Interaction of Norfloxacin with Super-Coiled DNA

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Norfloxacin, that inhibits the action of topoisomerase II, binds to wide variety of DNA. The binding mode of this drug to double- and super-coiled DNA (ds- and scDNA) is compared in this study by various spectroscopic methods, including absorption, fluorescence, and circular dichroism(CD) spectroscopy. Hypochromism in the absorption band, negative and positive induced CD bands (respectively in 240-260 nm and 270-300 nm region) are apparent for the norfloxacin that bound to both the dsDNA and scDNA. A decrease in fluorescence is also noticed in the presence of both DNAs. Since the spectroscopic characteristics are the same for both complexes, it is imperative that the binding mode of the norfloxacin is similar in ds- and scDNA. In the presence of Mg^{2+}, which is a cofactor in the topoisomerase II action, the fluorescence intensity of the scDNA-norfloxacin complex increased and the resulting fluorescence intensity and shape was identical to that in the absence of scDNA. Therefore, the addition of an excess amount of Mg^{2+} may result in the extrusion of norfloxacin from scDNA.

Key Words : Quinolone, Norfloxacin, DNA, Super-coiled DNA

Introduction

Norfloxacin, a member of quinolone antibiotics, is a clinically important antibacterial agent derived from nalidixic acid. It is a specific inhibitor of DNA gyrase, a bacterial type II topoisomerase, which unwinds the supercoiled DNA (referred to as scDNA in this study) prior to replication and transcription.[1] Norfloxacin also controls DNA superhelicity and plays important roles in various cellular processes. The efficiencies of replication, transcription and recombination are greatly affected by the inactivation of the enzymes.[2] In general, quinolones are extensively used as first-line treatments of many infections, but the drugs are not entirely safe and are liable to cause adverse reactions such as non-specific neurological effects.[3]

Binding studies of norfloxacin to DNA and to gyrase have revealed that drugs do not bind to enzymes but to DNAs.[4] Subsequent studies with various DNA substrates and DNA-enzyme complexes have led to the proposal of a cooperative drug binding model, in which the drug molecules bind to a single-stranded DNA (referred to as ssDNA in this study) pocket created by the enzyme DNA gyrase.[5-7] In the complex, norfloxacins are stabilized *via* self-association of the drug molecules and the hydrogen bonds between the drug and DNA phosphate. This proposal motivated our group and others to investigate the direct interaction between the quinolone antibiotics, including norfloxacin and ofloxacin, and native and various synthetic DNAs.[8-13]

From the through spectroscopic investigation, we concluded that (1) norfloxacin preferred to bind to ssDNA compared to double strand DNA (referred to as dsDNA in this work), (2) when the drug formed a complex with dsDNA, it preferred to bind in the minor groove of the guanine base and was stabilized by the hydrogen bonds between the carbonyl and carboxylic group of norfloxacin and the amine group of the guanine base with the possibility of partial intercalation. Other binding mode, that deviates from our model and Shen's model[1] have also been proposed. From the linear dichroism study, Baily and his coworkers[14] proposed the intercalative binding of norfloxacin. In the presence of Mg^{2+} ion, the Mg^{2+} bridged model, in which carbonyl and carboxylic group of norfloxacin and two phosphate groups of the scDNA were coordinated to the Mg^{2+} ion was proposed.[15]

In this study, we compared the various spectroscopic properties of norfloxacin in the presence of dsDNA and scDNA in order to understand the binding mode of norfloxacin to scDNA. The effect of the Mg^{2+} ion, which is a required cofactor for the enzymatic process, on the norfloxacin-scDNA binding process was also investigated.

Materials and Methods

Chemicals. Norfloxacin and other chemicals were purchased from Sigma and used without further purification. Double stranded *calf thymus* DNA, purchased from Sigma, was 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 DNA solution was then dialyzed several times against 5mM cacodylate buffer at pH 7.0. This buffer was used throughout this work. ScDNA was prepared from a pGEM3zf(+) plasmid. The concentrations of the DNAs and

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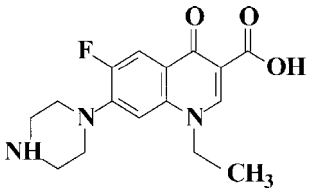

Figure 1. Chemical Structure of Norfloxacin.

norfloxacin were determined by the molar extinction coefficients: $\varepsilon_{258nm} = 6{,}700\ cm^{-1}\cdot M^{-1}$ for ds- and scDNA and $\varepsilon_{273nm} = 37{,}500\ cm^{-1}\cdot M^{-1}$ for norfloxacin. All measurements were performed at 10 °C except for CD, which was recorded at 4 °C.

Absorption and circular dichroism spectroscopy. Absorption spectra were recorded on either a Jasco V-550 or on a Hewlett Packard 8452A diode array spectrophotometer using a 1 cm optical path length quartz cell. Titrations were performed by adding aliquots of the drug solution to a constant volume of the DNA solution (*ca.* 100 μM) and appropriate volume corrections were made. CD spectra were recorded on a Jasco J-715 spectropolarimeter (displaying the CD in milidegrees ellipticity), equipped with a Jasco PTC-348 peltier temperature controller.

Fluorescence emission measurement. The fluorescence intensity of norfloxacin decreases in the presence of both dsDNA and scDNA. The quenching of fluorescence of norfloxacin by both DNAs has been known to be a static mechanism.[20] Consequently, the equilibrium constant of norfloxacin for both DNAs can be measured using the Stern-Volmer method.[16]

$$\frac{F_0}{F} = 1 + K_{SV}[Q]$$

Where F_0 and F denotes fluorescence intensity in the absence and presence of a quencher, $[Q]$ is the concentration of the quencher, in our case, DNA. The slope, K_{SV}, is the Stern-Volmer constant for the complex formation, which is considered to be an equilibrium constant for the static quenching process. All fluorescence measurements were performed on a Jasco FP-777 fluorometer with the excitation wavelength of 323 nm for norfloxacin.

Results and Discussion

Spectroscopic characteristics of norfloxacin complexed with ds- and scDNA. The absorption spectra of norfloxacin complexed with dsDNA and scDNA at various mixing ratios are depicted in Figures 2(a) and 2(b), respectively. These spectra were produced by subtracting the absorption spectrum of the corresponding DNA from the complexes and were normalized to the highest concentration. A marked hypochromism (up to 14-16%) was observed in both the 270 nm and 320 to 340 nm bands for both complexes. The interaction of the drug with both DNAs causes a weak red shift of 2 nm for the maximum at 270 nm, owing to the perturbation of the complexed chromophore upon binding to

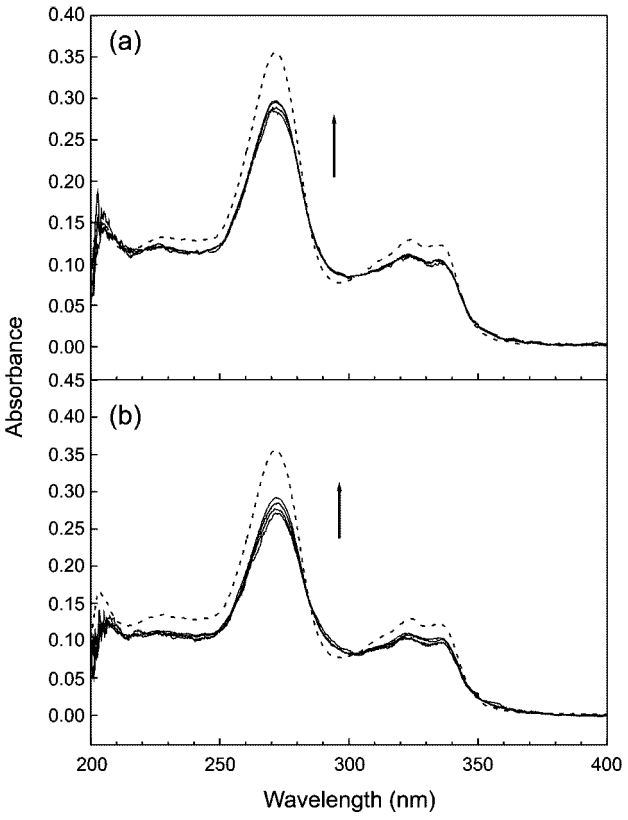

Figure 2. The absorption spectrum of norfloxacin (dashed curve) and that in the presence of dsDNA (panel (a)) and scDNA (panel (b)). In the direction of the arrow, the absorption spectrum was recorded for 4, 6, 8 and 10 μM of norfloxacin. [DNA] = 114 μM. [scDNA] = 117.5 μM.

both DNA bases, while no shift was observed for bands between 320 and 340 nm. Similar hypochromism and red shift for the norfloxacin in the presence of dsDNA have been reported.[8,13] The difference in the mixing ratio resulted in a small variation in the absorption spectrum. Two isosbestic points were observed at different binding ratios for both complexes, implying that the conformation of the norfloxacin molecule bound to dsDNA and scDNA were homogeneous, *i.e.*, both system consist only the DNA-free and DNA bound norfloxacin. In both complexes, the environment of norfloxacin and its interaction with DNA is similar since their absorption spectra are similar.

CD spectrum is induced even with the achiral drugs when bound to DNA. This CD spectrum is called the induced CD spectrum. The origin of the induced CD spectrum is believed to be the interaction of the electric transition of the drug and chirally arranged transition moments of DNA bases, and hence are expected to be very sensitive to the drugs environment.[17,18] Norfloxacin, which is an achiral molecule, therefore, it does not exhibit intrinsic optical activity by itself, and becomes optically active when it binds to a macromolecular template such as various DNAs.[8-13] Figure 3 depicts the induced CD spectrum of norfloxacin when bound to ds- and scDNA. The CD spectrum of the corresponding DNA was subtracted from those of the complex similarly

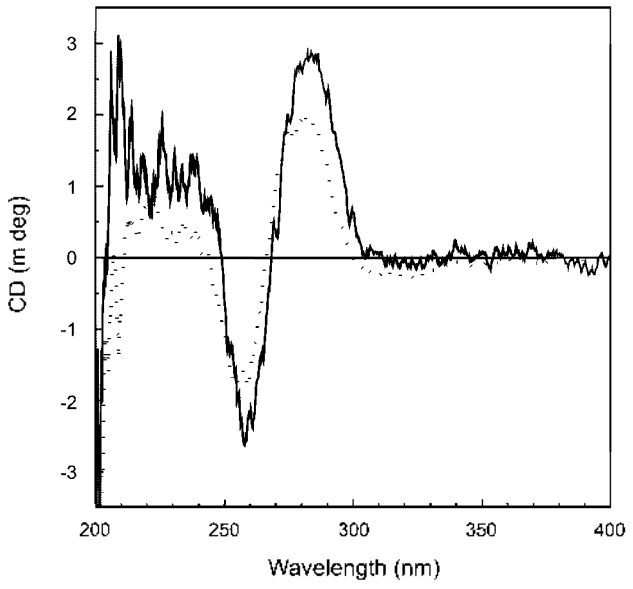

Figure 3. Induced CD spectrum of norfloxacin with dsDNA (dotted curve) and scDNA (solid curve) at R is 0.05. The CD spectrum of corresponding DNA was subtracted from that of the complex for an easy comparison. [DNA] = 100 μM, [scDNA] = 35.5 μM.

with absorption spectra. At a glance, both CD spectra of norfloxacin complexed with ds- and scDNA are almost identical, consisting of a strong positive band in the 270 to 300 nm region, and a strong negative band in the 240 to 260 nm region. A very weak negative band in the 315 to 320 nm region is also noticed. Since the CD spectrum is sensitive to the arrangement of the drug in DNA, the identical CD is evidence for the same binding mode of norfloxacin in both DNAs, in addition to the absorption spectrum.

Fluorescence emission spectra of norfloxacin in the presence and absence of dsDNA and scDNA are shown in Figures 4(a) and 4(b), respectively. The intensity of the emission spectrum of norfloxacin decreases with an increasing dsDNA concentration was reported (Fig. 3(a)).[12] The shape of the emission spectra and the decreasing pattern in the presence of scDNA (Fig. 3(b)) are very similar compared to that in the presence of dsDNA, again indicating that the binding modes of norfloxacin to both DNAs are similar. Utilizing the decrease in the fluorescence intensity, the Stern-Volmer plots for the norfloxacin-DNA binding was constructed and inserted in Figure 4(b). The slope in the plot for the norfloxacin-scDNA complexation is slightly higher than that of the DNA-norfloxacin complex formation. The equilibrium constant for these complex formations at 10 °C were calculated as 3.69×10^3 M^{-1} for dsDNA and 5.03×10^3 M^{-1} for scDNA. The former value for the norfloxacin-dsDNA complexation is in the same range with those of the published results.[12] From the fact that when norfloxacin is bound to scDNA it exhibits the same absorption, fluorescence emission, and CD spectrum with that complexed with dsDNA, the binding mode and the environment of norfloxacin is similar with these two DNAs.

Role of Mg^{2+} in the norfloxacin-scDNA complex formation. It was reported by Palù and his coworkers[15] that an increase of Mg^{2+} concentration linearly increased the intensity of the norfloxacin fluorescence emission at a Mg^{2+} concentration of 1-2 mM in the presence of DNA while no interaction was observed in the absence or in an excess amount of Mg^{2+}.[15,18] This observation indicated that an appropriate Mg^{2+} concentration probably modulated the norfloxacin binding to DNA. Consequently, they suggested a norfloxacin-scDNA binding model, in which the carbonyl and carboxylic group of the drug and two phosphate groups form a complex to a Mg^{2+} ion, here Mg^{2+} being a bridge between norfloxacin and DNA. We measured the fluorescence intensity of norfloxacin in order to confirm the role of the Mg^{2+} ion in the norfloxacin-scDNA complexation (Fig. 5). In the absence of Mg^{2+}, norfloxacins fluorescence intensity in the presence of various nucleic acids are different: it is the lowest in the presence of poly[d(G-C)$_2$], while that bound to poly[d(A-T)$_2$] is the highest and that bound to scDNA intermediate, indicating the interaction of norfloxacin with these polynucleotides are different, as it was previously observed.[9,12] Binding of norfloxacin to polynucleotides in the absence of Mg^{2+}, induced a significant fluorescence

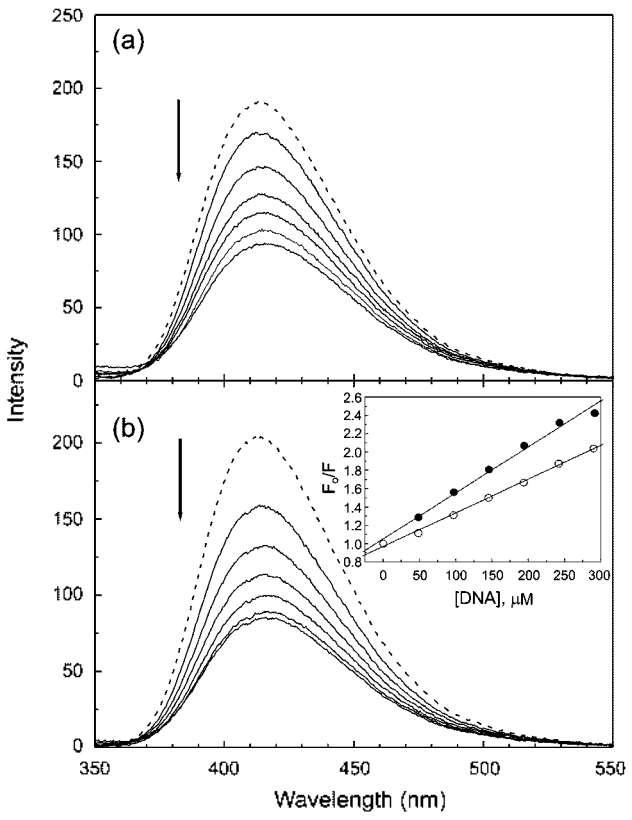

Figure 4. Fluorescence emission spectrum of norfloxacin in the absence (dashed curve) and presence of dsDNA (panel (a)) and scDNA (panel (b)). Emission spectra were recorded with 323 nm excitation. [Norfloxacin] = 1.0 μM, [dsDNA] = 48.3, 96.7, 145.0, 193.4, 241.7, 290.0 μM and [scDNA] = 48.5, 97.1, 145.7, 194.2, 242.8, 291.4 μM. *Insertion*: Stern-Volmer plot for the dsDNA-norfloxacin (opened circles) and scDNA-norfloxacin complex formation (closed circles). Fluorescence intensity was measured with 412 nm emission. Slit width 3.0/5.0 nm.

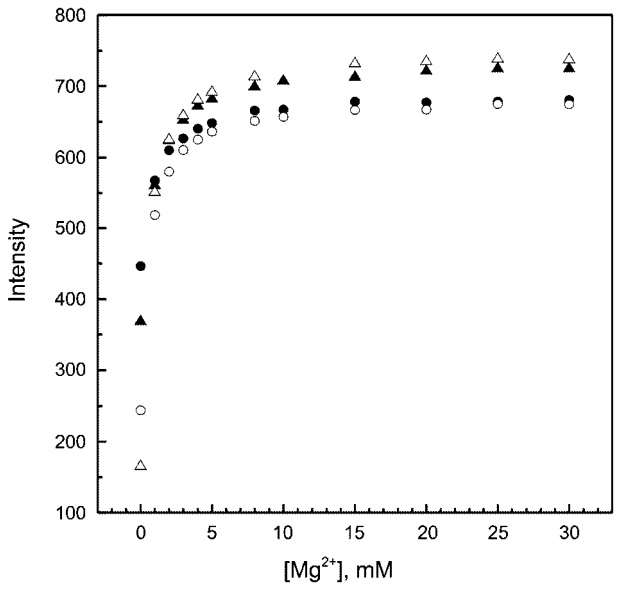

Figure 5. Changes in the fluorescence intensity with increasing Mg^{2+} concentration for the various DNA-norfloxacin complexes. The intensity was measured at 416 nm emission. R = 0.01. [DNA] = 100 μM. scDNA: open circles, poly[d(A-T)$_2$]: closed triangles, poly[d(G-C)$_2$]: open triangles, DNA-free norfloxacin: closed circles.

spectral change: quenching of norfloxacin fluorescence upon DNA and poly[d(G-C)$_2$] binding, while a spectral shift occurred to a higher wavelength upon poly[d(A-T)$_2$] binding.[9] As the Mg^{2+} concentration increased up to a few mM, the fluorescence emission intensity significantly increased, and then it reached a plateau at around 10 mM Mg^{2+} concentration. At the plateau, the intensity (Fig. 4) as well as the shape (data not shown) of the fluorescence emission spectra are identical disregarding the polynucleotide present in the system. This observation strongly suggests that norfloxacin can form a complex with a Mg^{2+} ion without polynucleotides. In the presence of polynucleotides, the high concentration of Mg^{2+} results in the extrusion of norfloxacin.

Conclusion

The binding mode of norfloxacin to dsDNA and scDNA are very similar. In the presence of access amounts of Mg^{2+}, norfloxacin is extruded from polynucleotide and directly forms a complex with this metal ion.

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