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Interaction between Norfloxacin and Single Stranded DNA

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We compared various spectroscopic properties of a norfloxacin-single stranded DNA complex with those of norfloxacin-double stranded DNA complex. Norfloxacin binds to both double- and single stranded DNA, and we observed the following spectroscopic changes for both complexes: hypochromism in the norfloxacin absorption region in the absorption spectrum, the characteristic induced CD spectrum consisting of a weak positive band at 323 nm and a strong positive band at 280-300 nm followed by a negative band in the 260 nm region, a strong decrease in the fluorescence intensity and a red-shift in the fluorescence emission spectrum, and shorter fluorescence decay times. These results indicate that the environments of the bound norfloxacin in both DNAs are similar, although the equilibrium constant of the norfloxacin-single stranded DNA was twice as high as the norfloxacin-double stranded DNA complex. Both complexes were thermodynamically favored with similar negative ΔG°. Negative ΔH° terms contribute to these spontaneous reactions; ΔS° term was unfavorable.

Introduction

Quinolones are synthetic antibacterial agents that have been widely studied in academic and industrial laboratories.[1-5] Considerable biological data indicates that type II DNA topoisomerase, a DNA gyrase,[6-17] is the functional target of the inhibital action of these drugs. Type II topoisomerase catalyzes the conversion of relaxed supercoiled DNA into a negatively supercoiled form and is essential for cellular life. Shen *et al.* reported that norfloxacin, one of the most potent DNA gyrase inhibitors of the quinolone family, does not directly bind to DNA gyrase but to DNA.[18] Following this discovery, norfloxacin was observed binding to supercoiled, relaxed, and double- and single stranded-DNAs by equilibrium dialysis and a membrane ultrafiltration method.[18-23]

Two binding modes of quinolone to DNA have been proposed.[21,24] In the presence of gyrase and non-hydrolyzable ATP analogue, gyrase induces a specific single stranded DNA pocket in which norfloxacins are bound and stabilized by hydrogen bonding, π-π stackings of the norfloxacin rings, and tail-to-tail hydrophobic interactions.[21] In contrast, norfloxacin reportedly bound to plasmid DNA in the presence of an appropriate amount of Mg^{2+} but exhibited no interaction in either the absence or excessive amount of Mg^{2+} ions when observed by fluorescence technique, electrophoretic DNA unwinding, and affinity chromatography techniques.[24] Based on these observations, a model for the ternary complex, in which the Mg^{2+} ion acts as a bridge between the phosphate groups of nucleic acids and the carbonyl and carboxyl moieties of norfloxacin, has been proposed.

In spite of the above reported binding modes, the interaction of quinolone antibiotics and DNA is not yet clearly understood. For example, we recently found that one of the fluorescent quinolones, norfloxacin, can form a complex with double stranded *calf thymus* DNA without ATP or Mg^{2+} mediation; this was determined by a red-shift and hypochromism in the normal absorption, a strong induced circular dichroism, and strong fluorescence quenching in the presence of DNA.[25] Furthermore, based on the linear dichroism measurement, the aromatic molecular plane of DNA-bound norfloxacin was concluded to be close to parallel relative to the DNA bases, which excluded the possibility of a groove binding mode.[25] In this work, we investigated the binding properties of norfloxacin to mixed sequence single stranded DNA using various spectroscopic techniques in the absence of ATP and Mg^{2+}. In particular, we compared the spectroscopic properties, including the absorption, fluorescence emission, induced CD, and thermodynamic parameters, of a norfloxacin-single stranded DNA complex with those of a norfloxacin-native double stranded DNA complex. This study may be an important key to und-

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erstanding the inhibition mechanism of norfloxacin, because the inhibition site has been reported to be the single-stranded part of a nicked supercoiled DNA-gyrase complex.[21]

Materials and Methods

Materials. We dissolved double stranded *calf thymus* DNA (purchased from Sigma) in a 5 mM cacodylate buffer at pH 7.0 containing 100 mM NaCl and 1 mM EDTA; the solution was centrifuged at 15,000 rpm for 90 minutes to remove any insoluble solids. NaCl and EDTA were removed by dialyzing several times against 5 mM sodium cacodylate buffer at pH 7.0, 4 °C. This buffer was used in this work. The single stranded DNA was prepared by simmering double stranded DNA for more than one hour, followed by rapid cooling in iced water. Norfloxacin was purchased from Sigma and used without further purification. The concentrations of norfloxacin and DNA were determined spectrophotometrically using the molar extinction coefficients ε_{258nm}=6,700 $cm^{-1}M^{-1}$ for the DNA and 37,500 $cm^{-1}M^{-1}$ at 273 nm for the norfloxacin. All experiments were performed at room temperature (25 °C) unless otherwise specified.

Normal Absorption. UV absorption can be used to study the denaturation and renaturation of DNA molecules. The aromatic bases attached to the deoxyribose-phosphates exhibit absorption maxima near 260 nm. Native double-stranded DNA absorbs less than denaturated (melted) DNA strands; denaturated DNA absorptivity increases in the 260 nm region by about 40%.[26-28] The binding of a drug to DNA generally produces hypochromism, broadening of the envelope, and a red-shift of the drug absorption band. These effects are particularly pronounced for intercalators; with groove binders, a large wavelength shift usually correlates with a conformational change in the drug upon binding to DNA or in drug-drug interactions.

We ran all absorption spectra on a Hewlett Packard 8452A diode array spectrophotometer. The aliquots of concentrated norfloxacin were added to a constant concentration of DNA solution; the absorption spectra were then corrected for volume changes and the corresponding DNA absorption spectrum was subtracted. The resulting absorption spectra were normalized to the highest norfloxacin concentration for easy comparison.

Measuring the Equilibrium Constant by the Fluorescence Emission. Two mechanisms, dynamic and static, may explain the simple fluorescence quenching process.[29] In the dynamic quenching mechanism, energy from the excited fluorophore transfers to the quencher when they collide. The static mechanism is fluorescence quenching through the formation of a non-fluorescent ground-state complex between the fluorophore and quencher. As the temperature of the system increases, the dynamic quenching rate increases, because the number of collisions increases. Decreasing the temperature enhances the static quenching efficiency because the complex is usually stable at lower temperatures. Assuming that the fluorescence intensity of the fluorophore-quencher complex (Φ_c) is negligible compared to an unquenched fluorophore, the intensity in the presence (F) and absence (F_o) of the quencher is expressed by the Stern-Volmer equation.[30]

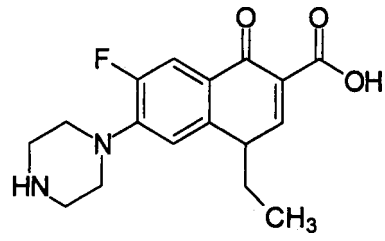

Figure 1. Molecular structure of norfloxacin and its extinction coefficient.

$$\frac{F_o}{F} = 1 + K_{SV}[Q] \quad (1)$$

Where K_{sv} is the Stern-Volmer constant, which is the equilibrium constant of the complex formation in the static quenching process. Since the fluorescence intensity of norfloxacin is strongly decreased by adding a polynucleotide, the Stern-Volmer approach is valid for approximating the equilibrium constant. If the fluorescence intensity is not negligible, the equation must be divided by the factor $(1+\Phi_c[Q])$.[31] We measured all fluorescence spectra and intensities on a Perkin Elmer LS50B fluorometer with careful temperature control.

Time-correlated Single Photon Counting. The fluorescence decay times of a given fluorophore are very sensitive to its environment. This property enables us to easily identify the number of fluorescent species in the system. Two representative techniques, single photon counting[29] and the phase modulation technique,[32] are usually utilized to measure the fluorescence lifetimes. We used the single photon counting technique in this work, using a single photon counting apparatus (FL900CD, Edinburgh, England) at Ewha Womans University. The flash lamp was filled with hydrogen gas at 0.43 atmosphere. The observed decay profile, $F(t)$, is generally fitted to the sum of the exponential curves.

$$F(t) = \sum a_i e^{-t/\tau} \quad (2)$$

Where a_i is the pre-exponential factor representing the fractional contribution to the time-resolved decay of the component with fluorescence lifetime τ_i. The long single decay time can be easily detected by a flash lamp setup and analyzed by plotting ln $F(t)$ *vs.* time, where the slope is $-1/\tau$. Many methods have been proposed to estimate the impulse response function for heterogeneous fluorescence decay profiles using the measured decay curve and lamp profile. We used the least square method in this work. This method calculates the expected value of a given decay curve until the best fit is obtained. The fit is judged by the χ^2 value and the weighed residuals.[30]

Circular Dichroism. CD is defined as the difference between absorbances measured with left and right circularly polarized light. Although norfloxacin is an achiral molecule, it acquires a CD signal when binds to polynucleotides. This CD signal is thought to be induced by the interaction between the bound achiral ligand and the chirally arranged base transition of the ligand with respect to the nucleobases.[33,34] All CD spectra were recorded on a Jasco J-720 spectropolarimeter using a 1 cm path quartz cell. In some

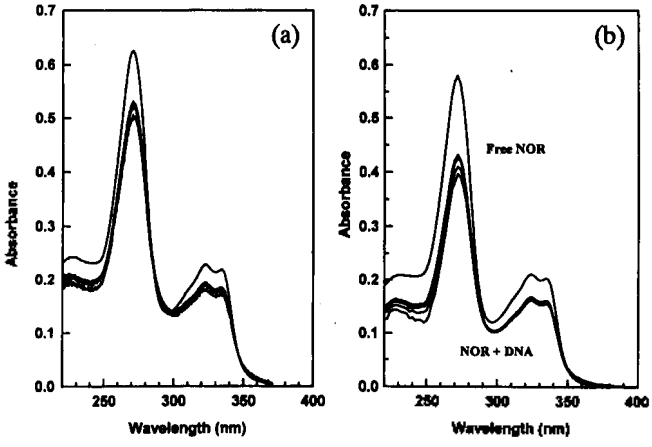

Figure 2. (a) Absorption spectra of norfloxacin in the absence (top curve) and presence of double stranded DNA. The data were collected for 197 μM DNA, and mixing ratios of 0.017, 0.050, and 0.084 from the bottom. The DNA absorption spectrum was subtracted from that of the mixtures and normalized to the norfloxacin concentration for easy comparison. (b) Absorption spectra of norfloxacin in the absence (top curve) and the presence of single stranded DNA. The data were collected for 143 μM DNA and mixing ratios of 0.024, 0.047, 0.070, 0.092 and 0.115 from the bottom. Data were treated as in (a).

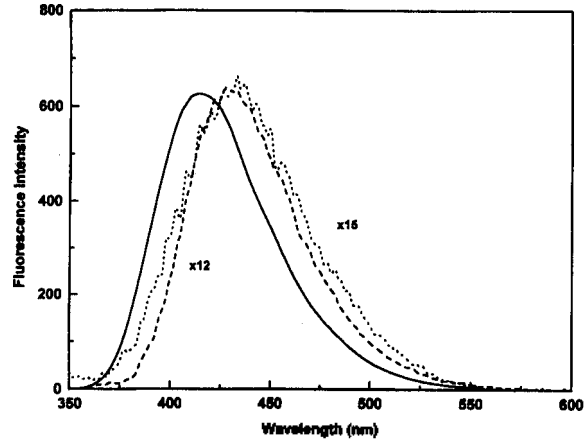

Figure 3. Fluorescence emission spectrum of norfloxacin in the absence and presence of single- and double stranded DNA. The dashed and the dotted curves represent 12 and 15 times enlarged emission spectra of norfloxacin bound to the single- and double stranded DNA. [norfloxacin]=1 μM, [single stranded DNA]=60 μM, [double stranded DNA]=316.7 μM. Excitation was at 323 nm. Slit widths are 7/9 nm for single stranded DNA and 4/7 nm for double stranded DNA.

cases, the spectra were averaged over an appropriate number of scans.

Results

Normal Absorption. The absorption spectra for different mixing ratios of norfloxacin with double- and single stranded DNA are shown in Figure 2. A 16-18% hypochromism in the 250-280 nm and the 310-340 nm region and isosbestic points were apparent for both double- and single stranded DNA, indicating that norfloxacin forms a complex with both single- and double stranded DNA. The mixing ratio-independent absorption spectra and isosbestic points suggest that the conformation of the DNA-bound norfloxacin is homogenous and independent of the mixing ratio. Furthermore, similar absorption spectra observed from the single- and double stranded DNA-bound norfloxacin suggest that the binding patterns and environments of norfloxacin in single- and double stranded DNA are similar.

Stern-Volmer Plot and Fluorescence Emission Spectra. Fluorescence emission spectra of norfloxacin in the presence and absence of single- and double stranded DNA are depicted in Figure 3. The emission spectra of the DNA-norfloxacin complexes were obtained by subtracting the emission spectrum of the unbound norfloxacin, which was calculated from the equilibrium constant (see below for equilibrium constant), from the emission spectrum of the norfloxacin-DNA mixture. A 15-20 nm red-shift and a strong decrease in the fluorescence intensity of the norfloxacin became apparent as the DNA concentration was increased. The shapes of the emission spectra of the single- and double stranded DNA-bound norfloxacin were similar. These observations again indicate that norfloxacin can interact with both single- and double stranded DNA in the absence of Mg^{2+} and ATP, and that the norfloxacin environments in single- and double stranded DNA are similar. The Stern-Volmer plots for the mixtures of single- and double stranded DNA and norfloxacin at various temperatures are depicted in Figure 4. The slopes in the Stern-Volmer plots for norfloxacin complexed with both DNAs increased as the temperature was lowered. With a hypochromism in the absorption spectrum in the presence of DNA, the decreases in the slopes with decreasing temperatures demonstrated that this quenching process occurs by a static mechanism; thus, the slope in the Stern-Volmer plot can be understood as the equilibrium constant for the norfloxacin-DNA complex. We calculated the equilibrium constant for norfloxacin-DNA complex formation at 25 °C to be as 3.77×10^3 M^{-1} for double stranded DNA and 6.54×10^3

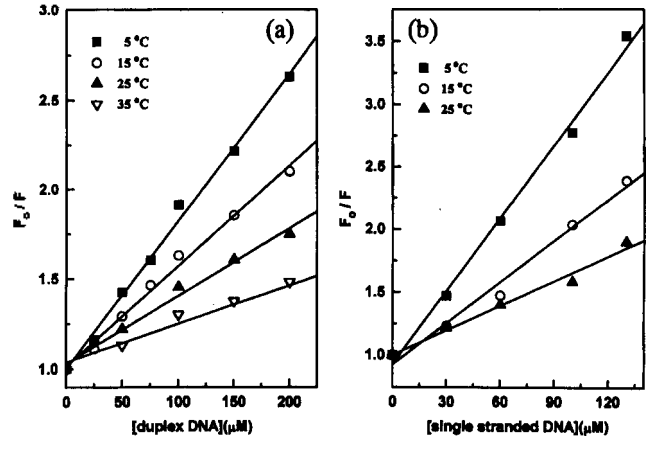

Figure 4. Quenching of norfloxacin fluorescence by double-(a) and single stranded DNA (b) at various temperatures. The concentration of norfloxacin was 1 μM. The samples were excited at 323 nm and the fluorescence was detected at 415 nm. Slit widths were 7/9 nm.

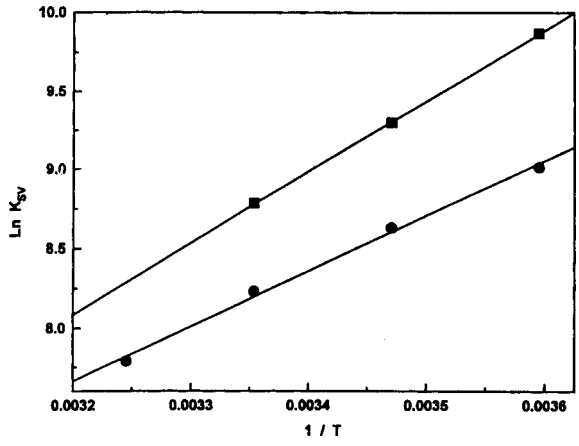

Figure 5. Van't Hoff plot for the complex formation between norfloxacin and single stranded DNA (squares) and double stranded DNA (circles). The equilibrium constants were measured by Stern-Volmer method.

M^{-1} for single stranded DNA, indicating that norfloxacin prefers to bind to single stranded DNA.

Thermodynamic Aspect of the Norfloxacin-DNA Complex Formation. Using the temperature-dependent K_{sv} values, the thermodynamic parameters can be obtained from the fundamental thermodynamic relationships.

$$\ln K_{SV} = -\frac{\Delta H^{o}}{RT} + \frac{\Delta S^{o}}{R} \quad (3)$$

$$\Delta G^{o} = -RT \ln K_{SV} \quad (4)$$

where ΔH^{o} is the standard enthalpy change for the complex formation, ΔS^{o} is the standard entropy change, and ΔG^{o} is standard Gibb's free energy of the reaction. R is the molar gas constant and T is the temperature in Kelvin. The resulting ln K_{SV} *vs.* $1/T$ plot for the norfloxacin-DNA quenching experiment is shown in Figure 5. The thermodynamic parameters are summarized in Table 1. In both cases, the complex formation was spontaneous with negative and similar ΔG^{o} values in spite of the unfavorable entropy. Therefore, the complex formation is driven by the enthalpy change, which represents the energy difference between the reactants and products, rather than the entropy effects, which mainly arise from solvent rearrangements, reducing the number of the species, and DNA conformational changes.

Salt Dependence of the Norfloxacin-DNA Complex Formation. The slope of the logK_{eq} *vs.* log[Na^{+}] plot for norfloxacin complexed with double stranded DNA was 1.5; that for single stranded DNA was 0.7 (Figure 6). These results suggest that one norfloxacin binding results in a one or one and half Na^{+} ion release from double stranded DNA, and the binding of two norfloxacins results in one sodium release from the DNA for single stranded DNA.

Table 1. Thermodynamic parameters for the norfloxacin-DNA complexation at 25 °C

DNA strand	K_{sv} (M^{-1})	ΔH^{o} (kJ·mol^{-1})	ΔS^{o} (J·mol^{-1}·K^{-1})	ΔG^{o} (kJ·mol^{-1})
single	6,540	−37.4	−52.6	−21.8
double	3,770	−29.0	−29.2	−20.4

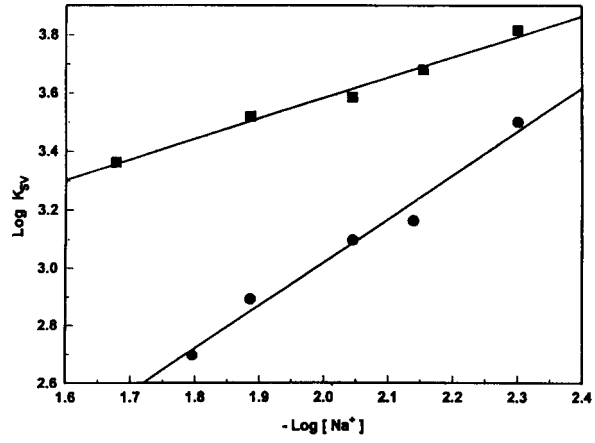

Figure 6. The logK_{eq} *vs.* −log[Na^{+}] plot for the norfloxacin-DNA complexation. (circles: double stranded DNA, squares: single stranded DNA)

Fluorescence Decay Profiles of Norfloxacin in the Presence and Absence of DNA. The fluorescence decay times were measured as explained in the experimental section. One representative result for the norfloxacin-single stranded DNA complex is depicted in Figure 7. The data

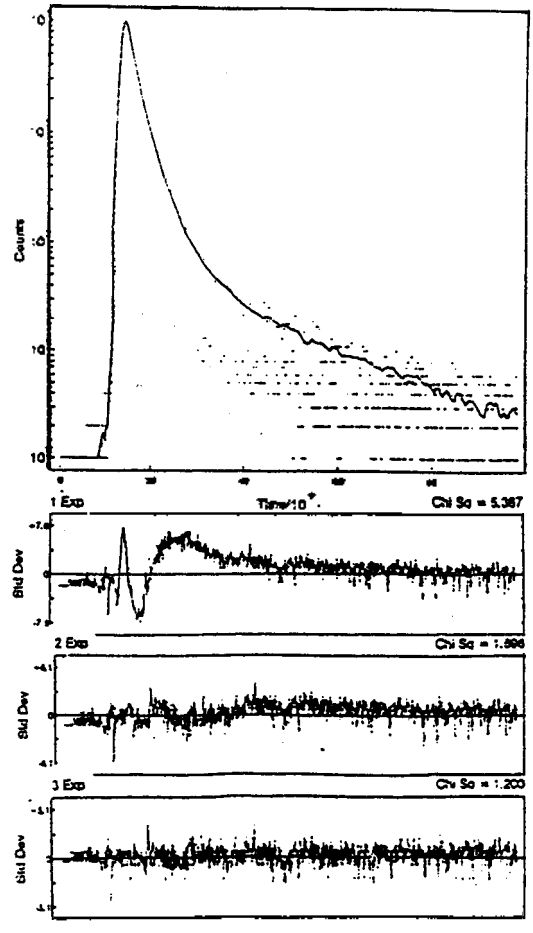

Figure 7. An example of a fluorescence decay profile obtained by the single photon counting technique. The residuals of single, double, and triple exponential fitting are shown in the lower panel. Excitation and emission wavelengths were 323 nm and 415 nm.

Table 2. Fluorescence decay times of norfloxacin in the absence and presence of single- and double stranded DNA. Excitation: 335 nm, Emission: 415 nm. [DNA]=1 mM, [norfloxacin] =1.0 μM. Relative amplitude are denoted in the parentheses

	Norfloxacin	with single-stranded DNA	with double-stranded DNA
single exponential fitting	1.4 (1.00) χ^2=2.33	1.9 (1.00) χ^2=6.39	1.5 (1.00) χ^2=4.76
double exponential fitting	1.3 (0.97) 8.4(0.03) χ^2=1.15	1.5 (0.81) 4.8 (0.19) χ^2=1.70	1.3 (0.94) 7.9 (0.06) χ^2=1.50
triple exponential fitting	1.3 (0.96) 6.2 (0.03) 26.7 (0.01) χ^2=1.13	1.3 (0.67) 3.4 (0.31) 23.7 (0.02) χ^2=1.20	1.2 (0.86) 3.8 (0.12) 26.7 (0.02) χ^2=1.10

validity was determined by the residuals and the χ^2 value. For this particular example, one and two component analyses yielded neither symmetrically distributed residuals nor good χ^2 values, which were 6.39 and 1.70 for the one and two component analyses. These results are not acceptable. Further analysis with three components results in well-distributed residuals (Figure 7, lower panel) and a χ^2 value of 1.20, which is acceptable. Other decay curves discussed here (data not shown) were similarly analyzed. The resulting fluorescence decay times and their amplitude for DNA-free norfloxacin and for that bound to single- and double stranded DNA are summarized in Table 2. Two fluorescent species were identified with decay times of 1.3 and 8.4 ns in the DNA-free norfloxacin. Their amplitudes were 0.97 and 0.03. This result is not surprising, because at pH 7.0 there are at least two differently protonated norfloxacins. The pK_a value at the carboxylic acid residue on the norfloxacin was 6.5 (data not shown). In the presence of single stranded DNA, the fluorescence decay profile of norfloxacin required three decay times (τ_1=1.3 ns, τ_2=3.4 ns, and τ_3=23.7 ns) with respective amplitudes of a_1=0.67, a_2=0.31, and a_3=0.02 to give a sufficiently good χ^2 value (χ^2=1.20) and residuals. When complexed with double stranded DNA, norfloxacin gave a similar results compared to that bound to single stranded DNA, except for the amplitude of the intermediate decay time, which was 0.31 for the single stranded DNA complex and 0.12 for double stranded DNA. The shortest component, 1.2-1.3 ns, was apparent for all conditions. Therefore, it is conceivable that the norfloxacin with the shortest decay time represents DNA-unbound norfloxacin. The intermediate component (τ=8.4 ns) of DNA-free norfloxacin gave a larger amplitude and shorter decay time (τ=3.0-4.0 ns) upon binding to single- and double stranded DNA.

Induced CD Spectra of Norfloxacin Bound to Single- and Double Stranded DNA. The norfloxacin used in this work does not posses any chiral center and therefore is optically inactive in the absence of DNA, but the CD spectra were induced in the presence of single- and double stranded DNA. The origin of this induced CD is thought to be the interaction between the transition moments of the achiral norfloxacin and chirally arranged DNA base transitions. The induced CD spectrum for a drug-DNA complex is very sensitive to the binding mode and the location and nature of the nucleo-bases of the bound drug. The induced CD of the norfloxacin observed in the presence of single stranded DNA, together with term "chirally arranged DNA base transitions", implies that some kind of secondary structure exists for the mixed sequence single stranded DNA, at least in the vicinity of the bound norfloxacin. Although this fact bears investigation, it is out of the scope of this work. The induced CD spectra for norfloxacin complexed with single stranded DNA and double stranded DNA are compared in Figure 8. The characteristics of the induced CD spectra of the norfloxacin in both complexes were similar, consisting of a weak positive CD band in the 300-350 nm region, a strong positive band in the 270-300 nm, and a negative band at 240-270 nm. The positive CD band at 300-350 nm was somewhat stronger in the single stranded DNA-norfloxacin complex than in that complexed with double stranded DNA. An observed isosbestic point at 270-280 nm indicated that the conformation of the bound norfloxacin was homogeneous in the corresponding DNA. Similar induced CD spectra of norfloxacin in both single- and double stranded DNA indicated that this drug binds similarly in both DNAs.

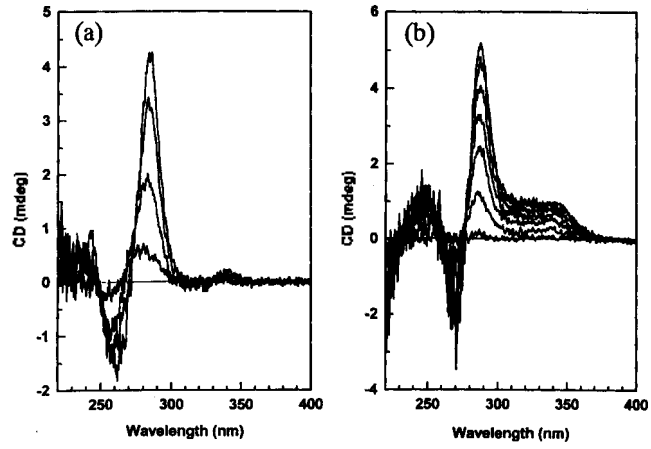

Figure 8. (a) Induced CD spectra of the norfloxacin-double stranded DNA complex at various mixing ratios. Only those for the mixing ratios of 0.07, 0.29, 0.60, and 0.93 from the bottom are shown. [DNA in base pairs]=50 μM. (b) Induced CD spectra of the norfloxacin-single stranded DNA complex at mixing ratios of 0.07, 0.29, 0.60, 1.02, 1.29, 1.47, and 1.56 from the bottom. [DNA in base]=50 μM.

Discussion

Absorption and Induced CD Spectra of Norfloxacin in the Presence of Single- and Double stranded DNA. The absorption spectra for norfloxacin upon binding to both single- and double stranded DNA showed 16-18% hypochromism in the 250-280 nm and 310-340 nm regions (Figure 2). Three isosbestic points (at 285 nm, 299 nm and 346 nm (Figure 2(a))) for double stranded DNA and one for single stranded DNA were apparent. Although shifts in the absorption band could not be observed in the presence of DNA, hypochromism in the absorption spectra represented definitive support for norfloxacin-DNA

interaction without ATP or Mg^{2+} mediation. The three observed isosbestic points in the absorption spectra were indicative of the homogeneous binding mode of norfloxacin to DNA. The shapes of the absorption spectra of norfloxacin bound to either DNA were similar, suggesting a similar binding mode for both DNAs. These observations contrast with previous reports by Shen *et al.*, in which one norfloxacin molecule per 6646 base pairs (of relaxed ColE1 DNA) was bound[18] and the drug did not bind to double stranded DNA *per se*, and by Palù *et al.*, in which no interaction between norfloxacin and plasmid DNA was evident in the absence of Mg^{2+}.[24] The gradual decrease in the 300-350 nm absorption band with a decreased mixing ratio suggested that a significant amount of norfloxacin in the system was not bound to DNA.

Induced CD is sensitive to the conformation of the DNA-bound drug and the nature of the nucleo-bases. The induced CD spectra shown in Figure 8 were alike regardless of the nature of the DNA, indicating that the norfloxacin conformations in the complexes were similar.

Fluorescence Emission and Binding Equilibrium. Fluorescence spectra are extremely sensitive to the environment of the fluorophore. Changes were apparent in both the shape and intensity of the norfloxacin emission spectrum after adding DNA. This observation is comparable to that reported for the norfloxacin-thermal denatured DNA mixture. In addition to a hypochromism in the absorption spectrum, changes in the fluorescence emission spectrum strongly indicated an interaction between norfloxacin and DNA. Furthermore, the fluorescence emission spectra of the bound species, obtained by subtracting the emission spectrum of the free norfloxacin from that of the mixture, were nearly the same when complexed with both single- and double stranded DNA. The absorption and induced CD spectrum, and the fluorescence decay profile (see following section) indicated that the environments of norfloxacin in the single stranded DNA and double stranded DNA are alike.

The fluorescence intensity of norfloxacin is quenched by adding either single- or double stranded DNA; the fluorescence quenching is more effective for single stranded DNA. We observed both changes in the absorption spectrum of norfloxacin upon adding DNA and an increase in the quenching efficiency as the temperature was decreased, which ensured a ground state complex formation between the norfloxacin and DNA. The equilibrium constants, equivalent to the Stern-Volmer quenching constant in the static quenching mechanism, were obtained from the slope of Figure 4 as 3.8×10^3 M^{-1} for double stranded DNA and 6.5×10^3 M^{-1} for single stranded DNA at 25 °C, which are lower than those of other intercalators by a factor of 10^2-10^4. This may explain why previous studies failed to detect any interaction between norfloxacin and double stranded DNA.

The binding of norfloxacin to thermal denatured DNA and single stranded synthetic polynucleotides has been studied.[18,19] The amount of bound norfloxacin reportedly increased more than ten times compared to double stranded DNA when the DNA was denatured. The drug binding level of homopolymer nucleic acids is the same as that of denatured DNA. Based on these observations, norfloxacin was proposed to bind to single stranded DNA through hydrogen bonds which become available when the bases are unpaired; the bound norfloxacin is further stabilized by norfloxacin-norfloxacin stacking. Our results indicate that the equilibrium constant for the norfloxacin-double stranded DNA complex formation is lower by only a factor of two than that for single stranded DNA. The norfloxacin may be stabilized in the intercalation pocket *via* electrostatic and stacking interactions between the norfloxacin and nucleobase(s) in double stranded DNA. The importance of the electrostatic interaction is supported by the Na+ ion effect on the equilibrium constant.

The ionic strength-dependence of the equilibrium offers an additional piece of information about drug-DNA interaction. Release of the Na^+ ion can be examined using the slope in the log K_{eq} *vs.* $-\log[Na^+]$ plot. For instance, mono- and di-valent cation ethidium and propidium intercalation results in slopes of 1.2 and 2.2 in the log K_{eq} *vs.* $-\log[Na^+]$ plot.[35,36] Considering the 0.2-0.3 Na^+ ion release due to the conformational change of DNA by intercalation, a 1 and 2 Na^+ ion release is expected for mono- and di-valent cationic intercalators. If norfloxacin is intercalated between the bases of DNA, the Na^+ ion release due to norfloxacin intercalation would be 1.0. In Figure 6, the log K_{eq} *vs.* $-\log[Na^+]$ plot exhibits a slope of 1.5 for the norfloxacin-double stranded DNA complex formation and 0.7 for the single stranded DNA. The value 1.5 is slightly higher for the classical intercalation.

Fluorescence Decay Profiles of Norfloxacin Complexed with DNAs. The fluorescence decay profile of DNA-free norfloxacin was well fitted by two exponential curves (a_1=0.97, τ_1=1.3 ns; a_2=0.03, τ_2=8.4 ns; χ^2=1.15), indicating that there were two fluorescent norfloxacin species in the aqueous solution. It was evident from the acid-base titration that two differently protonated norfloxacins existed at neutral pH-one protonated at the carboxylic residue and another deprotonated at the same site. However, it is noteworthy that the number of exponential curves do not necessary limit the number of species in the system, because the existence of a non-fluorescent norfloxacin species is always possible. A Previous study by Kim and his coworkers[25] demonstranted that the DNA-binding species of norfloxacin is the protonated one. When bound to single- as well as double stranded DNA, the fluorescence decay profiles of norfloxacin could be fitted by three exponential curves (a_1=0.67, τ_1=1.3 ns; a_2=0.31, τ_2=3.4 ns; a_3=0.02, τ_3= 23.7 ns; χ^2=1.20 for single stranded DNA, and a_1=0.86, τ_1= 1.2 ns; a_2=0.12, τ_2=3.8 ns; a_3=0.02, τ_3=26.7 ns; χ_2=1.10 for double stranded DNA) with good residuals and χ_2 values. In a comparison between the fluorescence decay profiles of DNA-free norfloxacin and that bound to both single- and double stranded DNA, the shortest component remained unchanged; the intermediate component became shorter, from 8.4 ns to 3.4-3.8 ns with increasing amplitude, indicating that this species probably was bound to DNA. Combining the acid-base titration and the single photon counting result indicated that the fluorescent species that exhibits the intermediate decay time is probably the protonated one. The relative amplitude of the intermediate species was larger in the single stranded DNA than in the double stranded DNA, which agrees with the quenching efficiency of the single stranded DNA being higher, *i.e.*, the relative population of the bound norfloxacin is larger in the presence of single

stranded DNA. The longest lived component apparent for both complexes (~25 ns) may be assigned to the noise, because the fluorescence measurement times for both complexes are very long since the fluorescence of the norfloxacin is very strongly quenched in the presence of DNA. For long measurement durations, it is possible that the noise increases and gives rise to a long-lived fluorescence component. The assumption that the longest fluorescence component is due to noise and not to norfloxacin with a long fluorescence decay time is supported by steady state spectroscopic observations. Isosbestic points in the absorption and the induced CD spectra support this assumption, as does the fact that the shapes of the absorption, induced CD, and fluorescence emission spectra of the DNA-bound norfloxacin were the same in the different mixing ratios.

Thermodynamics of Norfloxacin Complexed with Single- and Double stranded DNA. Different types of possible molecular interactions between bound drugs and DNA have been suggested; Van der Waals stacking interactions between bound drug molecules and adjacent base pairs are expected. These interactions should contribute negative enthalpy and negative entropy terms. Although negative contributions from the drug-base stacking interaction must offset positive ΔS° contributions arising from the disruption of stacked base (pairs) in native DNA, it is thought that the exothermic nature of the binding reaction mainly reflects drug-base stacking interactions. Hydrogen bonds may also be formed upon the binding of drug molecules to DNA. The formation of hydrogen bonds produces a more ordered stable state, resulting in negative enthalpy and negative entropy terms. The structure of the DNA itself is changed upon drug intercalation; the overall length increases and the helix flexibility decreases. Intercalation disrupts the normal base stacking interaction and contributes to a positive enthalpy term. Disruption of the base stacking probably also increases the DNA entropy (the less ordered state), leading to a positive entropy contribution. These positive contributions to both the enthalpy and the entropy may offset the negative enthalpy and entropy contributions derived from the drug intercalating into the DNA, causing an overall negative free energy for the complex formation. The effects of salts on the thermodynamic binding parameters have been elucidated for daunomycin and adriamycin-DNA complex formations. As the salt concentration is increased, the overall enthalpies and entropies become less negative. The dependence of the enthalpy on the ionic strength is well beyond the predictions of polyelectrolyte theory, so the physical bases of the ionic strength dependence of the enthalpy and entropy are unknown. However, CD measurements indicate that salts induce changes in the secondary structure of DNA. Various solvent rearrangements are expected upon drug-DNA complex formation: (1) the liberation of the hydration shells around the hydrophobic parts of the drug molecules gives rise to a positive contribution to the drug binding entropy; (2) the hydration spine that lies in the minor groove around the A-T rich sequences is disrupted by minor groove binding drugs, contributing a positive entropy; and (3) the disruption of the water structure surrounding the DNA upon drug binding contributes positive enthalpy and entropy terms.

The complexations of norfloxacin with single- and double stranded DNA are spontaneous reactions with similar magnitudes of negative free energy, at about 20.0 $kJ \cdot mol^{-1}$. The entropy of the complex formations is unfavorable, and that of a norfloxacin-single stranded complex formation is almost twice as high as that of a norfloxacin-double stranded DNA complex formation. Among the many factors which contribute favorable enthalpy, the base-norfloxacin stacking interaction and forming of the hydrogen bond are believed to be the main sources of the exothermic nature of the binding reaction. The similar ΔH° values for both complex formations therefore conceivably suggest that the extent of stacking with the nucleo-bases and the hydrogen bond formation of the norfloxacin molecule are similar in single- and double stranded DNA. If the base stacking and hydrogen bond formation are similar in both DNAs, then the difference in the entropy terms may be the result of stiffness and elongation of the double stranded DNA, which is not expected from single stranded DNA.

Binding Mode of Norfloxacin Complexed with Single- and Double stranded DNA. We compared the spectroscopic properties of the norfloxacin-single stranded DNA complex to those of the norfloxacin-double stranded DNA complex to investigate the conformation of norfloxacin in single stranded DNA.

Three binding modes are presently proposed for norfloxacin when complexed with DNA. According to Palù *et al.*, Mg^{2+} acts as a bridge between the phosphate groups of DNA and the carbonyl and carboxyl moieties of norfloxacin; maximum binding was observed at 1-2 mM Mg^{2+} ion concentration.[24] No interaction between norfloxacin and DNA was evident in either the absence or presence of an excessive amount of Mg^{2+}. However, Shen *et al.* proposed a cooperative quinolone-DNA binding mode in the inhibition site of DNA gyrase in the presence of ATP.[20,21] In this mode, norfloxacins were bound in a specific single stranded DNA pocket induced by gyrase and were stabilized by the π-π stacking of the norfloxacin rings and the tail-to-tail hydrophobic interactions.

A direct interaction model for the norfloxacin-double stranded DNA was recently proposed by Kim and his coworkers, base on the spectroscopic observations.[25] The spectroscopic properties of a norfloxacin-DNA complex in the absence of ATP and Mg^{2+} can be summarized as a 16-18% hypochromicity in the absorption spectrum, effective quenching of the fluorescence intensity, a red-shift in the fluorescence emission spectrum, and an angle of about 75.0° between the lowest electric dipole transition and the DNA helix axis. In addition to the spectroscopic results, they observed an unwinding of the supercoiled DNA by norfloxacin. Although a precise binding model cannot be deduced from these spectroscopic results, the possibility of groove binding and surface binding of norfloxacin in DNA has been excluded, because the planar part of the norfloxacin was almost parallel to the nucleo-bases and bound norfloxacin exhibited a strong π-π interaction with the nucleo-bases. While these observations support the intercalation binding mode in which the norfloxacin molecule sits between the base pairs of the DNA, the following deviations from classical intercalation should be noted: (1) the unwinding pattern of the super-coiled DNA by norfloxacin was very different from that of a classical intercalator, ethi-

dium, and (2) the magnitude of the linear dichroism in the DNA absorption region decreased upon norfloxacin binding (contrary to a classical intercalator). This model disagrees with that of Palù *et al.*[21] on several points; Mg^{2+} was not required for the norfloxacin to form a complex with a double helical DNA complex and the norfloxacin-phosphate interaction was not required for Kim's model.[25]

Utilizing Kim's model of the norfloxacin-double stranded DNA complex conformation, various spectroscopic properties of a norfloxacin-single stranded DNA complex were compared with those of a double stranded DNA complex. This study is important not only to understand the DNA gyrase inhibition mechanism by norfloxacin but also the physico-chemical standpoint, because norfloxacin is at least 2-10 times more likely to bind to single stranded DNA than to double stranded DNA. In a comparable study, the spectroscopic properties for norfloxacin complexed with single stranded DNA are the same as for double stranded DNA, indicating that the norfloxacin environments are similar in these two different nucleic acids. The only possible norfloxacin conformation in single stranded DNA, which results in observed spectroscopic change, is stacking. Either the molecular plane of the norfloxacin was inserted between the nucleo-bases of the single strand DNA or the norfloxacin itself was stacked along the single stranded DNA. Another possible conformation to consider is direct contact through the electrostatic interaction between the positively charged nitrogen atom and the negatively charged phosphate group of the DNA. However, this binding is not likely to occur, because in that case the distance between the norfloxacin molecule and the nucleo-base would not be close enough to yield the π-π interaction which causes the observed spectroscopic results.

Conclusion

According to our experimental results, the various spectroscopic properties for norfloxacin complexed with single stranded DNA are the same as for double stranded DNA, indicating that the norfloxacin environments are similar in these two different nucleic acids.

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Synthesis and Crystal Structure of Cobalt(III) Complex with Chiral Pentadentate Bis-Amide Ligand, 1,9-bis(*S*)-pyrrolidinyl-2,5,8-triazanonane-1,9-dione(*S,S*-prodienH_2)

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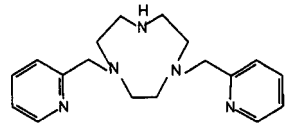
A chiral pentadentate bis-amide ligand, 1,9-bis(*S*)-pyrrolidinyl-2,5,8-triazanonane-1,9-dione(*S,S*-prodienH_2) has been synthesized from the reaction of bis(2-aminoethyl)amine(dien) and *S*-proline, and the structure of [Co(*S,S*-prodien)H_2O]ClO_4 has been determined by single crystal X-ray diffraction. The geometrical structure of the Co (III) complex has been an αβ-form, where the dien moiety of ligand chelates to a facial in metal center, and the aqua ligand coordinates a *cis* site to the secondary nitrogen of dien. The Co-N(1), Co-N(3) distances of two amide moiety in *S,S*-prodien are shorter than the other Co-N(2), Co-N(4), and Co-N(5) distances because of the increased basicity of nitrogen in amide. The complex crystallizes in the monoclinic space group $P2_1$(#4), with a=7.838(1), b=12.675(1), c=9.710(1) Å, β=100.39(1) and z=2. Refinement gives the final R and R_w values of 0.045 and 0.057, respectively for 2130 observed reflections. Based upon the CD and X-ray data, it is identified that the absolute configuration of the αβ-[Co(*S,S*-prodien)H_2O]ClO_4 has a Λ-form.

Introduction

Asymmetric synthesis and optical resolution of coordination compounds of multidentate ligands with nitrogen donor atoms are well known.[1] Particularly, chiral metal complexes with tetradentate ligands are well documented[2] but relatively few complexes have pentadentate ligands coordinated to metal ion. Recently, McLachlan *et al.*[3] have reported the coordination chemistry of pentadentate ligands derived from the tripod ligand tris(2-aminoethyl)amine(tren) and 1,4,7-triazacyclononane(tacn). Pyridyl arms have been attached to tren forming the pentadentate ligands, 3-[4-(2-pyridyl)-3-azabut-3-enyl]-3-azapentane-1,5-diamine(L1) and 3-[4-(2-pyridyl)-3-azabutyl]-3-azapen-tane-1,5-diamine(L2), which have been applied in the synthesis of mononuclear and azido bridged binuclear nickel(II) complexes.[4] The preparation and properties of Co(III) complexes with L1 and L2 ligand have been reported, along with the amide complex, $[Co(HL3)Cl]^{2+}$(HL3=3-{2-[hydroxy(2-pyridyl)methylene-amino]ethyl}-3-azapentane-1,5-diamine) was obtained through oxidation of $[Co(L1)Cl]^{2+}$. Amed *et al.*[5] have been reported the Co(III) complexes of pentadentate ligands, 1,9-bis(2'-pyridyl)-2,5,8-triazanonane(picdien) and 1,11-bis(2'-pyridyl)-2,6,10-triazaundecane(picditn), which have pyridyl arms in dien[=bis(2-aminoethyl)amine] and ditn[=bis(3-aminopropyl)amine]. The geometrical structure of the Co(III) complexes of pcdien and pcditn ligand have been an αβ-form which the dien moiety of ligand chelates to a facial site in metal center, and aqua ligand coordinates a *cis* site to the secondary nitrogen of dien. In αβ-Co(III) complexes of picdien both *syn* and *anti* forms are found, while only the *anti* forms in the picditn complexes have been isolated. The structures have been established by single-crystal X-ray diffraction. Also, Williams *et al.*[6] have prepared and investigated the stereoselectivity of the Co(III) complex of pentadentate ligand, 3*S*-di(2-picolyl)-amino-N-(2-picolyl) hexahydroazepine(*S*-ahazterpy), with one chiral center. [Co(*S*-ahazterpy)Cl]$(ClO_4)_2 \cdot 2H_2O$ has been obtained to single isomer(*trans*-equatorial) that the tertiary amine has fixed stereochemistry by the *S*-chirality in the heterocyclic ring of the *S*-ahaz(3*S*-aminohexahydroazepine) unit.

In this paper we present the preparation and structure of Co(III) complex with 1,9-bis(*S*)-pyrrolidinyl-2,5,8-triazanon-



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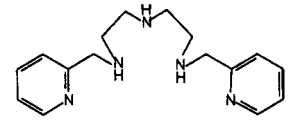
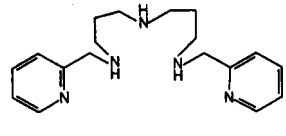


picdien



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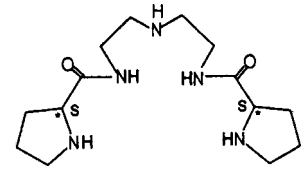

S,S-prodienH_2