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Characterization of ciprofloxacin binding to the linear single- and double-stranded DNA

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Abstract

The binding of ciprofloxacin to natural and synthetic polymeric DNAs was investigated at different solvent conditions using a combination of spectroscopic and hydrodynamic techniques. In 10 mM cacodylate buffer (pH 7.0) containing 108.6 mM Na^+, no sequence preferences in the interaction of ciprofloxacin with DNA was detected, while in 2 mM cacodylate buffer (pH 7.0) containing only 1.7 mM Na^+, a significant binding of ciprofloxacin to natural and synthetic linear double-stranded DNA was observed. At low ionic strength of solution, ciprofloxacin binding to DNA duplex containing alternating AT base pairs is accompanied by the largest enhancement in thermal stability (e.g. $\Delta T_m \approx 10$ °C for poly[d(AT)]·poly[d(AT)]), and the most pronounced red shift in the position of the maximum of the fluorescence emission spectrum (λ_{max}). Similar red shift in the position of λ_{max} is also observed for ciprofloxacin binding to dodecameric duplex containing five successive alternating AT base pairs in the row. On the other hand, ciprofloxacin binding to poly[d(GC)]·poly[d(GC)], calf thymus DNA and dodecameric duplex containing a mixed sequence is accompanied by the largest fluorescence intensity quenching. Addition of NaCl does not completely displace ciprofloxacin bound to DNA, indicating the binding is not entirely electrostatic in origin. The intrinsic viscosity data suggest some degree of ciprofloxacin intercalation into duplex.

Keywords: Fluoroquinolone; DNA-binding; Mixed mode of binding; Sequence selectivity; Spectroscopic and hydrodynamic technique

1. Introduction

Ciprofloxacin (Fig. 1) belongs to the family of fluoroquinolone antibacterial agents that also include enoxacin, norfloxacin, ofloxacin and some other molecules. These fluoroquinolone antibacterial agents are synthetic derivatives of 6-fluoro-4-oxo-quinoline-3-carboxylic acid. They are fluorinated at position 6 and mostly bear a piperazinyl moiety at position 7. Ciprofloxacin is one of the most potent quinolone derivatives in clinical use with a very broad spectrum of antibacterial activity and is often used as an antibacterial agent of last resort [1–4].

Binding of fluoroquinolones to DNA is relatively weak, thus it is unlikely that their binding to DNA triggers the formation of gyrase–DNA complex [5–8]. Likewise, binding of fluoroquinolone to gyrase is weak, even though the presence of mutated gyrase alleles in resistant bacteria clearly implicate gyrase in the interactions [9–13]. Numerous studies have shown that drug binding to DNA [14–16] and gyrase [17] is enhanced in the presence of Mg^{2+} ions and that Mg^{2+} is essential for antibacterial efficiency of drug–DNA interaction. Fluoroquinolone binding to the gyrase–DNA complex may prevent the religation step [14,18–21]. The mechanism of fluoroquinolone's inhibition of religation and the role of DNA in drug binding remains to be resolved. Understanding the interactions between fluoroquinolone and DNA may help to elucidate the mechanism of action of this important class of antibacterial agents, and may ultimately lead to the design of better, more potent antibacterial agents with less side effects.

To investigate the type of DNA–fluoroquinolone interactions in the absence of Mg^{2+} ions, several aspects of DNA

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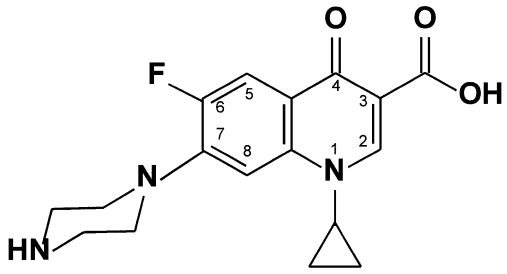

Fig. 1. Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinolinecarboxylic acid.

binding were examined. To this aim, we have investigated the binding of ciprofloxacin to a series of natural and synthetic polynucleotides at different solution conditions using a combination of spectroscopic (fluorescence and UV-spectroscopy) and hydrodynamic techniques (viscometry). Our results show that ciprofloxacin's apparent mode of binding, structure and sequence preferences significantly depend on solution conditions.

2. Materials and methods

2.1. Materials

Ciprofloxacin (1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline carboxylic acid) (Fig. 1) was purchased from Sigma Chemical, Ltd. (St. Louis, USA) and used without further purification. The drug was dried overnight at 130 °C [22]. Ten milligrams of ciprofloxacin was precisely weighted on high precision balance (Sartorius Analytic A 210P, Sartorius GmbH, Germany), dissolved in dimethylsulfoxide (DMSO) and diluted with triply distilled water to reduce the concentration of DMSO to 2% (v/v). The molar extinction coefficient of ciprofloxacin was determined spectrophotometrically at 275 nm. From the slope of the line, A_{275} vs. concentration (Beer's law), the molar extinction coefficient of ciprofloxacin at 275 nm, ε_{275}, was determined to be 35,900±500 M^{-1} cm^{-1} in 2% (v/v) DMSO solution at 25 °C.

Netropsin–HCl (Net) from Boehringer Mannheim GmbH (Germany) and ethidium bromide (EtBr) from Sigma were used without further purification. The concentration of the netropsin and ethidium bromide in solution was determined spectrophotometrically using extinction coefficients of ε_{296} (25 °C)=21,500 M^{-1} cm^{-1} and ε_{480} (25 °C)=5600 M^{-1} cm^{-1}, respectively.

2.1.1. Polynucleotides

Natural genomic DNA (calf thymus DNA) and three synthetic DNA polymers, poly[d(AT)]·poly[d(AT)], poly[d(A)]·poly[d(T)] and poly[d(GC)]·poly[d(GC)], were purchased from Pharmacia Biotech (Uppsala, Sweden). These polymers were of the highest grade commercially available. Before use they were thoroughly dialysed against corresponding buffer solution. Thermally denatured calf thymus DNA was prepared by heating the sample up to 95 °C and cooling it down to the room temperature. The concentrations of double-stranded polynucleotides were determined spectrophotometrically at 25 °C using the following molar extinction coefficients expressed in molar concentration of base pairs: poly[d(AT)]·poly[d(AT)], ε_{260}=13,300 M^{-1} cm^{-1}; poly[d(A)]·poly[d(T)], ε_{260}=12,000 M^{-1} cm^{-1}; poly[d(GC)]·poly[d(GC)], ε_{254}=16,800 M^{-1} cm^{-1}; calf thymus DNA, ε_{259}=12,800 M^{-1} cm^{-1}. These values were either provided by the manufacturer or taken from the literature [23]. For the fluorescence titration experiments the polymeric DNA concentration was ~500 μM (stock) in base pairs, while for UV-spectroscopic experiments the DNA concentration was 15 μM in base pairs. For viscometry the concentration of poly[d(AT)]·poly[d(AT)] and sonicated calf thymus DNA was 200 μM in base pairs. Unless otherwise stated, the buffer solution (pH 7.0) used in our experiments with polynucleotides consisted of 2 or 10 mM cacodylate containing 1.7 or 108.6 mM Na^+ and 0.1 mM Na_2EDTA.

2.1.2. Oligonucleotides

DNA dodecameric DNA duplexes were prepared by mixing the corresponding single strands synthesized using the standard cyanoethylphosphoramide chemistry [24]. The molar extinction coefficients at 260 nm for single-stranded oligomers, ε_{260}, were determined by phosphate analysis using enzyme degradation and colorimetric detection of free phosphate [25]. The following dodecameric oligonucleotides with the corresponding molar extinction coefficients expressed in molar concentration of single strand at 25 °C were used:

G1 5′-GTTAGTACTTGG-3′, ε_{260}=107,000 M^{-1} cm^{-1};
C1 5′-CCAAGTACTAAC-3′, ε_{260}=101,100 M^{-1} cm^{-1};
G2 5′-GTTAGTATATGG-3′, ε_{260}=106,900 M^{-1} cm^{-1};
C2 5′-CCATATACTAAC-3′, ε_{260}=101,700 M^{-1} cm^{-1}.

For all fluorescence measurements the oligomeric DNA concentration was between 0 and 500 μM (per single strand). Unless otherwise stated, the buffer solution (pH 7.0) used in our experiments with oligonucleotides consisted of 10 mM cacodylate containing 28.6 mM Na^+ and 0.1 mM Na_2EDTA.

2.2. Fluorescence measurements

Intrinsic fluorescence emission spectra of ciprofloxacin (titrated by HCl, NaOH, NaCl, or polymeric or oligomeric single- or double-stranded DNA) or at different ratio of ciprofloxacin to DNA (R) were performed at 20 °C either in a Perkin-Elmer Model LS-50 Luminescence spectrometer or in a Jasco FP-750 Fluorimeter equipped with a water thermostated cell holder using 1-cm path length quartz cuvette. The excitation wavelength used was 330 nm and

the emission spectra were recorded in the range from 350 to 625 nm. Fluorescence titrations profiles were measured by incrementally adding aliquots of reagent (HCl, NaOH, NaCl or corresponding DNA) in a cuvette containing a known and always constant concentration of ciprofloxacin (1 mM). The emission spectra of ciprofloxacin, corrected for the solvent blank, were multiplied for dilution factor and corrected for PM-tube response using a fluorescence spectrum of quinine sulfate (c=2.5×10^{-7} M) in 0.1 M perchloric acid as a standard.

2.3. Determination of the equilibrium constants by Stern–Volmer method

Son et al. [7,8] used the Stern–Volmer method [26] to estimate the equilibrium constants of norfloxacin binding to various synthetic polynucleotides. Norfloxacin, in comparison to ciprofloxacin, contains an ethyl- instead of cyclopropyl—group attached to N1 nitrogen atom of quinolone ring system (Fig. 1). In order to make the results comparable, we used the same procedure to estimate the equilibrium constants of ciprofloxacin binding to various synthetic polynucleotides.

2.4. UV spectrophotometry

UV-absorbance measurements were conducted using a Cary 1 UV–VIS spectrophotometer (Varian, Australia) and a matched set of 1-cm path length quartz cuvettes. The spectrophotometer was equipped with a thermoelectrically controlled cell holder. Absorbance versus temperature profiles (UV-melting curves) were measured at 260 nm. The heating rate was 1.0 °C min^{-1}. For each optically detected transition, the melting temperature (T_m) of DNA was determined as the transition midpoint. Melting experiments of polymeric DNA at different ratios of drug to DNA (R from 0 to 1) were performed at the same buffer conditions as described above. To correct for the contribution of ciprofloxacin to the absorbance spectrum of DNA, the reference cuvette was filled with the solution of ciprofloxacin at the same concentration and buffer as in the sample cuvette.

2.5. Viscometry

Viscosity measurements were performed using an Ubbelodhe Micro-Viscometers (Schott Glaswerke, Mainz, Germany) submerged in a water bath maintained at 20.0 (±0.1) °C. Flow times were measured with a stopwatch to an accuracy of ±0.2 s. Viscosity studies with sonicated calf thymus DNA [8] and poly[d(AT)]·poly[d(AT)] were conducted in 2 mM cacodylate buffer (pH 7.0). Aliquots of 1 mM Net or EtBr were titrated into viscometer containing 2.5 ml of 200 μM in base pair polynucleotide solution, and flow times in the range of 100–140 s were measured after each addition. Due to the low solubility of ciprofloxacin, the direct titration of polynucleotides by drug was not possible. A series of solutions at different ligand to DNA ratio (R=0 to 0.2) with a constant concentration of DNA (200 μM per base pair) were prepared. The viscosity of each sample was measured independently. The relative viscosity, η_{rel}, of the DNA solution was calculated as $\eta_{rel}=\eta/\eta_o=\rho t/\rho_o t_o$, where t and t_o are the flow times for the DNA or drug–DNA and solvent solution, respectively, ρ and ρ_o are densities of the DNA or drug–DNA and solvent, respectively. The specific viscosity was calculated as $\eta_{sp}=\eta_{rel}-1$. Reduced viscosities (η_{sp}/C_N) were evaluated at one concentration only (for each value of R), and intrinsic viscosities, [η], were calculated using a value of 0.53 for Huggins' coefficient k in Eq. (1) [27]:

$$\frac{\eta_{sp}}{C_N} = [\eta](1 + k[\eta]C_N). \quad (1)$$

The concentration C_N refers to the mole of nucleotide per liter, irrespective of the amount of drug bound. If DNA is approximated with a rodlike molecule and assuming negligible changes in its axial ratio upon ligand binding [27], the ratio of the intrinsic viscosities ([η]/[η]$_o$) depends on the relative ratio of the contour lengths (L/L_o) and is given by the expression [27–29]:

$$\frac{L}{L_o} \approx \sqrt[3]{\frac{[\eta]}{[\eta]_o}} \quad (2)$$

where L_o and [η]$_o$ denote the apparent molecular length and intrinsic viscosity of DNA in the absence of ligand.

2.6. High precision densimetry

The densities of all samples used for viscometry were measured at 20 °C with a precision of ±1.5×10^{-6} g cm^{-3} using a vibrating tube densimeter (DMA-60/602, Anton Paar, Austria).

2.7. pH measurements

The pH values of all solutions were measured separately for each experiment using Iskra model MA 5740 pH-meter (Slovenia) and Ag/AgCl combination microelectrode (Mettler Toledo, Switzerland). Absolute error in our pH measurements was ±0.01 pH unit.

3. Results and discussion

3.1. Intrinsic fluorescence properties of ciprofloxacin

Fig. 2 shows the fluorescence emission spectrum of ciprofloxacin at pH 7.0; pH-dependent changes in the fluorescence intensity of ciprofloxacin at 413 nm between pH 1 and 13, with the maximal intensity observed at pH 7.5 (Fig. 2—inset). It has been reported that ciprofloxacin exists as a cation below pH 5, as a mixture of anions, cations and

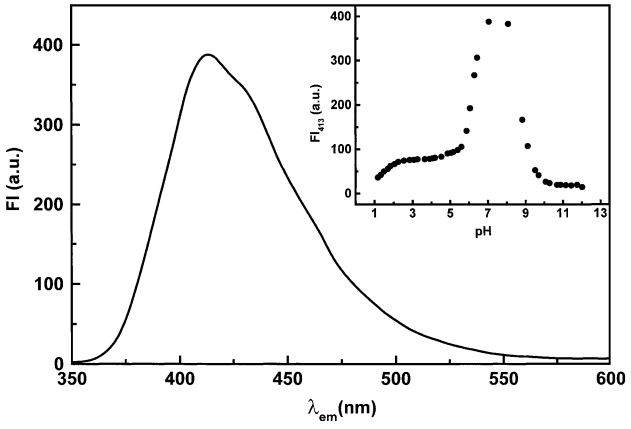

Fig. 2. The intrinsic fluorescence emission spectra of ciprofloxacin at pH 7.0. The pH dependency of ciprofloxacin single-wavelength fluorescence intensity, FI (●), at 413 nm (inset). λ_{ex}=330 nm, C_{cf}=1 μM, T=25 °C. a.u. stands for arbitrary units.

zwitterions at pH between 5 and 10, and as an anion at pH higher than 10 [30–33]. The molar fluorescence intensity and λ_{max} in the fluorescence emission spectra of ciprofloxacin do not change with its concentration in the concentration range from 0 to 1.1 μM in 2 or 10 mM cacodylate buffer (both pH 7.0). Also, no significant changes in the fluorescence emission properties of ciprofloxacin were observed with increasing NaCl concentration up to 1 M at the same solution conditions (data not shown). To summarize, under experimental solution conditions applied in fluorimetric measurements, ciprofloxacin does not self-associate or form a complex with sodium ions, and it is present in several different charged forms [30].

3.2. Fluorimetric characterization of ciprofloxacin binding to DNA at pH 7.0

Fig. 3A and B shows the fluorescence emission spectra of ciprofloxacin in the presence of different concentrations of calf thymus DNA and poly[d(AT)]·poly[d(AT)] at 20 °C in 2 mM cacodylate buffer (pH 7.0), respectively. A significant decrease in the fluorescence intensity of ciprofloxacin in the presence of calf thymus DNA has been observed with the shape and λ_{max} of the spectra remaining unaffected increasing ratio of base pairs to ligand, R^{-1}. Similar results were obtained in the presence of poly[d(GC)]·poly[d(GC)]. In contrast, as shown in Fig. 3B, a noticeable change in the shape of ciprofloxacin fluorescent spectra accompanied by a shift in the position of λ_{max} from 413 nm (R^{-1}=0) to 436 (±1) nm (R^{-1}=47.9) is observed upon titration with poly[d(AT)]·poly[d(AT)]. The appearance of an isosbestic point at 431 nm in the ciprofloxacin fluorescent spectra suggests the presence of two forms of ciprofloxacin, e.g. free and bound. Recently, it has been shown that the fluorescence emission spectrum of norfloxacin undergoes a red shift in the presence of poly[d(AT)]·poly[d(AT)], and a small decrease in its fluorescence intensity, while in the presence of poly[d(GC)]·poly[d(GC)] and calf thymus DNA a strong decrease in fluorescence intensity was observed [7,34].

Fig. 4A and B shows the fluorescence titration curves of ciprofloxacin at two different solvent conditions titrated by various DNAs at 413 nm expressed as a relative fluorescence emission intensity of ciprofloxacin $(FI/FI°)_{413}$ versus molar ratio of DNA base pairs to drug, R^{-1}, where FI° and FI stand for fluorescence emission intensity of ciprofloxacin in the absence and presence of DNA, respectively. In 2 mM

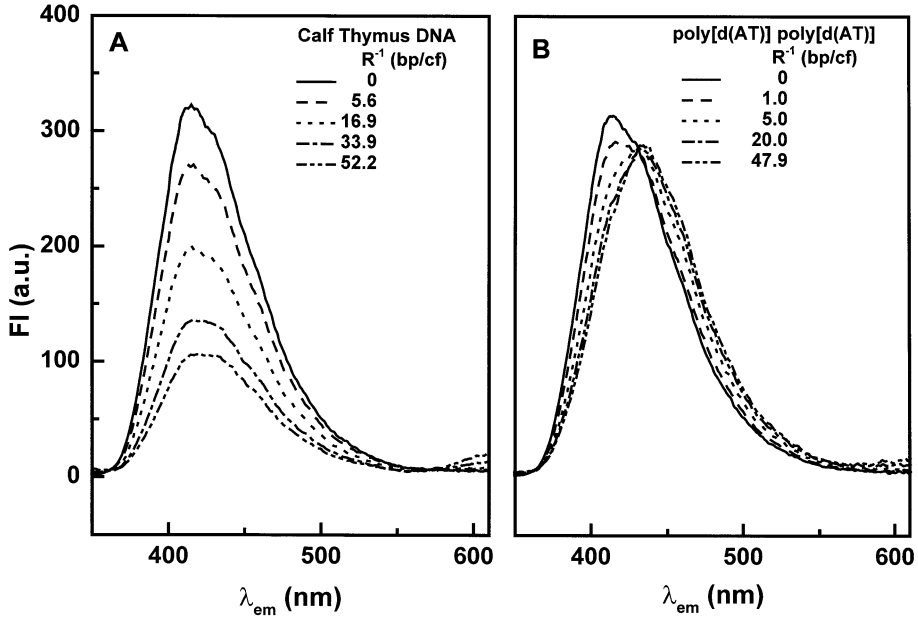

Fig. 3. Fluorescence emission spectra of ciprofloxacin in the presence of calf thymus DNA (A) and poly[d(AT)]·poly[d(AT)] (B) at different DNA base pairs to drug ratio, R^{-1}, as marked. λ_{ex}=330 nm, C_{cf}=1 μM, T=20 °C.

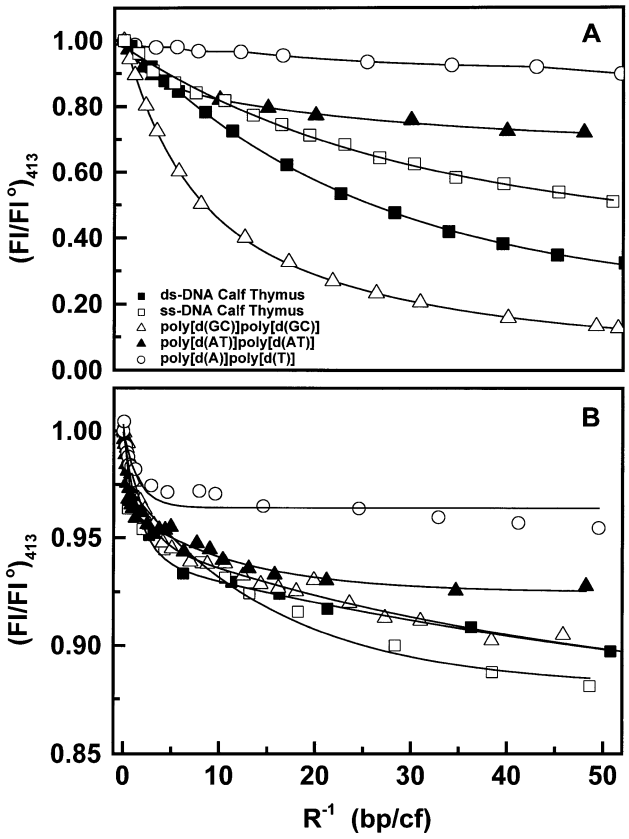

Fig. 4. Relative fluorescence emission intensity $(FI/FI^{\circ})_{413}$ of ciprofloxacin derived from multiple titration experiments at two different solution conditions: (A) 2 mM cacodylate (pH=7.0), 1.7 mM Na^+ and 0.1 mM Na_2EDTA, at 20 °C; (B) 10 mM cacodylate (pH=7.0) and 108.6 mM Na^+, at 25 °C. ds-DNA (■), thermally denatured calf thymus ss-DNA (□), poly[d(AT)]·poly[d(AT)] (▲), poly[d(GC)]·poly[d(GC)] (△) and poly[d(A)]·poly[d(T)] (○) at 413 nm. FI° and FI are fluorescence emission intensity of ciprofloxacin in the absence and presence of DNA, respectively. R^{-1} is the molar ratio of DNA (per base pairs) to drug. λ_{ex}=330 nm, C_{cf}=1 μM.

cacodylate buffer the magnitude of fluorescence quenching of double-stranded DNA follows the same order as that observed at higher ionic strength (10 mM cacodylate buffer containing 108.6 mM Na^+) with the exception of single-stranded calf thymus DNA (Fig. 4A and B): poly[d(GC)]·poly[d(GC)] > calf thymus DNA>poly[d(AT)]·poly[d(AT)]> poly[d(A)]·poly[d(T)]. Note, however, that fluorescence quenching by single-stranded calf thymus DNA (ss-DNA) is lower than by double-stranded calf thymus DNA (ds-DNA) at lower ionic strength (in 2 mM cacodylate buffer) and vice versa at higher ionic strength (10 mM cacodylate buffer containing 108.6 mM Na^+) (Fig. 4A and B).

The Stern–Volmer equilibrium constant (K_{SV}) for the formation of ciprofloxacin–polynucleotides complex can be obtained by plotting $(FI^{\circ}/FI)_{413}$ versus the concentration of polynucleotides. The K_{SV}'s of ciprofloxacin–DNA complex formation in the presence of single-stranded DNA, calf thymus DNA, and poly[d(GC)]·poly[d(GC)] were larger than those in the presence of poly[d(A)]·poly[d(T)] and poly[d(AT)]·poly[d(AT)] (Table 1). However, at the applied experimental conditions, K_{SV} values in the presence of poly[d(A)]·poly[d(T)] and poly[d(AT)]·poly[d(AT)] could have been underestimated due to the changes in fluorescence emission spectra of free versus bound ciprofloxacin. Similarly, a study of norfloxacin binding to polynucleotides yield the same order of K_{SV}'s [7,34]. The K_{SV} values for norfloxacin slightly differ from our values due to different solution conditions applied in the norfloxacin study [7,34]. The similar behaviour of ciprofloxacin and norfloxacin is not surprising since these two representatives of fluoroquinolone family differ only in the group attached to nitrogen atom of quinolone ring system.

The qualitative difference in the observed fluorescence emission spectra of ciprofloxacin in the presence of poly[d(AT)]·poly[d(AT)] compared to poly[d(GC)]·poly[d(GC)] or calf thymus DNA is likely to indicate the different way of interaction of ciprofloxacin with these polynucleotides. It is possible that ciprofloxacin fits better in the narrow groove of AT sequences, allowing for little or no rotation of the drug and thus smaller nonradiative deactivation of the excited state occurs [35]. The stronger quenching of ciprofloxacin fluorescence without significant shift in λ_{max} by GC sequences, compared to AT, could originate in guanine. The oxidation potential of guanine is the lowest in nucleobases. Also, guanine can be an effective quencher of fluorescence through electron transfer from DNA to photo-excited fluoroquinolone [26,33]. Furthermore, due to the difference in the local surface properties of the studied polynucleotides, factors such as differences in hydration, structural features [36], and surface charge density can affect ciprofloxacin binding. Surface pH may also play a role, as it has been shown that the fluorescence emission intensity of ciprofloxacin is pH-sensitive (Fig. 2—inset).

3.3. Electrostatic contribution to the binding of ciprofloxacin to polynucleotides

The quenching of the fluorescence emission intensity of ciprofloxacin was more efficient at lower salt concentra-

Table 1
Equilibrium constants for ciprofloxacin polynucleotide complex formation calculated with the Stern–Volmer method

Polynucleotide	K_{SV} (M^{-1})
2 mM Cacodylate buffer, 1.7 mM Na^+, 0.1 mM Na_2EDTA, pH 7.0	
Single-stranded calf thymus DNA	$(1.9\pm0.3)\times10^4$
Double-stranded calf thymus DNA	$(4.1\pm0.1)\times10^4$
poly[d(GC)]·poly[d(GC)]	$(1.3\pm0.1)\times10^5$
poly[d(AT)]·poly[d(AT)]	$(4.4\pm0.4)\times10^3$
poly[d(A)]·poly[d(T)]	$(1.9\pm0.4)\times10^3$
10 mM Cacodylate buffer, 108.6 mM Na^+, 0.1 mM Na_2EDTA, pH 7.0	
Single-stranded calf thymus DNA	$(5.6\pm0.5)\times10^2$
Double-stranded calf thymus DNA	$(5.7\pm0.6)\times10^2$
poly[d(GC)]·poly[d(GC)]	$(6.7\pm0.1)\times10^2$
poly[d(AT)]·poly[d(AT)]	$(3.4\pm0.4)\times10^2$
poly[d(A)]·poly[d(T)]	$(0.7\pm0.2)\times10^2$

tions, indicating that binding of ciprofloxacin is strongly salt-dependent (Fig. 4A and B). To determine the contribution of nonspecific (electrostatic) interactions to the binding, the ciprofloxacin–polynucleotides complex ($R^{-1} \cong 50$) base pairs) was back-titrated with NaCl. Fig. 5 shows the relative fluorescence emission intensities, FI/FI°, of ciprofloxacin at 413 nm and the changes in the maximum wavelength, λ_{max}, of ciprofloxacin emission spectra after titration by calf thymus DNA and poly[d(AT)]·poly[d(AT)] (A, B) and after titration of the final ciprofloxacin–duplex complex by NaCl (C, D). The fluorescence emission intensity of ciprofloxacin–duplex complexes increases with increasing NaCl concentration. At higher concentrations of NaCl (>10 mM), only about 5% of the total fluorescence signal remains quenched in the presence of calf thymus DNA, while about 25% in the presence of poly[d(AT)]·poly[d(AT)]. Since the fluorescence emission intensity of ciprofloxacin in the absence of DNA does not change significantly with increasing NaCl concentration (data not shown), it appears that at higher NaCl concentrations (>10 mM) some fraction of ciprofloxacin remains bound to all polynucleotides independent of salt concentration. This observation suggests that the electrostatic contribution to ciprofloxacin binding to DNA is significant. The majority of ciprofloxacin bound to double-stranded calf thymus DNA, poly[d(GC)]·poly[d(GC)] and poly[d(A)]·poly[d(T)] (data not shown) can be removed by increasing NaCl concentration to a value of less than 10 mM; however, the same concentration of NaCl removes

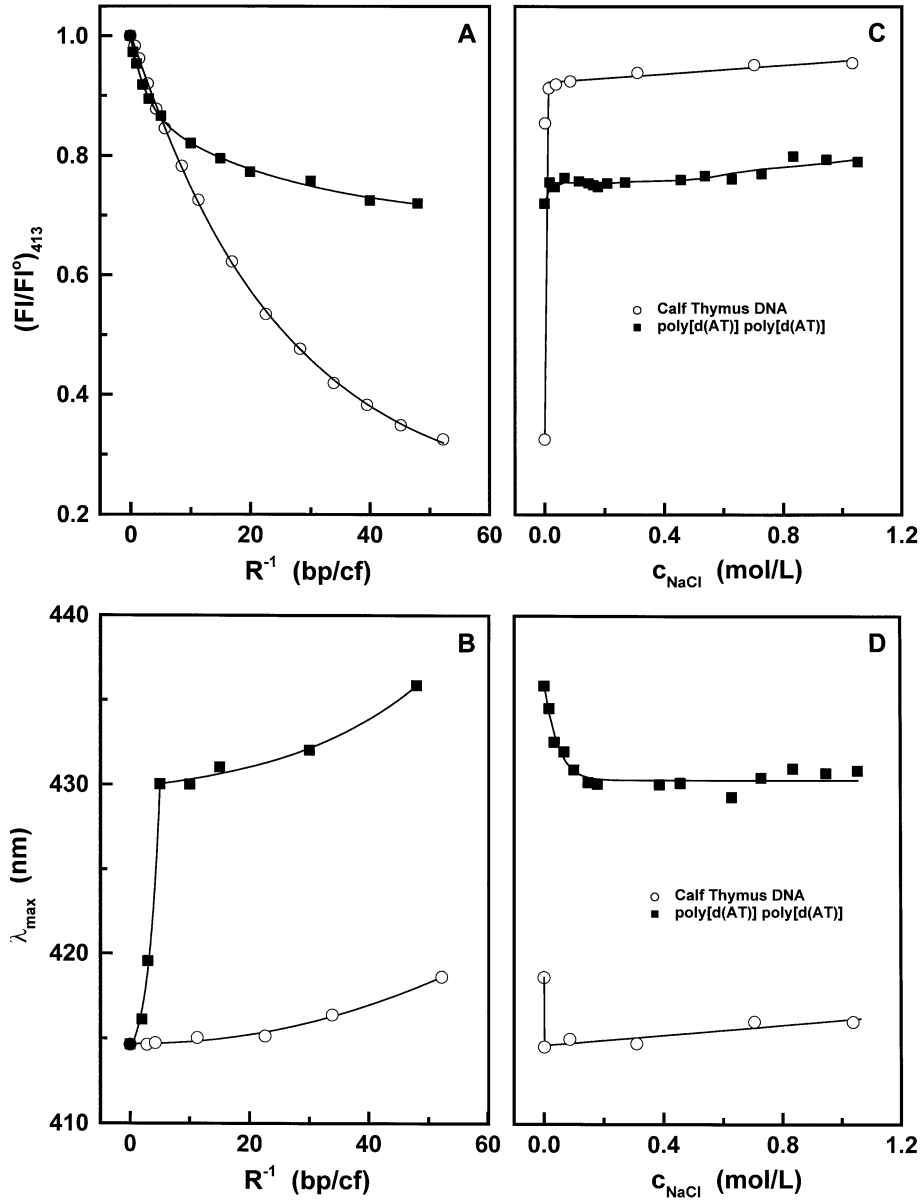

Fig. 5. The changes in the relative fluorescence emission intensity $(FI/FI°)_{413}$ and changes in maximum wavelength, λ_{max}, of the ciprofloxacin emission spectra after titration with native ds-DNA calf thymus DNA (○) (A and B), and poly[d(AT)]·poly[d(AT)] (■) and after NaCl back-titration of the final ciprofloxacin–DNA complex (C and D). Solution conditions were 2 mM cacodylate (pH=7.0), 1.7 mM Na^+ and 0.1 mM Na_2EDTA at 20 °C. C_{cf}=1 μM and λ_{ex}=330 nm.

only a small fraction of ciprofloxacin bound to poly[d(AT)]·poly[d(AT)], suggesting a qualitatively different kind of interaction of ciprofloxacin with poly[d(AT)]·poly[d(AT)] compared to the rest of the studied polynucleotides.

To summarize, these results suggest that ciprofloxacin has at least two different modes of binding to double-stranded DNA: (1) a nonspecific binding to all double-stranded DNA molecules which is electrostatically driven (e.g. ciprofloxacin interactions with negatively charged phosphate groups and electrostatic stacking interactions on the helix exterior), and (2) a specific nonelectrostatically controlled binding (e.g. intercalation, minor or major groove binding), which could depend on several different factors (e.g. DNA sequence, the geometry of minor or major groove, the extent of hydration [36], base stacking interactions, and not at least on the binding site size). From Fig. 5B the apparent stoichiometry of ciprofloxacin binding to poly[d(AT)]·poly[d(AT)] can be determined. It equals a ciprofloxacin molecule per five base pairs, while for all the other polynucleotides the stoichiometry of ciprofloxacin binding to DNA could not be accurately determined.

3.4. Ciprofloxacin binding to various single- and double-stranded oligomeric DNAs

In order to examine the origin of different behaviour of ciprofloxacin in the presence of poly[d(AT)]·poly[d(AT)], calf thymus DNA and poly[d(GC)]·poly[d(GC)], we studied the ciprofloxacin binding to two dodecameric DNA duplexes. Duplex D#1 (G1+C1) has a random sequence while duplex D#2 (G2+C2) contains a sequence of five consecutive AT base pairs, corresponding to the apparent binding site size of ciprofloxacin binding to poly[d(AT)]·poly[d(AT)]. The fluorescence emission spectra of ciprofloxacin in the presence of selected dodecameric DNA duplexes were measured at pH 7.0 in 10 mM cacodylate buffer (8.6 mM Na^+), 0.1 mM Na_2EDTA and 20 mM NaCl. The experiments could not be performed at the same ionic strength conditions used for poly[d(AT)]·poly[d(AT)] due to instability of dodecameric duplexes [37]. Melting experiments (data not shown) demonstrate that duplexes D#1 and D#2 are fully formed at 20 °C in the selected buffer at concentrations higher than 40 μM per single strand.

Upon titration with duplex D#2 containing TATAT sequence, the shape and the λ_{max} of ciprofloxacin fluorescence emission spectra change (Fig. 6B), while no significant changes were observed in the presence of duplex D#1 containing a random base sequence at the same position, TACTT (Fig. 6A). The magnitude of quenching is higher with duplex D#1 containing the mixed base sequence. These results are in qualitative agreement with the results obtained for poly[d(AT)]·poly[d(AT)] and calf thymus DNA described above. The quenching of ciprofloxacin fluorescence emission intensity by oligonucleotides follows the same pattern as previously observed for polynucleotides: (i) the fluorescence quenching is higher by double-stranded DNA than by single-stranded DNA; (ii) the fluorescence quenching is becoming more pronounced with increasing amount of GC base pairs (Supplementary material, Fig. S1A and B) and (iii) the red shift in the position of λ_{max} in the fluorescence emission spectra of ciprofloxacin is observed only for DNA containing AT base sequences.

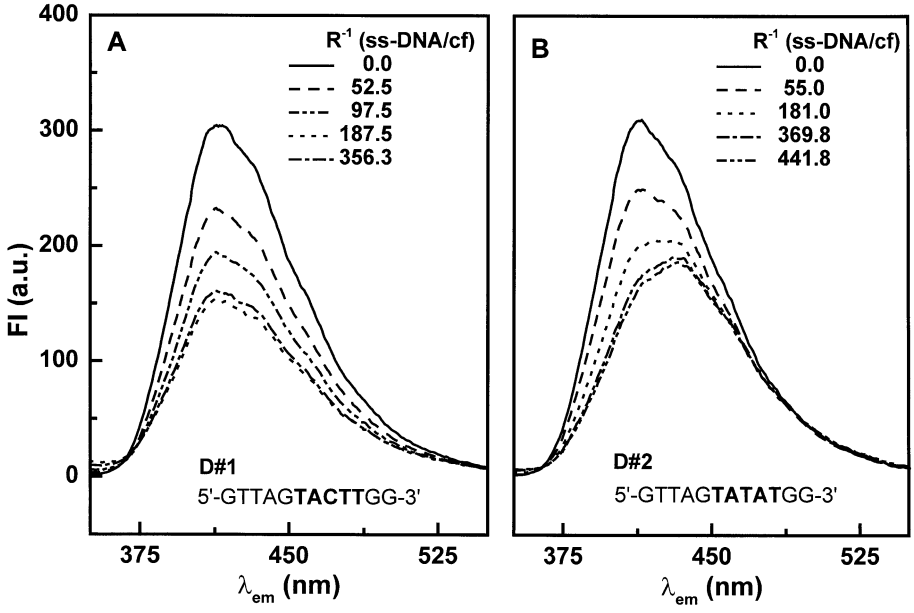

Fig. 6. Fluorescence emission spectra of ciprofloxacin in the presence of two dodecameric duplexes, (A) D#1 (G1+C1) and (B) D#2 (G2+C2), at different DNA (per single strand) to drug ratio, R^{-1}, as marked. Solution conditions were 10 mM cacodylate (pH=7.0), 28.6 mM Na^+ and 0.1 mM Na_2EDTA at 20 °C. C_{cf}=1 μM and λ_{ex}=330 nm.

3.5. Electrostatic contribution to the binding of ciprofloxacin to oligonucleotides

Fig. 7A shows the changes in fluorescence emission intensity of ciprofloxacin upon titration by D#1 and D#2, while the corresponding changes in λ_{max} are shown in Fig. 7B. Additionally, the corresponding changes in ciprofloxacin fluorescence emission intensity and λ_{max} upon back-titration of the ciprofloxacin–D#1 and ciprofloxacin–D#2 complexes with NaCl are shown in Fig. 7C and D. Increasing NaCl concentration does not completely displace ciprofloxacin from ciprofloxacin–D#1 and –D#2 complexes. At higher concentrations of NaCl (>100 mM), only 10% of the total fluorescence signal remains quenched in the presence of D#1 and ~20% in the presence of D#2 containing five AT base pairs (Fig. 7C). Almost complete restoration of ciprofloxacin fluorescence was observed after NaCl titration of each single-stranded oligonucleotide separately, except G1 (Supplementary material, Fig. S1C and D). The stronger quenching of the ciprofloxacin fluorescence by double-stranded DNA compared to single-stranded DNA could be due to the electrostatically enhanced outside binding of ciprofloxacin to double-stranded DNA. After back-titration of ciprofloxacin–D#2 complex with NaCl, the observed shift in λ_{max} to its initial value suggests that most of ciprofloxacin dissociated from dodecameric duplex containing five consecutive AT base pairs (Fig. 7D). Slightly different behaviour observed for ciprofloxacin binding to oligomeric DNA duplex containing five consecutive AT base pairs compared to poly[d(AT)]·poly[d(AT)] (Fig. 5A–D) suggests that the site size and/or other structural parameters are important for binding.

3.6. UV-melting profiles of polymeric double-stranded DNA in the presence of ciprofloxacin

Melting experiments of various double-stranded DNA at different drug to DNA ratios were performed at pH 7.0 at different buffer conditions (2 mM cacodylate, 1.7 mM Na^+ and 0.1 mM Na_2EDTA or 10 mM cacodylate, 108.6 mM Na^+ and 0.1 mM Na_2EDTA) (Fig. 8A and B). Ciprofloxacin shifts the melting temperature, T_m, of all studied double-stranded DNA to higher values at low ionic strength of the buffer (2 mM cacodylate, 1.7 mM Na^+ and 0.1 mM Na_2EDTA) (Table 2). The most pronounced increase in T_m of 9.8 (±1) °C was observed at ciprofloxacin to DNA base pairs ratio (R) of 1 for poly[d(AT)]·poly[d(AT)] (Table 2). The observed thermal stabilization, ΔT_m ($=T_m-T_m^\circ$), for calf thymus DNA at R=1 is 3.3 (±1) and 2.3 (±1) °C for poly[d(GC)]·poly[d(GC)]. To test the reversibility of ciprofloxacin binding to synthetic double-stranded DNA, we cooled the samples down and reheated the same sample the second time. The melting curves obtained upon reheating were completely superimposable onto the first heating scans (data not shown), suggesting that ciprofloxacin does not inhibit reannealing by associating irreversibly with the single strand. Interestingly, our data reveal that even at the highest R (=1) value, the complete saturation of binding of ciprofloxacin to poly[d(AT)]·poly[d(AT)] was not achieved (Fig. 8). These results further support our fluorescence results that at low ionic strength, ciprofloxacin binds specifically to double-stranded DNA and shows higher sequence preferences for alternating AT base pairs.

It should be mentioned that in the buffer containing 10 mM cacodylate, 8.6 mM Na^+ and 0.1 mM Na_2EDTA, insignificant thermal stabilization of poly[d(AT)]·poly[d(AT)] by ciprofloxacin and destabilization of calf thymus DNA are observed. In contrast, in high ionic strength buffer (10 mM cacodylate, 108.6 mM Na^+, 0.1 mM Na_2EDTA) ciprofloxacin shifts the melting temperature, T_m, of both native and different synthetic DNAs to lower values (Table 2). The most pronounced decrease in T_m (ΔT_m=−3.5 (±1) °C) was observed for calf thymus DNA compared to ΔT_m value of −1.7 (±1) °C for poly[d(AT)]·poly[d(AT)] (Table 2). The decrease in thermal stability of the different double-stranded DNAs upon addition of ciprofloxacin would suggest that the ligand preferentially interacts with single-stranded DNA rather than double-stranded DNA. At these experimental conditions, poly[d(GC)]·poly[d(GC)] is too stable to melt in the examined temperature range.

At low ionic strength of solution drug stabilizes duplex rather than single-stranded DNA (Table 2). The increase in T_m is expected due to the outside stacking and electrostatic interactions between DNA and ciprofloxacin. At neutral pH and in the absence of salt, protons may promote ciprofloxacin binding to DNA by neutralizing the negative charge on the carboxylate group of different drug species [8]. The observed increase in thermal stability of poly[d(AT)]·poly[d(AT)] of 9.8 (±1) °C is too high to be explained only by electrostatic interactions. The salt-dependent UV-melting, fluorescence NaCl back-titration and viscometry data (see below) suggest that ciprofloxacin exhibits another mode of binding in addition to electrostatically driven outside binding of the drug to DNA that occurs under conditions of high drug loading and is enhanced at low ionic strength. Such a binding mode, in fact, is common among DNA intercalating ligands [38]. Many studies using the linear dichroism have shown that the molecular plane of norfloxacin was near parallel to the DNA bases (perpendicular to the DNA helix axis) in the binding complex [6–8], and would support the intercalative binding mode. However, the observed identical T_m of DNAs in the absence and presence of norfloxacin and the negligible unwinding of supercoiled DNA by norfloxacin at higher ionic strength did not classify the norfloxacin as a classical intercalator or a minor groove binder [8,34].

3.7. Viscometry results show that ciprofloxacin has some properties of an intercalative binder

The primary mode of binding by which a ligand interacts with a polymeric host nucleic acid structure may be investigated by viscometry. In general, intercalators cause an

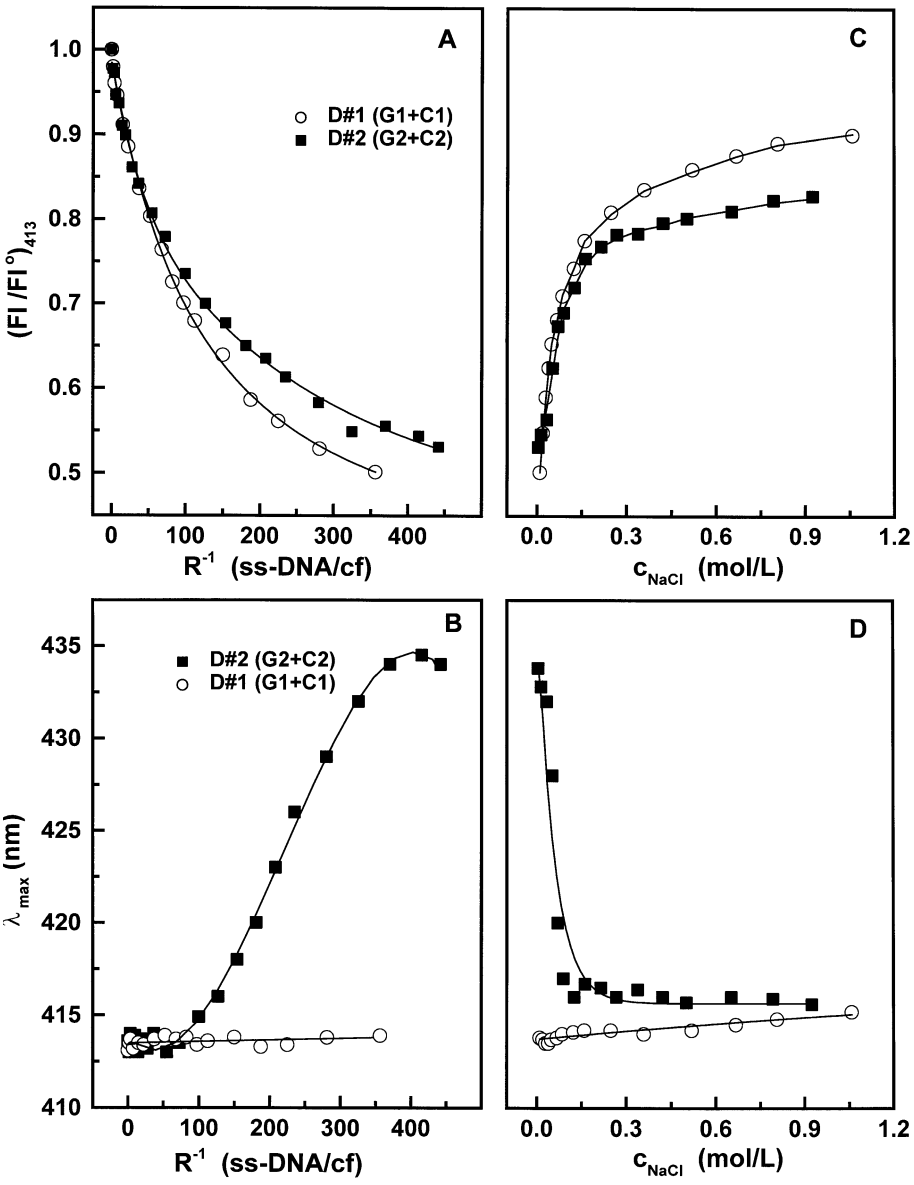

Fig. 7. The changes in the relative fluorescence emission intensity $(FI/FI^{\circ})_{413}$ (A) and in the position of the maximum wavelength, λ_{max} (B) in the ciprofloxacin emission spectra after titration by dodecameric DNA duplexes D#1 (G1+C1) (○) and D#2 (G2+C2) (■) and NaCl back-titration of the final ciprofloxacin–dodecameric duplex complex (C and D). Solution conditions were 2 mM cacodylate (pH=7.0), 1.7 mM Na^+ and 0.1 mM Na_2EDTA at 20 °C. C_{cf}=1 μM and λ_{ex}=330 nm.

increase in intrinsic viscosity due to an increase in apparent molecular length of the DNA helix. Ligand interaction between stacked bases within a linear host duplex (intercalation) is associated with the lengthening of the nucleic acid. Thus, a ligand-induced increase in the viscosity of a duplex nucleic acid solution is consistent with (but does not absolutely prove) an intercalative mode of binding [39].

Fig. 9 shows the effect of ciprofloxacin, ethidium bromide (a typical intercalator) and netropsin (a typical minor groove binder) binding on the apparent relative contour lengths of two host duplexes. Inspection of Fig. 9 reveals that upon addition of ciprofloxacin to calf thymus DNA and poly[d(AT)]·poly[d(AT)] duplexes, each undergoes substantial increase in apparent contour length. The extent of increase in DNA contour length in the presence of ciprofloxacin is lower than by ethidium bromide but much higher than by netropsin under the same experimental conditions, suggesting some degree intercalation of ciprofloxacin into double-stranded DNA under solution conditions employed (Fig. 9). Several factors may account for the quantitative differences in apparent contour length between ciprofloxacin and ethidium bromide binding to two different duplexes. First, competing nonintercalative-binding modes of ciprofloxacin, such as groove binding and electrostatically facilitated stacking at the helix exterior, may reduce the amount of intercalative binding and therefore the extent of helix lengthening. Second, structural differences between the intercalated complexes (e.g., degree of helix unwinding)

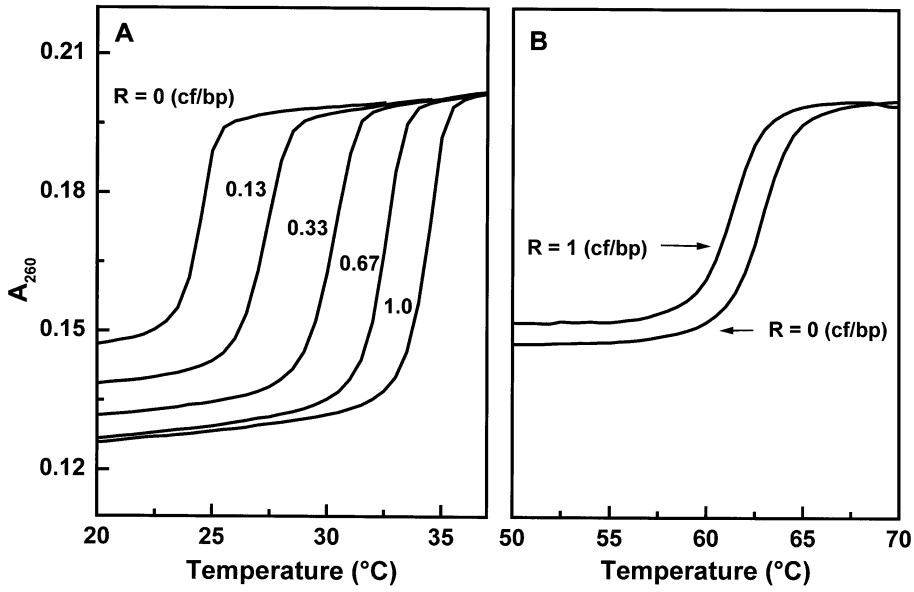

Fig. 8. Absorbance versus temperature profiles of poly[d(AT)]·poly[d(AT)] and their ciprofloxacin complexes (at indicated values of *R*) at 260 nm. Solution conditions were: (A) 2 mM cacodylate (pH=7.0), 1.7 mM Na^+ and 0.1 mM Na_2EDTA; (B) 10 mM cacodylate (pH=7.0), 108.6 mM Na^+ and 0.1 mM Na_2EDTA. C_{DNA}=15 μM in base pairs. The arrows indicate the melting curves of poly[d(AT)]·poly[d(AT)] in the absence (*R*=0) and presence (*R*=1) of ciprofloxacin, respectively.

of ciprofloxacin with DNA and ethidium with DNA [40], which differentially alter the effective hydrodynamic length and stiffness of the target duplex, may contribute to the observed difference apparent contour length. Third, differences in binding site size and binding affinity between ciprofloxacin and ethidium bromide should affect helix lengthening. Fourth, as our results suggest, there are qualitative differences between ciprofloxacin and ethidium bromide binding to poly[d(AT)]·poly[d(AT)] and random sequence genomic DNA. Thus, the binding-induced hydrodynamic differences, we observed between duplexes, might reflect the presence of some partially intercalated drug molecules, which manifest additional interactions within the groove(s). Not, at least, another potential contribution to the differential effect we observed could be ciprofloxacin-induced conformational changes in the target duplexes independent of intercalation. In general, such an effect could cause a single binding mode to reflect properties characteristic of multiple binding modes [39]. Due to these undetermined factors, the observed change in intrinsic viscosity does not provide absolute proof of intercalative binding. However, the similarity between the effect of ethidium bromide and ciprofloxacin on the change in intrinsic viscosity compared to the effect of netropsin, for example, at least strongly suggests that ciprofloxacin is also an intercalator at conditions applied here.

Table 2
The melting temperatures of the nucleic acid duplexes at pH 7.0 (at different solution conditions) in the absence and presence of ciprofloxacin

Nucleic acid duplex	T_m^o (°C)	T_m (°C)	ΔT (°C)
2 mM Cacodylate buffer, 1.7 mM Na^+, 0.1 mM Na_2EDTA			
Calf thymus DNA	53.6±0.5	56.9±0.5	3.3±1.0
poly[d(AT)]·poly[d(AT)]	24.4±0.5	34.2±0.5	9.8±1.0
poly[d(GC)]·poly[d(GC)]	81.7±0.5	84.0±0.5	2.3±1.0
10 mM Cacodylate buffer, 8.6 mM Na^+, 0.1 mM Na_2EDTA			
Calf thymus DNA	67.5±0.5	64.6±0.5	−2.9±1.0
poly[d(AT)]·poly[d(AT)]	39.8±0.5	41.1±0.5	1.3±1.0
10 mM Cacodylate buffer, 108.6 mM Na^+, 0.1 mM Na_2EDTA			
Calf thymus DNA	86.4±0.5	82.9±0.5	−3.5±1.0
poly[d(AT)]·poly[d(AT)]	63.0±0.5	61.3±0.5	−1.7±1.0

T_m^o is the melting temperature of the nucleic acid duplex in the absence of ciprofloxacin. T_m is the melting temperature of the ciprofloxacin–DNA complex at the *R* value of 1.0. $\Delta T=T_m-T_m^o$.

4. Concluding remarks

The present study of ciprofloxacin binding to various polymeric and oligomeric DNAs clearly shows that ciprofloxacin can bind to single- and double-stranded DNA. At low ionic strength, ciprofloxacin binds specifically to double-stranded DNA and shows higher sequence preferences for alternating base sequences. Similar results were published before for norfloxacin, another representative of fluoroquinolone family, which is structurally very similar to ciprofloxacin and differs only in the ethyl-group attached to N1 nitrogen atom of quinolone ring system [6–8,34]. Furthermore, our results highlight the importance of solvent conditions in determining ciprofloxacin–DNA interactions and contribute to the broad-based effort to define the molecular recognition patterns that control the affinities and specificities of nucleic-acid-binding fluoroquinolone. In addition, we have observed, for the first time, the slightly

different behaviour of ciprofloxacin binding to oligomeric DNA duplex containing five consecutive AT base pairs compared to poly[d(AT)]·poly[d(AT)], suggesting that the size and/or other structural parameters are important for binding. The reversibility of ciprofloxacin binding to double-stranded DNA, as observed from UV-melting curves, suggests that ciprofloxacin does not inhibit the reannealing by associating irreversibly with the single strands. Furthermore, the experimental observations from UV-melting curves, fluorescence emission spectra and fluorescence NaCl back-titration of ciprofloxacin–DNA complexes suggest that ciprofloxacin has at least two different binding modes: a nonspecific binding to single- and double-stranded DNA molecules, which is electrostatically driven, and a specific non-electrostatically controlled binding. The effect of ciprofloxacin on the change in intrinsic viscosity strongly suggests that ciprofloxacin has the properties of an intercalative binder.

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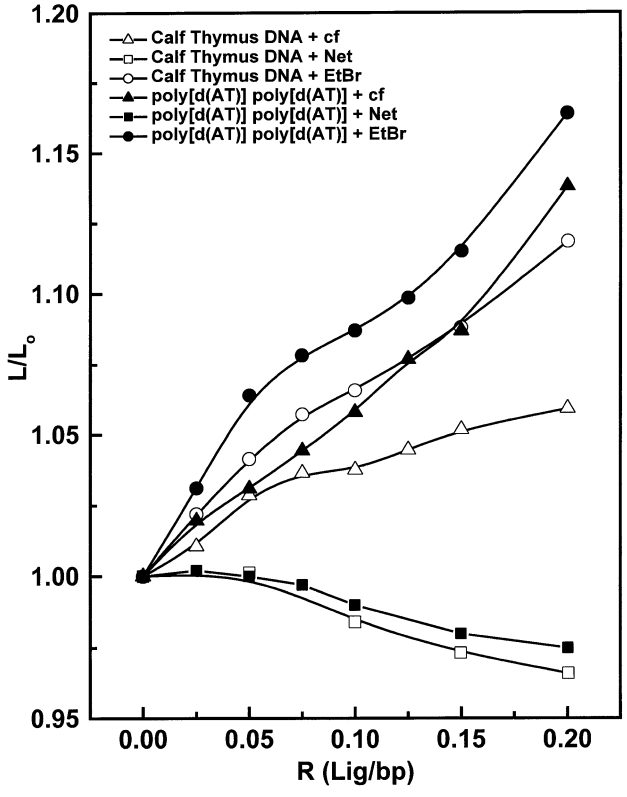

Fig. 9. Viscometric titration of sonicated calf thymus DNA (open symbol) and poly[d(AT)]·poly[d(AT)] (solid symbol) with ethidium bromide (○, ●), netropsin (□, ■) and ciprofloxacin (△, ▲) at 20.0 °C. L/L_o is plotted as a function of moles of drug added per mole of DNA base pairs, *R*. Solution conditions were 2 mM cacodylate (pH=7.0), 1.7 mM Na^+ and 0.1 mM Na_2EDTA.

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