta\phi$ and δm are the experimental uncertainties in the measured phase and modulation values and were set at 0.2° and 0.005, respectively.

The FD anisotropy decays were also analyzed in terms of the multiexponential model using nonlinear least squares analysis (Lakowicz and Gryczinski, 1991; Lakowicz *et al*., 1993; Kang *et al*., 2002a,b,c):

$$r(t) = \sum_i r_0 g_i e^{-t/\theta_i} \tag{10}$$

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 phase shift (Δ_ω) at the modulation frequency ω is given by

$$\Delta_\omega = \phi_{VH} - \phi_{VV} \tag{11}$$

where ϕ_{VH} and ϕ_{VV} are the perpendicular (*VH*) and parallel (*VV*) components of the emission. The modulation ratio Λ_ω is

$$\Lambda_\omega = m_{VV}/m_{VH} \tag{12}$$

where m_{VV} and m_{VH} are the amplitudes of the parallel (*VV*) and the perpendicular (*VH*) components of the modulated emission, respectively. The modulated anisotropy r_ω was calculated by:

$$r_\omega = \frac{\Lambda_\omega - 1}{\Lambda_\omega + 2} \tag{13}$$

The calculated values of Δ_ω and Λ_ω are given by

$$\Delta_{c\omega} = \arctan\left(\frac{D_{VV}N_{VH} - N_{VV}D_{VH}}{N_{VV}N_{VH} + D_{VV}D_{VV}}\right) \tag{14}$$

$$\Lambda_{c\omega} = \left(\frac{N_{VV}^2 + D_{VV}^2}{N_{VH}^2 + D_{VH}^2}\right)^{1/2} \tag{15}$$

where

$$N_k = \int_0^\infty I_k(t)\sin\omega t dt \tag{16}$$

$$D_k = \int_0^\infty I_k(t)\cos\omega t dt \tag{17}$$

In this expression the subscript k indicates the orientation, parallel (*VV*) or perpendicular (*VH*). The parameters describing the anisotropy decay are obtained by minimizing χ_R^2 values:

$$\chi_R^2 = \frac{1}{\nu}\sum_\omega \left[\left(\frac{\Delta_\omega - \Delta_{c\omega}}{\delta\Delta}\right)^2 + \left(\frac{\Lambda_\omega - \Lambda_{c\omega}}{\delta\Lambda}\right)^2\right] \tag{18}$$

where $\delta\Delta$ and $\delta\Lambda$ are the experimental uncertainties in the differential phase and modulation ratio values and were set at 0.2° and 0.005, respectively.

Results and Discussion

Fig. 2 shows the chemical structure of RuPD. The emission spectra of RuPD intercalated into the supercoiled and linear forms of pBS II SK(+) phagemids are shown in Fig. 3. There was no difference in the emission spectra for both forms under our experimental conditions. RuPD showed an emission peak at about 620 nm. As expected, we observed lower steady-state anisotropy values of RuPD than in our previous studies using RuBD (Kang *et al*., 2002a,b) because of its longer lifetime (Fig. 4). Steady-state anisotropy measurements also showed no difference in the anisotropy values between 4 and 45°C for both phagemid forms (Fig. 4).

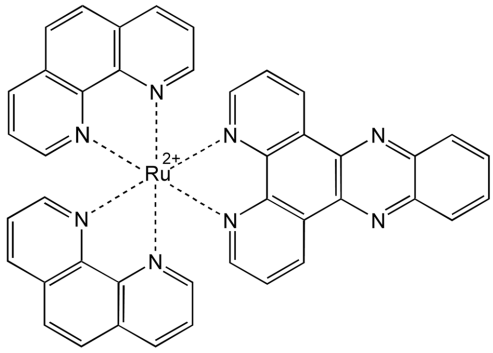

Fig. 2. Chemical structure of $[Ru(phen)_2(dppz)]^{2+}$ (RuPD).

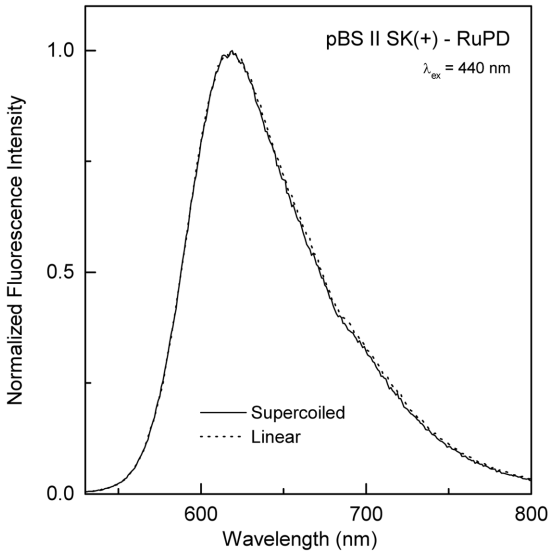

Fig. 3. Emission spectra of RuPD intercalated into supercoiled and linear pBS II SK(+) phagemids.

The FD intensity decays of RuPD intercalated into supercoiled and linear pBS II SK(+) phagemids are shown in Fig. 5. The intensity decays were best fit by a triple exponential decay. Table 1 shows the intensity decay results for both phagemid forms. The mean lifetime values for the supercoiled and linear phagemids were 489.7 and 506.4 ns, respectively. The mean lifetime for the supercoiled phagemids was somewhat shorter than that for the linear phagemids. This result suggests that the RuPD MLC was more efficiently shielded from water in the linear phagemids than in the supercoiled phagemids, resulting in a longer lifetime. The observation of shorter lifetimes for the supercoiled phagemids agrees with our previous studies using supercoiled, linear, and relaxed pTZ18U plasmids (Kang *et al*., 2002a,b). Thus, it seems that the linear form adopts more favorable conformations for RuPD intercalation than supercoiled form. The lifetime values of RuPD (Table 1) are larger than reported previously (Jenkins *et al*., 1992; Murphy and Barton, 1993; Malak *et al*., 1997; Holmin *et al*., 1998; Kang and Lakowicz, 2001). The earlier values ranging from 170 ns to 380 ns were

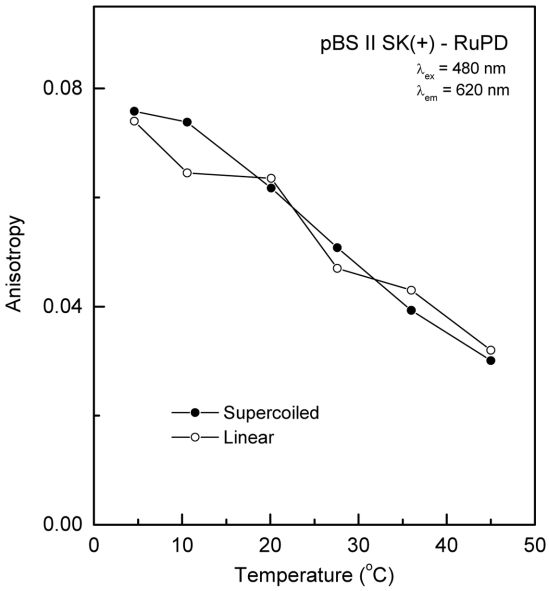

Fig. 4. Temperature-dependent steady-state anisotropy of RuPD intercalated into supercoiled and linear pBS II SK(+) phagemids. The steady-state anisotropy values were calculated by Eq. (1).

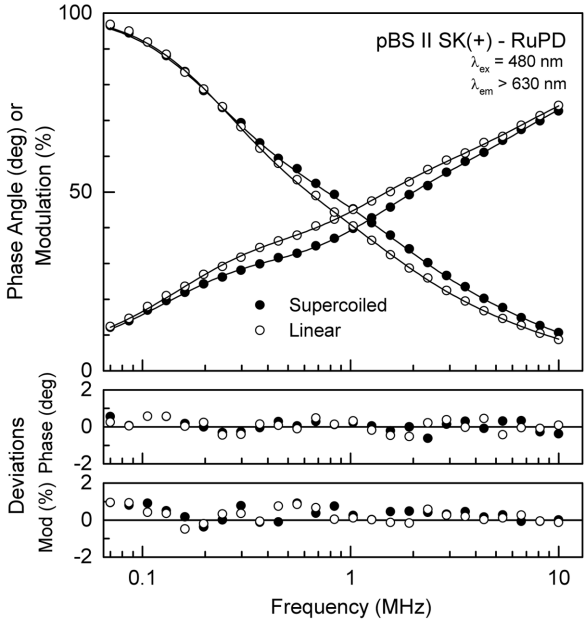

Fig. 5. Intensity decays of RuPD intercalated into supercoiled and linear pBS II SK(+) phagemids. The symbols represent the measured phase and modulation values. The solid lines show the best multiexponential fits to the data according to Eq. (9). The middle and lower panels show plots of the residuals between the experimental data and the fitted curve.

mostly measured by time-correlated single photon counting (Jenkins *et al*., 1992; Murphy and Barton, 1993; Malak *et al*., 1997; Holmin *et al*., 1998), which weights the shorter decay times more heavily than does the FD measurements. In addition, resolution of a triple exponential decay is difficult

Table 1. Multiexponential intensity decay analyses of $[Ru(phen)_2(dppz)]^{2+}$ (RuPD) intercalated into supercoiled and linear pBluescript II SK(+) phagemids

Phagemid type	τ_i (ns)	α_i	f_i [a]	$<\tau>$ [a] (ns)	χ_R^2 [b]
Supercoiled	823.7 ± 4.5	0.089 ± 0.001	0.541 ± 0.003	489.7 ± 1.9	1.9 ± 0.4
	115.2 ± 1.9	0.421 ± 0.006	0.356 ± 0.004		
	28.4 ± 0.6	0.490 ± 0.006	0.102 ± 0.002		
Linear	760.6 ± 2.6	0.132 ± 0.001	0.611 ± 0.002	506.4 ± 1.3	1.9 ± 0.3
	127.3 ± 3.5	0.397 ± 0.004	0.307 ± 0.001		
	28.6 ± 1.0	0.471 ± 0.004	0.082 ± 0.002		

Values are represented as the mean ± SEM of 5 determinations.
[a]Fractional intensities f_i and mean lifetimes $<\tau>$ were calculated using Eqs. (4) and (3), respectively.
[b]The χ_R^2 values were calculated by Eq. (9), and the standard errors of phase angle and modulation were set at 0.2° and 0.005, respectively.

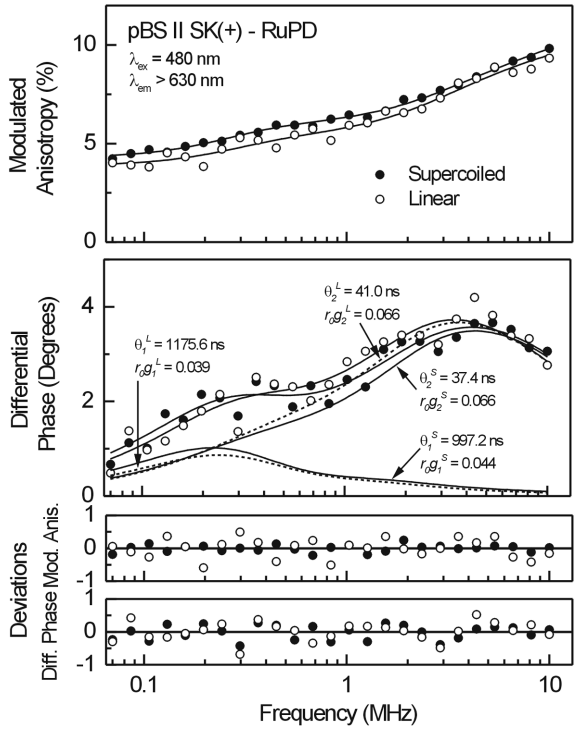

Fig. 6. Anisotropy decays of RuPD intercalated into supercoiled and linear pBS II SK(+) phagemids. The symbols in the first and second panels represent the modulated anisotropy and the measured phase shift values, respectively. The modulated anisotropy values were calculated from measured Λ_ω using Eq. (13). The solid lines show the best multiexponential fits to the data according to Eq. (18). The differential phase data in the second panel are based on the rotational correlation times and amplitudes shown in Table 2. The superscripts *S* and *L* denote supercoiled and linear pBS II SK(+) phagemids, respectively. The lower two panels show plots of the residuals between the experimental data and the fitted curve.

(Lakowicz, 1999), and the individual decay times are subject to significant uncertainty.

In addition to the intensity decay measurements, we also measured the anisotropy decays of RuPD intercalated into the two forms of the pBS II SK(+) phagemids (Fig. 6), and the results are summarized in Table 2. 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 consistent with the bending (flexure of the helix axis) and torsional (twisting of bp) motions of phagemids, respectively. We observed longer rotational correlation times for the linear phagemids than for the supercoiled phagemids, reflecting a higher degree of internal flexibility in the supercoiled phagemids than in the cut ones. The obtained rotational correlation times were 997.2 ns for the supercoiled and 1175.6 ns for the linear phagemids. This result is also in agreement with what we observed with the supercoiled, linear, and relaxed plasmids (Kang *et al*., 2002a,b). The resolution of the differential phase values (Fig. 6, second panel) clearly shows that RuPD is a good probe for measuring the bending and torsional dynamics of the supercoiled and linear phagemids. Fig. 6 also shows the modulated anisotropy values for RuPD intercalated into both phagemid forms (Fig. 6, first panel). We observed slightly higher modulated anisotropy values for supercoiled phagemids. This is not consistent with our steady-state anisotropy data, which showed little difference between the two forms (Fig. 4). In our previous study (Kang *et al*., 2002a), we also observed slightly higher modulated anisotropy values for the supercoiled plasmids. We interpreted that this may be at least partly due to a significantly reduced torsion elastic constant for linear plasmids because of relaxation of the superhelical strain in the linear plasmids (Kang *et al*., 2002a).

In this study, we demonstrated the usefulness of RuPD, a long-lifetime MLC for probing the bending and torsional dynamics of supercoiled and linear pBS II SK(+) phagemids. The most important observation of this report is that the use of RuPD, which has a lifetime of about 500 ns (Table 1), enabled us to extend the measurable time scale of DNA dynamics to microsecond. We could measure the rotational correlation time larger than 1 μs. However, as in our previous studies (Kang *et al*., 2002a,b), it has to be pointed out that the lifetime of RuPD is still short to measure the slower bending motions or end-over-end tumbling motions of the plasmids. The

Table 2. Multiexponential anisotropy decay analyses of $[Ru(phen)_2(dppz)]^{2+}$ (RuPD) intercalated into supercoiled and linear pBluescript II SK(+) phagemids

Phagemid type	θ_i (ns)	$r_0 * g(i)$	$\Sigma(r_0 * g(i))$	$\chi_R^{2\,a}$
Supercoiled	997.2 ± 14.7	0.044 ± 0.003	0.110 ± 0.003	3.2 ± 0.9
	37.4 ± 2.0	0.066 ± 0.002		
Linear	1175.6 ± 19.6	0.039 ± 0.002	0.105 ± 0.002	4.9 ± 0.5
	41.0 ± 1.6	0.066 ± 0.001		

Values are represented as the mean ± SEM of 5 determinations.
[a]The χ_R^2 values were calculated by Eq. (18), and the standard errors of phase angle and modulation were set at 0.2° and 0.005, respectively.

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 still 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).

The use of long-lifetime MLCs to measure DNA dynamics is just beginning (Lakowicz *et al*., 1995, 2000; Malak *et al*., 1997; Terpetschnig *et al*., 1997; Kang *et al*., 2002a,b). Barton and coworkers (Jenkin *et al*., 1992) showed that 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 phen derivative RuPD. However, RuPD clealy showed the differences in the torsional and bending dynamics of supercoiled and linear pBS II SK(+) phagemids (Table 2 and Fig. 6). The phen derivative RuPD has some advantages over the other bpy derivative RuBD. The lifetimes of RuBD bound to DNA are about 100 ns (Jenkins *et al*., 1992; Murphy and Barton, 1993; Lakowicz *et al*., 1995, 2001; Malak *et al*., 1997; Kang *et al*., 2002a,b). RuPD showed still longer lifetimes bound to DNA, with a mean decay time near 500 ns (Table 1). Besides, the quantum yield of RuPD (Q = 0.017) (Kang and Lakowicz, 2001) is more than twice that of RuBD (Q = 0.008) (Lakowicz *et al*., 2001). Because of longer lifetime and higher quantum yield than the bpy derivative, RuPD is expected to have numerous applications in studies of nucleic acid structure and dynamics.

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