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Base specific complex formation of norfloxacin with DNA

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Abstract

We examined the base specificity of the norfloxacin–DNA interaction by measuring the binding constant of norfloxacin to various synthetic polynucleotides, using the Stern–Volmer and the Benesi–Hildebrand methods. The equilibrium constants were largest for poly[d(G–C)$_2$] and poly(dG)·poly(dC), suggesting that norfloxacin binds preferentially to the G–C bases of calf thymus DNA. We also found that norfloxacin has a greater affinity for purine than for pyrimidine. The binding mode of norfloxacin to double-stranded polynucleotide was studied using circular and linear dichroism (CD and LD). When the norfloxacin was complexed to poly[d(G–C)$_2$], poly(dG)·poly(dC) and DNA, all of the complexes exhibited a similar weak, positive CD band and negative LD in the 300–350-nm region. A closer examination of the LD spectra suggests that the molecular plane of norfloxacin is near perpendicular relative to DNA helix axis that excludes the groove binding mode or surface binding of norfloxacin.

Keywords: Norfloxacin; Quinolone; DNA; Base specificity; Polynucleotide; Optical spectroscopy

1. Introduction

Quinolones are known to inhibit the action of the essential bacterial enzyme topoisomerase II, a DNA gyrase which mediates reduction of the DNA linking number via a double-strand breakage–passage–resealing process [1]. This process is essential for efficient replication of DNA and for separation of daughter DNA during cell division. To clarify the mechanism of quinolone action, studies have been conducted on the direct binding of quinolones to DNA or DNA gyrase, which are the logically potential targets for quinolones. An earlier attempt to investigate the radioactive nalidixic acid–DNA interaction using an equilibrium dialysis method failed to detect any direct interaction between the drug and DNA [2]. However, the binding of this class of antibiotic to

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DNA has been re-investigated, since a membrane ultrafiltration technique with [^{3}H] norfloxacin demonstrated that quinolones do not bind to gyrase [3].

Whether quinolones interact directly with native DNA has been a matter for debate. Equilibrium dialysis, fluorescence measurements and a ^{19}F NMR study failed to reveal any evidence of direct interaction between quinolone and DNA [4,5]. The first evidence to support the direct interaction of quinolone with single, double and supercoiled DNA appeared in 1985 [3]. The fact that norfloxacin can form a complex with various DNAs was revealed using a membrane ultrafiltration technique, and subsequent studies from the same group [6,7] have demonstrated that norfloxacin binds preferentially to single-stranded DNA; when DNA is paired, the norfloxacin binding is weak and exhibits no base specificity. They also found that the binding of the drug to relaxed DNA is enhanced in the presence of gyrase and non-hydrolyzable ATP analogue. A cooperative quinolone–DNA binding model based on these findings was proposed, in which DNA-bound gyrase induces a specific single-stranded quinolone binding site in the relaxed DNA substrate in the presence of ATP [8,9]. The direct binding of quinolones to DNA is also supported by the fact that quinolones can unwind DNA in the presence of Mg^{2+} ions [10,11]. A ternary complex model for the quinolone–DNA complex was proposed based on the observation that norfloxacin binds to plasmid DNA in the presence of an appropriate amount of Mg^{2+} ions but exhibits no binding in an absence or excess amount of Mg^{2+} ions. In the complex, the Mg^{2+} ions act as a bridge between the phosphate group of nucleic acids and the carbonyl and carboxyl moieties of quinolone [11].

Despite its importance, the interaction of quinolone antibiotics and DNA is not yet clearly understood. For example, we recently found that norfloxacin (Fig. 1), an antibiotic belonging to the quinolone family, can interact with the base (pairs) of double-stranded *calf thymus* DNA without Mg^{2+} or ATP mediation, which contrasts with previous reports. In a long-term effort to clarify the mechanism of quinolone antibiotics and DNA–gyrase interaction, we investigated the base selectivity and base-specific binding mode of norfloxacin when forming a complex with double-stranded *calf thymus* DNA and synthetic polynucleotides. We used established conventional optical spectroscopic methods in our study, including electric absorption, circular and linear dichroism spectroscopy, and fluorescence techniques.

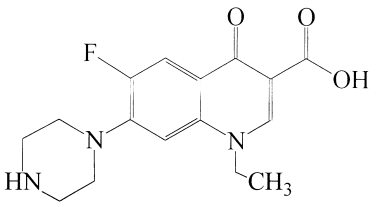

Fig. 1. Molecular structure of norfloxacin.

2. Materials and methods

2.1. Materials

Polynucleotide, purchased from Pharmacia, and *calf thymus* DNA (referred to as DNA), from Sigma were dissolved in 5 mM cacodylate buffer containing 100 mM NaCl and 1 mM EDTA at pH 7.0 by exhaustive stirring at 4°C. The polynucleotide solutions were then dialyzed several times against 5 mM cacodylate buffer at pH 7.0. This buffer was used throughout this work. Nucleotides and norfloxacin were purchased from Sigma and used without further purification. Single-stranded DNA was prepared by simmering the double-stranded DNA, followed by rapid cooling in iced water.

2.2. Measuring equilibrium constant by Stern–Volmer method

Fluorescence quenching refers to any process that the fluorescence intensity of a given fluorophore decreases upon adding quencher [12]. Assuming that the fluorescence intensity of the fluorophore–quencher complex (Φ_o) is negligible, compared to an unquenched fluorophore, the in-

tensity in the presence (F) and absence (F_o) of the quencher is expressed by the Stern–Volmer equation

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

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 upon adding polynucleotide (see Results), the Stern–Volmer approach is valid to approximate the equilibrium constant. If Φ_o is not negligible, Eq. (1) must be divided by the factor $(1 + \Phi_o K_{SV}[Q])$ [13]. All fluorescence spectra and intensities were measured on a Perkin Elmer LS 50B fluorometer.

2.3. Absorption measurements

In general, binding of a drug to DNA produces hypochromism, broadening of the envelope, and a red-shift in the drug absorption region. The ground state association constant, K_{BH} for 1:1 norfloxacin:nucleotides complexes can be represented by the following equilibrium

$$\text{norfloxacin} + M \rightleftharpoons [\text{norfloxacin}...M] \quad (2)$$

where M denotes the macromolecules (in our case, DNA, polynucleotide or nucleotides). The equilibrium constant of the complex formation may be estimated from the changes in absorbance at a fixed wavelength, using the Benesi–Hildebrand equation [14].

$$\frac{1}{\Delta A} = -\frac{1}{(\varepsilon_b - \varepsilon_f)[L_t]} + \frac{1}{(\varepsilon_b - \varepsilon_f)[L_t]K_{BH}}\frac{1}{[M]} \quad (3)$$

where ϵ is the extinction coefficient, the subscripts b, f and t denote bound, free and total drugs. $[L]$ is the drug (norfloxacin) concentration $[M]$ is the concentration of the macromolecule, and ΔA is the change in the absorbance at a given wavelength. By plotting the reciprocal absorbance vs. the reciprocal concentration of the macromolecules, the Benesi–Hildebrand association constant for complex formation, K_{BH}, can be calculated from the ratio of the slope to the intercept. The Benesi–Hildebrand plot was constructed by the change in the absorbance at 323 nm. Absorption spectra were measured on a Jasco V-550 or a HP8452A diode array spectrophotometer.

2.4. Linear dichroism and reduced linear dichroism

Linear dichroism (LD), which is determined by the differential absorption between plane polarized light with the polarization parallel and perpendicular to the reference axis, is a powerful technique to determine the binding geometry of a drug–DNA complex [15–17]. The measured LD spectrum is divided by isotropic absorption spectrum $[A_{iso}(\lambda)]$ to give the reduced linear dichroism spectrum (LD^r).

$$LD^r(\lambda) = \frac{LD(\lambda)}{A_{iso}(\lambda)} \quad (4)$$

The magnitude of the LD^r spectrum depends on the orientation factor (S) and optical factor (O) [15,16].

$$LD^r(\lambda) = S \times O = 3S\frac{(\langle 3\cos^2\alpha \rangle - 1)}{2} \quad (5)$$

The optical factor depends on the angle, α, that the transition dipole moment of the drug makes with the flow direction, therefore the DNA helix axis. The brackets denote an ensemble average over the angular distribution. The orientation factor, S, which depends on the contour length and flexibility of the DNA, the flow rate, viscosity and temperature of the medium may be determined from the DNA dichroism at 260 nm, assuming an effective angle of 86° between the $\pi \rightarrow \pi^*$ transition moments of the nucleo-bases and the DNA helix axis. The drug α values close to 90° are consistent with intercalative binding; the LD^r for the intercalated drug is therefore negative and similar in magnitude to that of the DNA bases. However, if the drug binds to the groove, then the

transition dipole moments of the drug lie along a groove, exhibiting α values of 40–50° and positive LD^r signal [16]. The LD spectra of the norfloxacin–DNA complex were measured on a Jasco 500C spectropolarimeter equipped with an Oxley prism to convert the circularly polarized light into linearly polarized light [15]. The orientation of the norfloxacin–DNA complex in the buffer solution is achieved using a flow Cuvette cell with an outer rotating cylinder. The LD signal was averaged over appropriate number of scans when necessary.

In the LD^r calculation under our conditions, two factors must be cautiously considered. The first is that the measured LD only 'sees' the bound norfloxacin in the drug absorption region. The norfloxacin molecule which is not bound to the DNA can not be oriented in the solution, and consequently can not contribute to the LD signal. In contrast, the measured absorption spectrum is the sum of the DNA-bound and unbound norfloxacin. The unbound portion of the drug in the solution, therefore, must be calculated from the equilibrium constant (which we measured using the Stern–Volmer method) and subtracted from the measured absorption spectrum to obtain the true $A_{iso}(\lambda)$ in Eq. (4). Secondly, the magnitude of the LD^r at 260 nm does not necessarily represent the true LD^r of the DNA, since the bound norfloxacin also absorbs in this region. However, the relative magnitude of LD^r at 260 nm and 280 nm was similar in the presence and absence of norfloxacin (see Results), even though the norfloxacin had a strong absorption band near 275 nm, suggesting that norfloxacin does not affect the shape of the LD^r signal in the DNA absorption region and that the LD^r magnitude at 260 nm of the complex can be used to calculate the S value.

2.5. Circular dichroism

Norfloxacin does not display circular dichroism (CD) spectrum in the absence of polynucleotide, but CD spectrum is induced when it forms the complex with polynucleotide (ICD). Although the origin of ICD of the achiral drug–DNA complex is not clear, it is believed to be induced by the coupling between the transition moments of achiral drug and chirally arranged nucleo-base transitions or by excitonic interaction of the DNA-bound drug. The shape and magnitude of ICD depends on the binding mode and location of the drug, and the nature of the nucleo-bases [18,19]. All CD spectra were measured on a Jasco J715 spectropolarimeter.

3. Results

3.1. Association of norfloxacin with various polynucleotides: fluorescence measurements

The addition of any polynucleotide into the norfloxacin solution results decreases in the fluorescence intensity of the norfloxacin. The gradual decreases observed upon increasing the polynucleotide concentration are evidence of direct interaction between the drug and a double-stranded polynucleotide. Since the fluorescence intensity of the bound norfloxacin is small compared to the polynucleotide-free norfloxacin [it decreases to zero when bound to poly(dG)·poly(dC) and poly[d(G–C)$_2$] and to approx. 7% when bound to DNA (Fig. 2a)], the Stern–Volmer method of Eq. (1) can be used to measure the quenching constant. The quenching efficiency decreases with an increase in the temperature of the mixture (4.1×10^3 M^{-1} at 15°C and 2.8×10^3 M^{-1} at 25°C for the norfloxacin–DNA mixture, data not shown) and the absorption spectrum changes upon adding the polynucleotide. These observations support that the fluorescence quenching occurs by a static process, i.e. by forming a non-fluorescent complex. The slope in the Stern–Volmer plot, therefore, is equivalent to the equilibrium constant for the norfloxacin–polynucleotide complex formation. Fig. 2b shows the Stern–Volmer plot of norfloxacin in the presence of various polynucleotides. The equilibrium constant of the norfloxacin is the highest for the single-stranded DNA, poly[d(G–C)$_2$] and poly(dG)·poly(dC), which is calculated to be $6.0 \sim 7.0 \times 10^3$ M^{-1}. That for poly(dG)·poly(dC) is slightly higher than for poly[d(G–C)$_2$]. The equilibrium constant for the norfloxacin–poly[d(A–T)$_2$] complex formation is the lowest, at 6.1×10^2 M^{-1}, which is 10 times

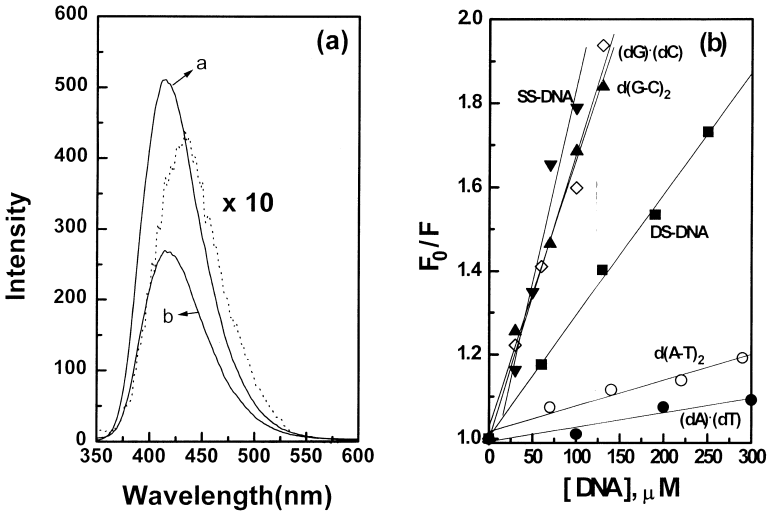

Fig. 2. (a) Fluorescence emission spectra of norfloxacin in the presence of DNA (curve b) and that of DNA-free norfloxacin (curve a). That for the DNA-bound norfloxacin (dotted curve, 10 times enlarged) is obtained by subtracting emission spectrum of free norfloxacin [which is calculated from the equilibrium constant (2.8×10^{-3} M^{-1})] from that of the norfloxacin–DNA complex. Excitation measured at 323 nm. Slit width: 4/7 nm for excitation and emission. [norfloxacin] = 1 μM and [DNA] = 323 μM. (b) Stern–Volmer plots of the fluorescence ratio F_o/F for norfloxacin as a function of the polynucleotide concentration. [norfloxacin] = 1 μM. Excitation and emission wavelength is 323 nm and 415 nm. Slit width: 4/7 nm.

lower than for poly[d(G–C)$_2$]. DNA exhibits an intermediate value of 2.8×10^3 M^{-1}. This results demonstrates that norfloxacin has a strong affinity to poly[d(G–C)$_2$] and poly(dG)·poly(dC).

The fluorescence emission spectrum of the free norfloxacin in the norfloxacin–DNA mixture, which is estimated from the equilibrium constant, is subtracted from that of the mixture in order to obtain the pure emission pattern of the DNA-bound norfloxacin. The resulting emission spectrum of the pure DNA-bound norfloxacin is depicted in Fig. 2a. Although the full width at half maximum of the DNA-bound norfloxacin is similar to that of DNA-free norfloxacin, the emission maximum is red-shifted by approx. 20 nm, indicating that the environment of norfloxacin in an aqueous solution and in DNA is quite different. The origin of the observed red-shift is not clear at this stage, but we believe that it is related to the different protonation state of the DNA-bound norfloxacin since a large red-shift in the fluorescence emission spectrum was observed at low pH (data not shown). The fluorescence emission spectrum of the poly[d(G–C)$_2$]-bound norfloxacin vanishes as the concentration of poly[d(G–C)$_2$] increases, indicating that the fluorescence quantum yield of the poly[d(G–C)$_2$]-bound species is extremely small. This observation indicates that the fluorescence intensity of norfloxacin bound to the AT rich region of DNA does not vanish although it is significantly small compared to polynucleotide-free norfloxacin. The small emission seen in the norfloxacin–DNA complex probably originates from norfloxacin which is bound to the AT rich region.

3.2. Association of norfloxacin with mononucleotide

The observed sequence-dependent equilibrium constant may be due to the different association properties of norfloxacin with individual bases — namely adenine, guanine, cytosine and thymine. Associations of norfloxacin with various mononucleotides have been tested. Fig. 3a shows the changes in the absorption spectrum of norfloxacin in the presence of AMP as an example; the absorbance in the norfloxacin absorption region (> 300 nm) decreased gradually upon adding AMP. Two isosbestic points, at 310 nm and 343 nm, were apparent. Based on the observed isos-

bestic points and the assumption that at a relatively low concentration of norfloxacin and nucleotides the formation of higher order complexes is unlikely, it can be concluded that norfloxacin forms a 1:1 homogeneous ground state complex with AMP. The typical Benesi–Hildebrand plot for AMP was constructed from the changes in absorbance at 324 nm (insertion in Fig. 3a). The association constant, K_{BH}, for the AMP was obtained from the intercept/slope ratio according to Eq. (3), which reveals to be 227 M^{-1}. A similar change was observed for the GMP with a K_{BH} of 146 M^{-1}. For pyrimidine derivatives CMP and TMP, K_{BH} were 52 M^{-1} and 50 M^{-1}. The fluorescence intensity ratio, F_o/F, increased with increasing concentrations of nucleotides (Fig. 3b). There is a clear difference between purines and pyrimidines; the purines, AMP and GMP, are efficient quenchers, while the pyrimidines, CMP and TMP, are not (Fig. 3b and Table 1).

Table 1
Binding constants of norfloxacin to mononucleotides obtained from the Benesi–Hidebrand plot and the Stern–Volmer plot

	K_{BH} (M^{-1})	K_{SV} (M^{-1})
AMP	227	75
GMP	146	90
CMP	52	7
TMP	50	11

3.3. Salt dependence of a norfloxacin–polynucleotide complex formation

When the log of equilibrium constant at various sodium concentration is plotted with respect to the log of sodium concentration (log K vs. log [Na^+]), the slope of resulting plot is 1 and 2 for the mono- and divalent cation ethidium and propidium. These results are understood to be the 1 and 2 Na^+ ions released when these drugs bind to DNA [20]. The slope of the log K vs. log [Na^+] plot for norfloxacin complexation with DNA is 1.2, and 1.3 for poly[d(G–C)$_2$] and poly(dG)·poly(dC) (Fig. 4). Considering that 0.2–0.3 sodium ions should be released in the intercalation conformational change [21], norfloxacin may possess one positive charge upon complexation with DNA, poly[d(G–C)$_2$] and poly(dG)·poly(dC).

3.4. Absorption, circular dichroism and linear dichroism

Only the conformations of norfloxacin complexed with DNA, poly[d(G–C)$_2$] and poly(dG)·poly(dC) were investigated by CD and LD, because the spectroscopic changes in norfloxacin upon binding to poly[d(A–T)$_2$] and poly(dA)·poly(dT) were too small to be measured. The absorption spectra of norfloxacin bound to poly[d(G–C)$_2$] is depicted in Fig. 5 as an example. The shape of the absorption spectra of the norfloxacin–DNA, norfloxacin–poly[d(G–C)$_2$] and norfloxacin–poly(dG)·poly(dC) complexes are similar, suggesting that the norfloxacin conformations in these polynucleotides are similar. Binding of a drug to DNA generally produces red-shift and hypochromism in the drug absorption region. In the norfloxacin case, a hypochromism in the 310–340-nm region (drug absorption region) is apparent upon binding to these polynucleotides, indicating that norfloxacin interacts directly with DNA, poly[d(G–C)$_2$] and poly(dG)·poly(dC) without ATP or Mg^{2+} mediation. Two absorption peaks, around 323 nm and 338 nm of the free norfloxacin, are somewhat smeared.

The ICD spectra of norfloxacin complexed with DNA, poly(dG)·poly(dC) and poly[d(G–C)$_2$] are depicted in Fig. 6a–c. In Fig. 6, the CD spectrum of polynucleotide is subtracted from the CD of the corresponding mixture for easy comparison. The nature of the ICD spectra of norfloxacin in all the complexes is similar, consisting a weak positive CD band in the 300–350-nm region, a strong positive band in the 270–300-nm region and a negative band in the 240–260-nm region. Observed isosbestic points (around 245 nm and 262 nm for the DNA mixture, 277 nm for the poly(dG)·poly(dC) mixture and 258 nm and 268 nm for the poly[d(G–C)$_2$] mixture) indicate that the conformation of the bound norfloxacin is homogeneous in the corresponding polynucleotide. The CD intensities are not directly proportional to the norfloxacin concentration in the mixture because the binding constants are small.

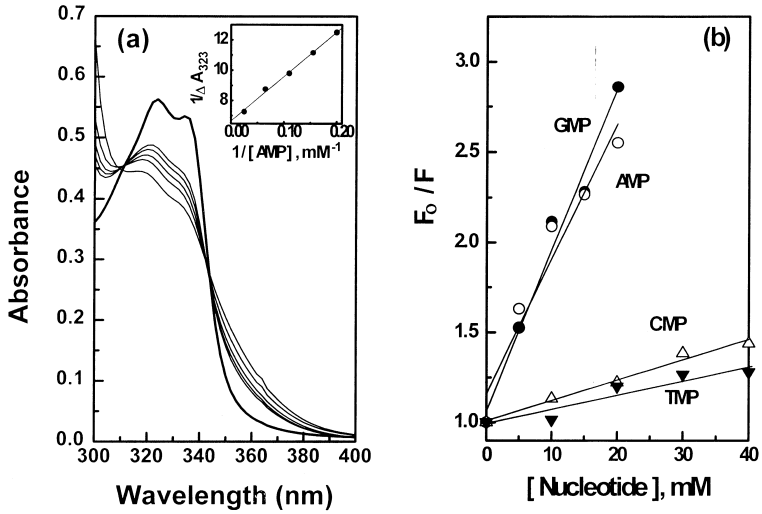

Fig. 3. (a) Absorption spectra of norfloxacin in the presence of various concentrations of AMP. From top at 330 nm: [AMP] = 0.0, 5.0, 6.5, 9.1, 15.4 and 40.0 mM. A typical Benesi–Hildebrand plot for the norfloxacin–AMP complex formation is inserted. Changes in the absorbance were measured at 324 nm for the plot. (b) Increases in the fluorescence intensity ratio, F_o/F, with increasing concentrations of nucleotides. Excitation and emission wavelengths were 323 and 415 nm.

The LD^r spectra of norfloxacin complexed with DNA, poly(dG)·poly(dC) and poly[d(G–C)$_2$] are determined from the measured LD divided by the absorption spectra. Since the equilibrium constants for the norfloxacin–polynucleotide complex formation were small, the absorption spectrum of the drug–polynucleotide mixtures may be contaminated by that of the unbound norfloxacin. Therefore the absorption spectrum of the free norfloxacin was subtracted from that of the mixture in order to determine the absorption spectrum of a pure norfloxacin–polynucleotide com-

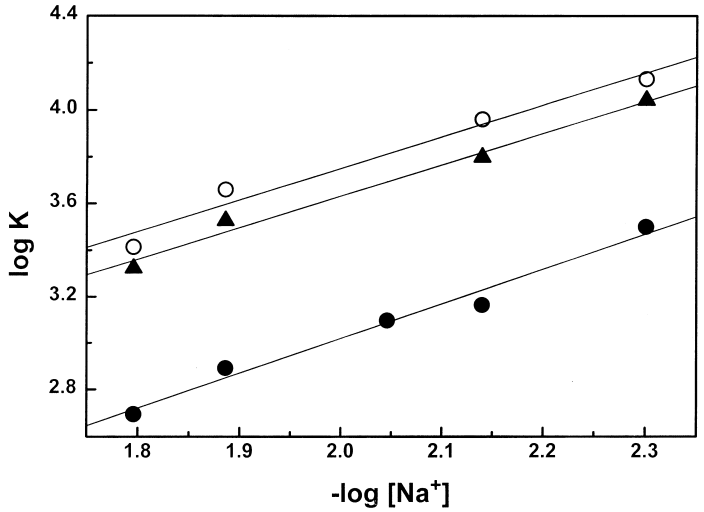

Fig. 4. Salt concentration dependence of the equilibrium constant for the norfloxacin–polynucleotide complex formation. The equilibrium constants were measured using the Stern–Volmer method — open circle: poly(dG)·poly(dC); triangle: poly[d(G–C)$_2$]; closed circle: DNA.

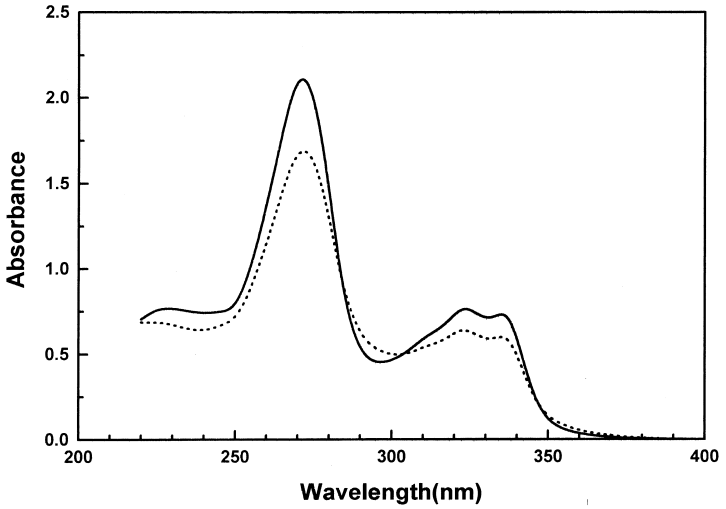

Fig. 5. Absorption spectra of norfloxacin in the absence (thick solid curve) and presence (dotted curve) of poly[d(G–C)$_2$]. The concentration of poly[d(G–C)$_2$] was 200 μM. Absorption spectrum of poly[d(G–C)$_2$] was subtracted from that of the mixtures for easy comparison.

plex. The LD^r spectra of the complexes were negative outside the DNA absorption region of the spectrum (Fig. 7); norfloxacin therefore cannot be inserted in the (minor) groove. The LD^r varied over the drug absorption region (300–350 nm), indicating that the angles between the transition moments of the drug and the polynucleotide helix axis are not a constant. If the orientation factor in Eq. (5) is calculated from the LD^r value of the polynucleotide at 260 nm, assuming that the angle of the bases is 86° relative to the helix axis [16], then the angle α between the drug transition moment and the helix axis can be calculated. The fluorescence anisotropy and the LD^r in the stretched polyvinyl alcohol film measurements for the polynucleotide-free norfloxacin indicated that two transition moments are responsible for the absorption in the 300–360-nm region; one lies along the nitrogen atom of the piperazine ring and the carbon atom of the carboxylic acids (transition I), which absorbs the light at 352 nm, and the other (transition II), which is 40–45° away towards the ethyl group, absorbs the light at 314 nm (to be published elsewhere). The angle of transition I for the norfloxacin–DNA complex is calculated at 75.0° relative to the DNA helix axis. The upper and lower limit of the angle between the transition I and the DNA helix axis, which were determined from the standard deviation of the slope of the Stern–Volmer plot, were 78° and 73°. The LD^r magnitude for transition II for this complex is larger than that at 260 nm, indicating that the transition II is a little tilted while the transition I is parallel to plane of the nucleo-bases (Fig. 7a). These angles of norfloxacin complexed with poly[d(G–C)$_2$] are 63.0 ~ 69.0° and 64.0 ~ 70.0° for the transitions I and II, with respect to the polynucleotide helix axis. The angle between the transition II of the norfloxacin molecule and the polynucleotide helix axis is 65.0 ~ 70.0° for the norfloxacin–poly(dG)·poly(dC) complex while the LD^r magnitude at 352 nm is larger than that at 260 nm. The larger LD^r magnitude in the drug absorption region compared to that in the polynucleotide absorption region is often observed for the intercalators. It must be noted that the absorption, as well as the LD magnitude, especially at 352 nm is small, and therefore some error in the angle calculation may exist in addition to stochastic error from the slope calculation. However, it is clear that the possibility of a groove binding mode for norfloxacin can be ruled out

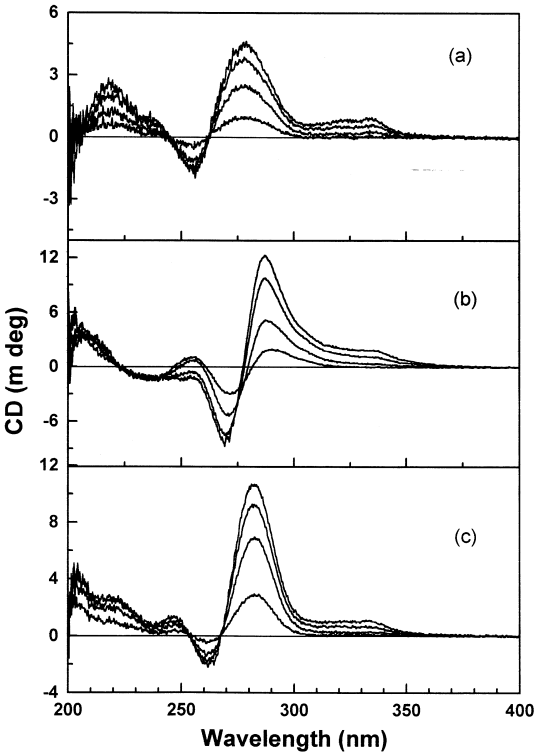

Fig. 6. ICD spectra of norfloxacin in the presence of (a) DNA, (b) poly(dG)·poly(dC) and (c) poly[d(G–C)$_2$]. Corresponding CD spectrum of the polynucleotide was subtracted. [Polynucleotide] = 50 μM. [Norfloxacin] = 3.5, 13.8 26.7 and 38.9 μM from the bottom at 324 nm.

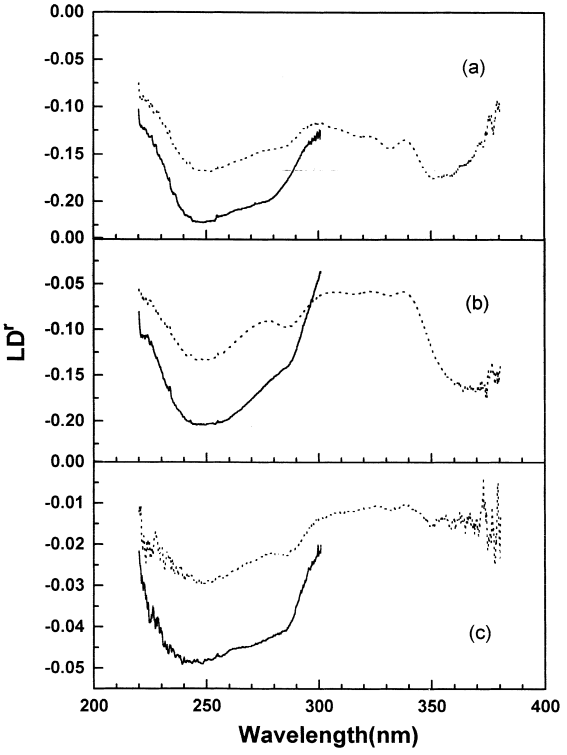

Fig. 7. LD^r spectrum for the (a) norfloxacin–DNA, (b) norfloxacin–poly(dG)·poly(dC) and (c) norfloxacin–poly[d(G–C)$_2$] complexes, obtained from the ratio of measured LD and absorption spectrum according to Eq. (4). Absorption spectra of the pure norfloxacin–polynucleotide complex for this calculation were corrected by equilibrium constant (see text). Solid curves denote LD^r of norfloxacin-free polynucleotide and dotted curve the norfloxacin–polynucleotide complexes. [Polynucleotide] = 200 μM, [norfloxacin] = 55 μM.

from the calculated angles of 65.0–90° between the transition moments and the polynucleotide helix axis for all complexes.

4. Discussion

4.1. Fluorescence quenching of norfloxacin by polynucleotide

The fluorescence intensity of norfloxacin is quenched by adding a polynucleotide (Fig. 2). Quenching of the fluorescence of any fluorophore may be explained by two process — the dynamic and static quenching mechanisms [12]. In the former process, energy of the excited fluorophore transfers to the quencher when they collide; in the latter mechanism, the fluorescence is quenched through the formation of a non-fluorescent ground state complex. The dynamic quenching process is characterized by: (1) a shortening of the fluorescence lifetime through an increase in the quencher concentration (collision occurs during the time the fluorophore is excited); (2) enhancement of the quenching efficiency through a temperature increase (molecules move faster at higher temperatures, resulting in increases in the number of collisions per time unit); and (3) the absorption or fluorescence excitation spectrum of the fluorophore in the presence and absence of quencher is the same because only the excited state fluorophore is responsible for the quenching process. In contrast, in the static

quenching process: (1) the fluorescence lifetime does not vary (only the ground state fluorophore forms a non-fluorescent complex with the quencher); (2) a temperature increase results in enhancement of the quenching efficiency (generally, a ground state complex formation is exothermic, and therefore the complex is more stable at lower temperature); and (3) changes in the absorption and fluorescence excitation are observed. The Stern–Volmer quenching constant in Eq. (1), therefore, denotes the product of the fluorescence lifetime and collision rate in the dynamic process and the equilibrium constant in the static process, when the fluorescence intensity of quenched fluorophore is negligible.

In the norfloxacin case, enhancement in the quenching efficiency upon a temperature increase and changes in the norfloxacin absorption spectrum in the presence of a polynucleotide are observed, indicating that the fluorescence of norfloxacin is quenched by forming a non-fluorescent ground state complex between the norfloxacin and polynucleotide. The Stern–Volmer constant — the equilibrium constant for the norfloxacin–polynucleotide complex formation — is highest when poly(dG)·poly(dC) and poly[d(G–C)$_2$] are used as quencher, indicating that complex formation with these polynucleotides is the most efficient. The fluorescence of the norfloxacin in the presence of a high concentration of poly(dG)·poly(dC) and poly[d(G–C)$_2$] vanishes because the fluorescence of the complex is extremely small. In contrast, the fluorescence of the norfloxacin–DNA mixture decreases to a constant intensity ($\sim 7\%$ of free norfloxacin), indicating that the norfloxacin binding site is not only at the two consecutive GC base pairs. Our finding that norfloxacin can form a complex most efficiently with double-stranded poly(dG)·poly(dC) and poly[d(G–C)$_2$] contrasts with a report by Shen et al. in which the binding of norfloxacin to double-stranded DNA was weak and exhibited no base preference [6,7]. Our results also differ from those of Palù et al. [11] in which they proposed an Mg^{2+} mediated norfloxacin binding. We presently have no explanation for this discrepancy.

The binding of norfloxacin to thermal denatured DNA and single-stranded synthetic polynucleotides has been previously studied [3]. The amount of bound norfloxacin was reported to be increased more than 10 times (compared to double-stranded DNA) when the DNA is 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 were further stabilized by norfloxacin-norfloxacin stacking [7]. Our results indicate that the equilibrium constant for the norfloxacin–double-stranded DNA complex formation is smaller only by a factor of two, compared to that with single-stranded DNA. The norfloxacin may be stabilized at the binding site by 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 (see below).

4.2. Mechanism of quenching of norfloxacin by 2′-deoxynucleotides

Norfloxacin forms weak ground-state complexes with mono-nucleotides. To clarify the interaction mechanism of norfloxacin with polynucleotide, we examined the formation of ground state complexes formed between norfloxacin and various 2′-deoxynucleotides and the efficiency of the static mechanism in quenching norfloxacin. The K_{BH} values were larger for the purine than for the pyrimidine derivatives. This suggests that the dispersion force and polarizability effects (which are greater for purines) play an important role in the association of norfloxacin with DNA bases. In the case of GMP, the values of the ground-state association constant, K_{BH}, and the Stern–Volmer quenching constant, K_{SV}, were similar (Table 1), as expected when the fluorescence of the complexes is strongly quenched. For AMP, $K_{BH} > K_{SV}$, which indicates that the photoexcited singlet norfloxacin–AMP complex is at least partially emitting. This is consistent with the fluorescence emission of the norfloxacin–poly-

nucleotide complex being negligible, but that complexed with DNA, in which norfloxacin binds not only to the GC base pairs, exhibiting a characteristic emission spectrum. Overall, the nucleotides' ability to form a complex with norfloxacin was not directly related to the observed quenching efficiency of the corresponding polynucleotide, indicating that other factors, such as the stacking interaction, electrostatic force and the conformation of the polynucleotide may be involved.

4.3. Conformation of norfloxacin in polynucleotide

DNA can provide three distinctive binding sites for norfloxacin namely, groove binding, binding to phosphate group and intercalation. If norfloxacin is located along either the minor or major groove, a positive LD^r, corresponding to an angle of 45° between the norfloxacin transition dipole moments and the DNA helix axis, is expected. The angle of 45° (indicating a wavelength-independent positive LD^r in the drug absorption region) between the transition moment of the drugs and the DNA helix axis for the minor groove binding drugs, 4′,6-diamidino-2-phenylindole and Hoechst 33258, has recently been reported [22–24]. When norfloxacin is bound to the phosphate group of the DNA stem, it is free to rotate, resulting in a random orientation; therefore, a zero LD^r in the 300–360-nm region is expected. These possibilities can be eliminated based on the observed LD^r for all the complexes, which indicate the transitions I and II is near perpendicular to the helix axis (Fig. 7). Since the association of norfloxacin with single-stranded DNA is more efficient than with double-stranded DNA, it is possible that the norfloxacin is concentrated at the denatured part of the duplex. This mode somewhat agrees with that of Shen et al. [7]. But this is not likely for double helical DNA, because in that case the norfloxacin would not be oriented (template single-stranded DNA is not oriented in our system) and the LD^r in the norfloxacin absorption region would be zero. The remaining possible binding mode is intercalation. However, deviations in the LD^r spectrum from classic intercalation should be noted. Since the intercalation of a drug between the nucleo-bases results stiffening and elongation of DNA, hence, resulting in increased orientability, the LD^r magnitude in the DNA absorption region is expected to be increased. Contrarily, what we observed is decreases in the LD^r magnitude upon norfloxacin binding, implying the orientability of DNA and polynucleotide is decreased. Therefore it is conceivable that the DNA and polynucleotide is bent and/or tilt near drug binding site with partial insertion of norfloxacin molecule between base pairs. ICD is sensitive to the conformation of the polynucleotide bound drug and the nature of the nucleo-bases [18,19]. ICD spectra represented in Fig. 6 are similar each other disregarding the nature of the polynucleotide, indicating the conformation of norfloxacin in the complexes are similar. ICD of the norfloxacin–DNA complex at the low mixing ratio is essentially the same as that of the norfloxacin–poly[d(G–C)$_2$] complex.

At the binding site, norfloxacin may possess one positive charge because the slope of the log K vs. log [Na^+] plot for norfloxacin complexation with DNA is 1.2, and 1.3 for poly[d(G–C)$_2$] and poly(dG)·poly(dC) (Fig. 4). Considering that 0.2–0.3 sodium ions are released due to the intercalation conformational change, 1.0 and 1.1 Na^+ ion are released, respectively, from the DNA, and poly[d(G–C)$_2$] and poly(dG)·poly(dC). These results agree with the finding that one Na^+ ion is released when a monovalent drug intercalates to DNA [20]. Therefore norfloxacin may possess one positive charge at the binding site in DNA, poly[d(G–C)$_2$] and poly(dG)·poly(dC).

5. Conclusion

Our conclusions from this study are:

1. norfloxacin can form a complex with a double-stranded polynucleotide without ATP or Mg^{2+} mediation;
2. the association of norfloxacin is efficient for poly[d(G–C)$_2$], poly(dG)·poly(dC) and single-stranded DNA, suggesting that norfloxacin preferentially binds to GC rich region or single-stranded part of duplex DNA;
3. when bound to double-stranded DNA, the

possibility of groove binding and surface binding mode of norfloxacin may be ruled out and the molecular plane is closer to perpendicular than parallel relative to the DNA helix axis; and

4. bound norfloxacin may be single protonated.

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