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Determination of Yeast DNA Based on Its Quenching the Fluorescence Emission of Norfloxacin

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ABSTRACT

The interaction between norfloxacin and yeast double-stranded DNA (dsDNA) was studied using UV spectrometry and fluorescence spectrometry. Norfloxacin exhibited a fluorescence excitation at 272 nm and a fluorescence emission at 415 nm in pH 7.0 phosphate buffer solution. The addition of yeast dsDNA to the norfloxacin solution resulted in a strong static fluorescence quenching due to the binding of the norfloxacin by yeast dsDNA. The binding constant was $(2.90 \pm 0.03) \times 10^4\,M^{-1}$ (15°C). Based on the fluorescence

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quenching, a novel method for sensitive determination of yeast dsDNA concentration ranging from 77 nM to 43.2 μM was developed. The relative standard deviation for eleven detections of 28.8 μM dsDNA was 3.0%. The fluorescence spectrum of the norfloxacin bound covalently to single-stranded DNA (ssDNA) showed an obvious change after the ssDNA hybridized with its complementary DNA, producing a qualitative detection method for DNA sequence.

Key Words: DNA; Norfloxacin; Fluorescence quenching; Genetic analysis; Yeast DNA.

INTRODUCTION

The studies on the affinity of DNAs to small molecules and their interaction have attracted considerable interest in recent years. The small molecules used for this purpose include drugs,[1–3] coordination complexes[4,5] and some fluorescent dyes.[6,7] Investigation on the interaction between DNA and drug is significant for the prevention of diseases, the improvement of the medicinal efficacy and the design and sieving of drugs. The techniques used for this purpose mainly include fluorescence,[7,8] UV spectrometry,[2,9] and electrochemical methods.[10,11] Among these methods, fluorometric technique has become a very popular tool for determination of DNA due to its convenience, sensitivity and high-speed.

Spectrometric method is the most popular procedure for DNA concentration determination, which is based on a strong UV absorption of the nucleic acids at 260 nm,[12] but its sensitivity is relatively low. The detection limit is about 1 μM. Several efforts have been made to develop a more sensitive method for the determination of lower DNA concentration. Some fluorescent dyes such as ethidium bromide,[13–15] Hoechst 33258[16,17] and acridine dyes[18–20] have been used for the determination of calf thymus DNA based on the enhancement or quenching of fluorescence intensity upon DNA addition. Those works suffered from a long-time analytical procedure or using toxic substances, for example, ethidium bromide is a strong carcinogen and can lead to skin cancer when it touches the skin. Here a novel method for the determinations of yeast DNA concentration and sequence is described by using norfloxacin as a fluorescence probe.

Norfloxacin is a widely used antibacterial drug and causes a side effect on liver only from the over use. Its efficacy is brought into play

by inhibiting the activity of DNA gyrase and preventing the duplication of DNA.[21] So the study on the interaction between norfloxacin and DNA plays a very important role in improving the absorption and antibiotic activity of norfloxacin in body. The interaction between norfloxacin and calf thymus dsDNA has been well studied by Son et al.[2,22] They concluded that the molecular plane of norfloxacin was near perpendicular relative to the DNA helix axis with a strong possibility of bending of the DNA stem near the norfloxacin binding site. We here study the interaction mode between norfloxacin and yeast dsDNA, an AT enriched DNA molecule.[23] This interaction results in a strong fluorescence quenching of norfloxacin, which is used for the determination of yeast DNA concentration. At the same time, a procedure to detect qualitatively native complementary DNA (cDNA) sequence is proposed by detecting the change of the fluorescence spectrum of the norfloxacin-bound ssDNA upon its hybridization with its cDNA.

MATERIALS AND METHODS

Apparatus and Reagents

Fluorescence spectra were recorded with an LS-50B spectrofluorometer (Perkin–Elmer, USA) with a quartz cell (1×1 cm cross-section). UV spectra were recorded with a UV-3100 spectrometer (Shimadzu, Japan). Commercially available yeast dsDNA (Changyang Pharmaceutical Factory, Shanghai) and calf thymus DNA (from Sino-American Biology Company) (UV determination: $A_{260}/A_{280} > 1.8$) were directly dissolved in water without further purification to give a final concentration of 1.0 mg/mL and stored at 4°C. Concentrations of DNA (per nucleotide phosphate) were determined spectrophotometrically with $\varepsilon_{260} = 6600\,M^{-1}\,cm^{-1}$.[24] Norfloxacin and 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC) were from Sigma. Zero point zero one molar pH 7.0 phosphate buffer solution (PBS) was used to control the acidity. Zero point one molar NaCl solution was used to adjust the ionic strength. Other reagents were of analytical reagent grade. Doubly distilled water was used throughout.

Methods

ssDNA and cDNA solutions were prepared by heating native dsDNA solution in a boiling water bath for 8 min and then rapidly cooling in an

ice-water bath. When hybridization, the solution containing ssDNA and its cDNA was incubated in a 60°C water bath for 1 h and then cooled gradually down to about 15°C to form dsDNA. As a control, calf thymus ssDNA was used as a substitute of cDNA with the same procedure.

Half milliliter of PBS, various amounts of DNA and 0.2 mL 1.0×10^{-4} M norfloxacin were put into a 5.0 mL calibrated tube. The mixture was then diluted with water to the mark and kept at 15 ± 2°C for 5 min. The fluorescence intensities of norfloxacin (F_0) and the mixture (F) were measured at 415 nm with an excitation wavelength of 272 nm. All measurements were carried out at 15 ± 2°C (or 31°C for the study on the temperature effect) with a slit width of 5.0 nm for both excitation and emission monochromators.

RESULTS AND DISCUSSION

Influence of pH on Fluorescence Emission of Norfloxacin

Norfloxacin is an amphoteric molecule with a piperazinyl and a quinolone ring (as shown in Fig. 1). Its isoelectric point is at pH 7.37.[25] The solution pH has a significant influence on its fluorescence emission intensity, its maximum emission wavelength (λ_{ex}) and its maximum excitation wavelength (λ_{em}). Figure 2 shows the fluorescence emission spectra of the norfloxacin at different pHs. In acidic solution (pH 3.0), the norfloxacin shows a strong fluorescence emission at λ_{em} of 445 nm. The protonation of 4-N on piperazinyl[26] produces a positive substitute, which results in a greater emission intensity and a longer λ_{em} when comparing with those in neutral or alkaline solutions. With an increasing pH, the protonation extent decreases, thus the intensity of fluorescence emission decreases with a blue-shift. When the solution pH is at 7.4, the blue-shift reaches the maximum value and a turning point of λ_{em} occurs at 410 nm. With the continuous increase of pH, the emission intensity further decreases and λ_{em} becomes longer. Obviously, this is due to the dissociation of more

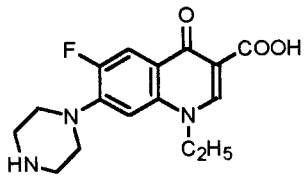

Figure 1. The structure of norfloxacin.

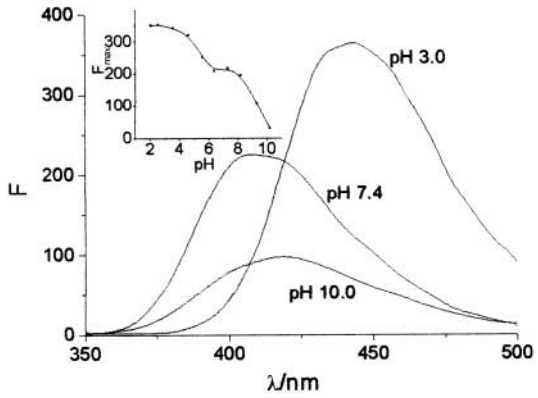

Figure 2. Fluorescence emission spectra of norfloxacin at different pHs. Inset: plot of the maximum emission intensity of norfloxacin vs. pH value.

carboxyl groups in the alkaline solution, which enlarges the π-conjugate system of the quinolone ring and decreases the energy difference between the ground state and the excited state. A relatively stable intensity of fluorescence emission occurs in the range of pH 6.0–8.0 (shown in inset of Fig. 2), around the isoelectric point of norfloxacin. Considering the stability of the emission intensity and the pH value of a body environment, pH 7.0 PBS was selected for the following experiments.

Fluorescence Spectra of Norfloxacin–Yeast dsDNA System

In pH 7.0 PBS the norfloxacin displays a maximum excitation at 272 nm and a maximum emission at 415 nm (Fig. 3). Both the excitation and emission intensities decrease upon the addition of yeast dsDNA, but their peak positions do not change. The decreases in the excitation and emission intensities indicate an interaction between norfloxacin and yeast dsDNA, which results in a fluorescence quenching of norfloxacin.

The influence of norfloxacin concentration on the quenching extent by DNA was investigated at a yeast dsDNA concentration of 28.8 μM in pH 7.0 PBS. Figure 4 shows the plot of $F/F_0 - 1$ vs. norfloxacin concentration, where F is the fluorescence emission intensity in presence of DNA, and F_0 is the intensity in absence of DNA. The extent of the fluorescence quenching increases with an increasing norfloxacin concentration and the maximum fluorescence quenching occurs at a norfloxacin concentration of 4.0 μM. So we chose 4.0 μM norfloxacin for the following experiments.

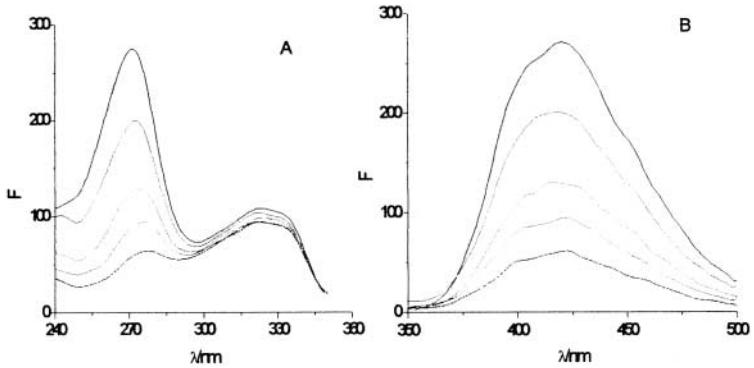

Figure 3. Excitation (A) and emission (B) spectra of 4.0 μM norfloxacin in the presence of DNA with different concentrations. $C_{DNA} = 0.0$, 4.8, 9.6, 14.4, 19.2, 24.0, 28.8, 33.6, and 38.4 μM from top to bottom.

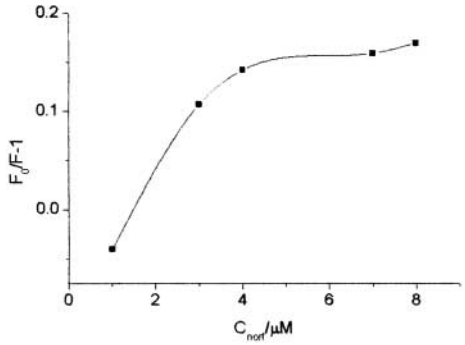

Figure 4. Influence of norfloxacin concentration on the quenching efficiency. $C_{DNA} = 28.8\,\mu M$.

The fluorescence quenching data are analyzed according to the Stern-Volmer equation[27]:

$$F_0/F - 1 = KC_{DNA} \tag{1}$$

where K is the Stern-Volmer constant. Figure 5 gives the plots of $F_0/F - 1$ vs. DNA concentration at 15 and 31°C. The K values are obtained to be $(2.90 \pm 0.03) \times 10^4\,M^{-1}$ and $(2.44 \pm 0.04) \times 10^4\,M^{-1}$ at 15°C and 31°C, respectively. They are much larger than $4.1 \times 10^3\,M^{-1}$ for that between norfloxacin and calf thymus dsDNA at 15°C.[2,22] This indicates that the

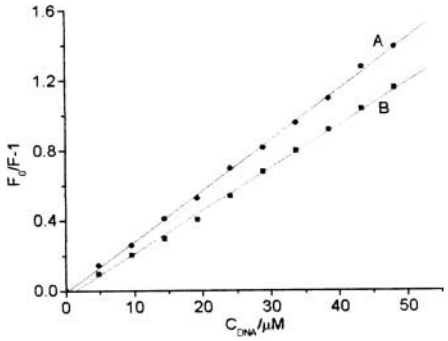

Figure 5. Fluorescence quenching Stern-Volmer plot of norfloxacin by DNA at 15°C (A) and 31°C (B). $C_{norf} = 4.0\,\mu M$.

interaction mechanism between the norfloxacin and yeast dsDNA is possibly different from that between the norfloxacin and calf thymus dsDNA.

The fact that the K value at 31°C is larger than that at 15°C suggests that the fluorescence quenching is a static process by forming a complex of norfloxacin with DNA at the experimental temperatures[28]:

$$\text{Norfloxacin} + \text{DNA} \rightleftharpoons [\text{Norfloxacin} - \text{DNA}] \tag{2}$$

So the quenching constant can be considered as the equilibrium constant for the formation of complex.

Interaction Mechanism of Norfloxacin with DNA

The influence of ion strength on the fluorescence quenching is shown in Fig. 6. In order to avoid the competitive interaction of other ions known to interact with DNA by a nonelectrostatic mode, we used NaCl to change the ion strength of solution. Although the emission intensity of the norfloxacin in absence and presence of dsDNA displays a slight decrease with the increasing ion strength, the change in the quenching efficiency F_0/F is very small. So it can be concluded that the interaction of the norfloxacin with DNA is not an electrostatic mode. Otherwise, the quenching efficiency would decrease greatly due to a weaker electrostatic interaction of norfloxacin with DNA when a positive atmosphere surrounds the DNA strand, in which Na^+ ions electrostatically bind to the phosphates of DNA.[29]

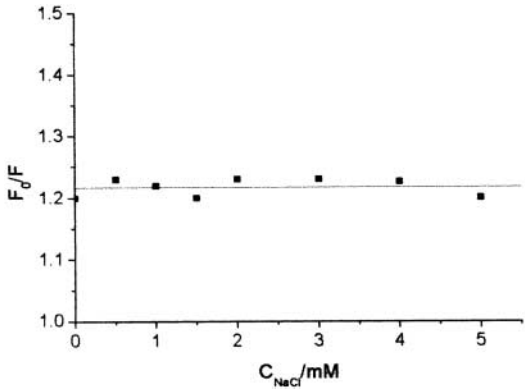

Figure 6. Influence of ionic strength on quenching efficiency. $C_{\text{norf}} = 4.0\,\mu\text{M}$, $C_{\text{DNA}} = 28.8\,\mu\text{M}$.

UV spectra of norfloxacin in absence and presence of DNA were studied for explaining the interaction mode between the norfloxacin and dsDNA. In pH 7.0 PBS, the maximum UV absorption of the norfloxacin is at 270 nm. Here the solutions containing the same concentrations of dsDNA are used as the blank solutions to observe the UV spectra of the norfloxacin complex with dsDNA. Both intensity and the position of UV absorption band do not change upon addition of DNA. So there is no intercalation between yeast dsDNA and the norfloxacin.[7] This result is similar to that between norfloxacin and calf thymus dsDNA concluded from the unwinding data.[22]

There are generally three interaction mechanisms between small molecules and nucleic acids[30]: (1) electrostatic interaction, small molecules electrostatically absorb on the phosphates of DNA chain, (2) intercalative binding, small molecules intercalate into the base pairs of the double stranded structure of DNA, (3) groove binding, small molecules act with the minor or major grooves of DNA chain. Above results have gotten rid of the electrostatic interaction and the intercalative binding mechanisms. The groove binding usually takes place in the AT enriched strands.[31] As known, yeast DNA is an AT enriched molecule,[23] therefore, the interaction between the norfloxacin and yeast dsDNA is possibly a groove binding. This mechanism is different from the interaction between norfloxacin and calf thymus dsDNA,[2,22] which is not an AT-enriched molecule.[23]

To better understand the interaction mechanism between norfloxacin and DNA, the interaction between the norfloxacin and yeast ssDNA was

studied. The experimental results indicated that yeast ssDNA possessed the same quenching ability as that of dsDNA, and the λ_{em} of norfloxacin did not change upon addition of yeast ssDNA, which also suggested that the interaction was a groove binding mode, not an electrostatic interaction or an intercalative binding.[32]

Quantitative Determination of DNA

Based on the fluorescence quenching of norfloxacin by DNA, a calibration plot for the determination of DNA concentration is constructed at a norfloxacin concentration of 4.0 μM. The plot of $(F_0/F-1)$ vs. the concentration of yeast dsDNA is linear ranging from 0.077 to 43.2 μM. The linear equation at 15°C is expressed as follows

$$F_0/F - 1 = 2.90 \times 10^4 C_{\mathrm{DNA}} - 0.0094 \tag{3}$$

The correlation coefficient r is 0.9995. The detection limit is 77 nM at which the fluorescence emission peak signal of norfloxacin shows a decrease of three times noise (3×0.36) of the spectrum of norfloxacin without presence of DNA. Eleven replicate analyses of a test solution containing 28.8 μM yeast dsDNA with the general procedure at different days give a relative standard deviation of 3.0%. ssDNA has the same quenching effect on the norfloxacin, thus the simultaneous determination of the contents of ssDNA and dsDNA is impossible.

Influence of Hybridization on Fluorescence Spectrum of Norfloxacin Labeled to ssDNA

With EDC as a linkage, the carboxyl group on the quinolone ring of norfloxacin can react with the 3′-hydroxyl group of DNA.[33]

$$\mathrm{NORF}-\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}-\mathrm{OH} + 3'\mathrm{HO}-\mathrm{ssDNA} \xrightarrow{\mathrm{EDC}} \mathrm{NORF}-\overset{\overset{\mathrm{O}}{\|}}{\mathrm{C}}-\mathrm{O}-\mathrm{ssDNA} \tag{4}$$

After incubating a solution containing 1.0 μM norfloxacin, 1.0×10^{-4} M EDC and 4.0 μM yeast ssDNA in a 25°C water bath for 8 h, norfloxacin can be labeled to ssDNA molecule. Because of the presence of excessive ssDNA in comparison with norfloxacin and the large equilibrium constant of above reaction, the norfloxacin in the mixture may be considered to label to ssDNA thoroughly. Thus a separation step was unnecessary.

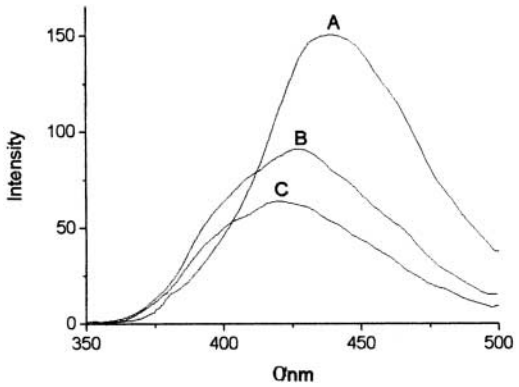

Figure 7. Fluorescence spectra of norfloxacin dsDNA mixture (A), norfloxacin-labeled ssDNA (B) and the hybridization product of norfloxacin labeled ssDNA with its cDNA (C) at the same concentration of DNA and a total norfloxacin concentration of 1.0 μM.

Figure 7 shows a shift of λ_{em} from 440 nm of the norfloxacin bound in dsDNA chain by a groove binding mode (curve A) to 420 nm of norfloxacin labeled to ssDNA (curve B). The shift is due to the reaction of the carboxyl group on the quinolone ring with the 3′-hydroxyl group of DNA, which reduces the π-conjugate system of norfloxacin, thus increases the energy difference between the ground state and the excited state and makes the norfloxacin molecule exhibit a shorter fluorescence emission wavelength. This change is similar to the shift of fluorescence emission wavelength caused from the decreasing protonation degree of 4-N on piperazinyl due to the increase of solution pH. The increase in the energy difference also decreases the emission intensity as shown in Fig. 7.

The hybridization was carried out by adding the cDNA to the solution including norfloxacin-labeled ssDNA and then incubating the mixture in a 60°C water bath for 1 h to form dsDNA. After the hybridization step, the λ_{em} of the norfloxacin labeled to the hybridization product shifts further from 420 nm of the norfloxacin labeled to ssDNA to 415 nm (curve C in Fig. 7) and the intensity decreases further. As discussed above and shown in Fig. 3B, the interaction of norfloxacin with DNA does not change its λ_{em}, thus the possibility of that norfloxacin in the conjugate interacts with free ssDNA can be excluded and the blue-shift of the emission peak indicates the hybridization even of ssDNA in the conjugate with free ssDNA. In other words, the hybridization event results in a shift of the λ_{em} of the norfloxacin labeled to ssDNA from 420 nm to 415 nm. The hybridization

step produces dsDNA helix, which squeezes norfloxacin in the stereo hindrance and changes its conjugate structure, resulting in a fluorescence hypochromicity. After the yeast ssDNA labeled with the norfloxacin reacts with calf thymus ssDNA in the same procedure, as a control, no change of the fluorescence emission spectrum is observable. Thus, the non-complementary sequences do not affect the spectrum of the norfloxacin labeled to ssDNA, and the cDNA sequence can be qualitatively detected by the shift of λ_{em} of the norfloxacin labeled to ssDNA.

The norfloxacin-labeled calf thymus ssDNA also demonstrates a fluorescence hypochromicity when it hybridizes with its cDNA. Thus, norfloxacin, as a native DNA hybridization indicator, could be applied to the detection of other native DNA chains.

CONCLUSIONS

Yeast DNA can strongly quench the fluorescence emission of norfloxacin. The interaction between norfloxacin and yeast DNA is suggested to be a groove-binding mode with a binding constant of $2.90 \times 10^4\,M^{-1}$ at 15°C. The difference between the binding constants at 15°C and 31°C indicates a static quenching mode. Based on the fluorescence quenching, native yeast dsDNA can be quantitatively determined according to Stern-Volmer equation. With EDC as a linkage, norfloxacin is labeled to ssDNA. The norfloxacin-labeled ssDNA behaves a different fluorescent character after it hybridizes with cDNA, producing a method to detect qualitatively DNA sequence.

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