THE DNA – CINOXACIN INTERACTION CHARACTERIZATION

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Cinoxacin is a quinolone antibiotic used for the treatment of urinary tract infections. It inhibits to DNA gyrase, causing in double-stranded DNA breaks and cell death. The interaction of cinoxacin with salmon sperm DNA has been studied using UV-Vis absorption and fluorescence spectroscopy. The quenching mechanism was studied, and the results suggested that both dynamic and static quenching processes were responsible for the positive deviation in the Stern-Volmer plot. The binding mode was investigated in terms of different models, such as Benesi and Hildebrand, Scott and Scatchard, supposing that a 1:1 complex between cinoxacin and DNA was formed. The results of the spectroscopic measurements suggested that the fluorescence quenching of cinoxacin by salmon sperm DNA is due to an electron transfer from the DNA to the excited-state of the quinolone antibiotic. These results contribute to deciphering the intimate action mechanism of cinoxacin on the bacterial DNA level.

Keywords: cinoxacin, DNA, UV-Vis absorption spectroscopy, fluorescence spectroscopy

1. Introduction

Cinoxacin (Fig. 1) is a synthetic compound with antimicrobial activity related to oxolinic and nalidixic acids, one of first-generation quinolones [1].

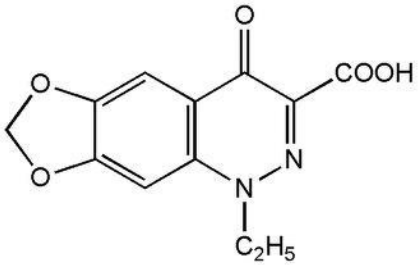

Fig. 1. The structure of cinoxacin

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The name of cinoxacin is derived from the basic ring structure which is a cinnoline (or 1-2 benzodiozine). Chemically, it is 1-ethyl-1,4-dihydro-4-oxo-(1,3)-dioxolo-(4,5-g)cinnoline-3-carboxylic acid [2].

Cinoxacin is one of the quinolone drugs which were introduced in the 1970s. It was patented in June 13, 1972 and assigned to Eli Lilly & Company [3]. Over time, it was found out that cinoxacin is effective in the treatments of several bacterial infections, such as ~~that~~ those of *Escherichia coli*, *Proteus mirabilis*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* [1, 4, 5].

Evidence exists that cinoxacin binds to DNA, interfering with synthesis of RNA and, consequently, with protein synthesis. It appears to also inhibit to DNA gyrase, causing in double-stranded DNA breaks and cell death [2, 4, 5].

The fluorescence quenching requires molecular contact between the fluorophore and quencher. This contact can be due to diffusive encounters, which is dynamic quenching or to complex formation, which is static quenching [6]. There are different types of chemical and/or electronic interactions that cause quenching. These interactions include intersystem crossing, electron exchange and photoinduced electron transfer. Since quenching occurs over short distances, the diffusion is needed for efficient quenching [6].

In this paper, we have used the steady-state fluorescence spectroscopy to investigate the fluorescence quenching of cinoxacin antibiotic with salmon sperm DNA. Various parameters responsible for fluorescence quenching have been determined by different models (the formation of ground state complex, the sphere of action static quenching and the finite sink approximation). Based on these parameters, possible fluorescence quenching mechanisms are discussed. Moreover, by viscosity measurements, the binding mode of cinoxacin to salmon sperm DNA is estimated.

2. Materials and methods

Cinoxacin was obtained from Sigma Aldrich, Germany and salmon sperm DNA was obtained from Ogata Research Laboratory, Chitose, Hokkaido, Japan. The stock solutions of cinoxacin and DNA were prepared in 0.15 M NaCl aqueous solution.

The UV-Vis absorption spectra of cinoxacin were recorded in the absence and in the presence of DNA using a Lambda25 UV-Vis spectrophotometer from Perkin-Elmer, USA, at room temperature, with quartz cells. The steady state fluorescence spectra in the cinoxacin - DNA system was recorded by exciting the samples at 357 nm corresponding to the longer wavelength of absorption band of cinoxacin using a Jasco FP-6500 spectrofluorometer.

Experimental data were processed with the OriginPro9 program, determining the binding parameters and the quality regression parameters.

All experimental data are reproducible within 5-7 % of experimental error.

3. Results and discussion

The UV-Vis absorption spectra of cinoxacin with various amounts of DNA are presented in fig. 2 and they reveal the presence of two absorption peaks located at 256 nm and 357 nm. By increasing the polymer/drug concentration ratio (P/D), a gradual increase in absorbance of the strong peak from 256 nm, accompanied with a decrease of the small peak from 357 nm was observed. At the same time, the occurrence of an isosbestic point located at ~305 nm indicates the existence of bound and free cinoxacin in equilibrium.

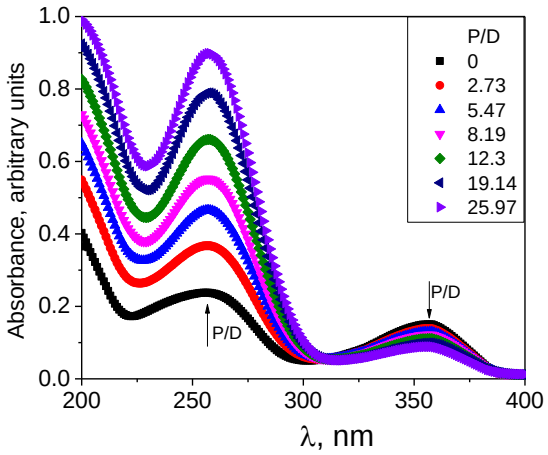

Fig. 2. Absorption spectra in cinoxacin – DNA system, at different polymer to drug ratios (P/D)

Fig. 3 shows the fluorescence emission spectra of cinoxacin with various amounts of salmon sperm DNA following an excitation at 357 nm.

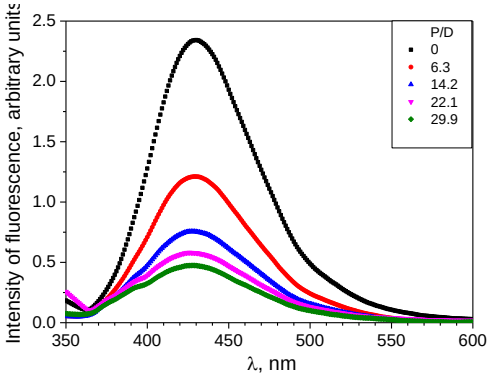

Fig. 3. Fluorescence emission spectra (λ_{ex} = 357 nm) in cinoxacin – DNA system, at different polymer to drug ratios (P/D)

It is observed that cinoxacin exhibits a strong fluorescence emission band located at 430 nm. The intensity of this band decreases gradually with the addition of

DNA. Moreover, there is a slight hypochromic displacement of 3-4 nm of this band with increasing of the polymer/drug concentration ratio (P/D).

In order to determine the binding mode of cinoxacin to salmon sperm DNA, the flow times for NaCl aqueous solution, DNA stock solution and cinoxacin - DNA mixtures were measured. It is known that an intercalative binding causes an increase in the viscosity of DNA solution since it requires a large space of the adjacent base pairs to accommodate the drug and to elongate the double helix [7]. The experimental data are presented as plots $(\eta/\eta_0)^{1/3}$ against the drug/polymer concentration ratio (D/P) (Fig. 4), where η and η_0 represent the viscosity of DNA in the presence and in the absence of drug.

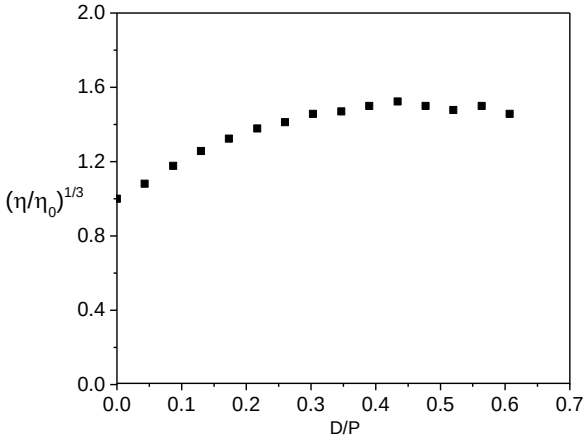

Fig. 4. Effect of increasing amounts of cinoxacin on the relative viscosity of DNA at room temperature

It can be observed the increase of the viscosity of DNA solutions after the drug addition, result which indicates the intercalation of cinoxacin between the pairs of nitrogenous bases from the nucleic acid structure. Similar results were presented in literature [8-10], this variation representing a strong evidence for cinoxacin molecules intercalation between the adjacent DNA bases pairs.

The fluorescence data from cinoxacin – DNA system were analyzed by the Stern–Volmer equation:

$$\frac{I_0}{I} = 1 + K_{SV} \cdot [Q] \tag{1}$$

where I_0 is the fluorescence intensity in the absence of quencher, I is the fluorescence intensity in the presence of quencher, K_{SV} is the Stern–Volmer quenching constant ($K_{SV}=k_q \cdot \tau_0$), k_q is the bimolecular quenching constant, τ_0 is the lifetime of fluorophore (cinoxacin in our case), in the absence of quencher and $[Q]$ is the concentration of quencher (DNA in our case).

Equation (1) is true as long as the experimental results show linear variation. However, often negative or positive deviations from linearity in Stern-Volmer plots from different fluorophore – quencher molecular systems were observed. These deviations from linearity in Stern-Volmer plots are indications on different mechanisms of quenching which are usually classified as either dynamic or static quenching [10-12].

Fig. 5 shows the Stern-Volmer plot in cinoxacin - DNA system.

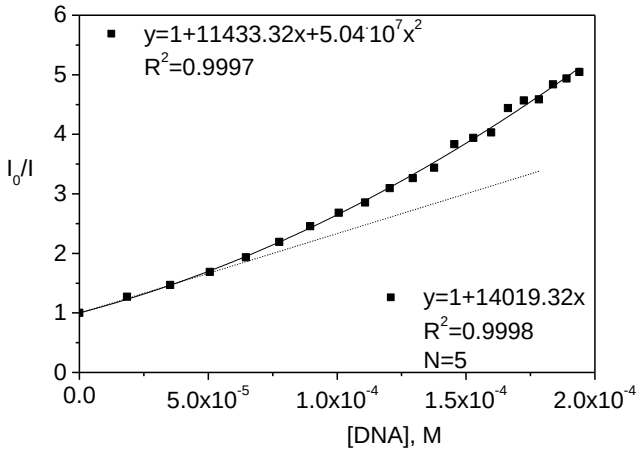

Fig.5. The Stern–Volmer plot in cinoxacin - DNA system

It can be observed that the Stern-Volmer plot in cinoxacin – DNA system is linear only at low levels of concentration of DNA ($<7 \cdot 10^{-5}$ M) and shows a positive deviation from linearity for higher concentrations of DNA. These observations suggest that the dynamic quenching process operates only at low concentration of quencher.

By linear regression of the first experimental points, the Stern–Volmer quenching constant (K_{SV}= 13800 M^{-1}) characteristic for the dynamic quenching process was obtained. Knowing the lifetime of cinoxacin (τ_0=1.4 ns), the bimolecular quenching constant k_q ($k_q=9.8 \cdot 10^{12}$ $M^{-1}s^{-1}$) has been determined. At the same time, it is observed that the value for the bimolecular quenching constant k_q is much greater than the maximum diffusion collision quenching rate constant ($2 \cdot 10^{10}$ $M^{-1}s^{-1}$) of a variety of quenchers with biopolymer [13, 14]. This result eliminates the hypothesis of pure dynamic quenching in the cinoxacin - DNA system.

In order to understand the complex nature of quenching process of cinoxacin with salmon sperm DNA, two models were used: i) formation of ground state complex, and ii) action sphere of static quenching.

The first model uses the following extended Stern-Volmer equation:

$$(\frac{I_0}{I}-1)\cdot\frac{1}{[Q]}=(K_{SV}+K_a)+K_{SV}\cdot K_a\cdot[Q] \quad (2)$$

while the second model uses the following modified form of Stern-Volmer equation:

$$(1-\frac{I}{I_0})\cdot\frac{1}{[Q]}=K_a\cdot\frac{I}{I_0}+\frac{1-w}{[Q]} \quad (3)$$

where K_a *is* the association (or static quenching) constant and *w* is the fraction of the excited state which is quenched by the collisional mechanism.

Fig. 6 shows the plot of $(\frac{I_0}{I}-1)\cdot\frac{1}{[Q]}$ versus *[Q]* in cinoxacin – DNA system, in accordance with assumptions from the first model.

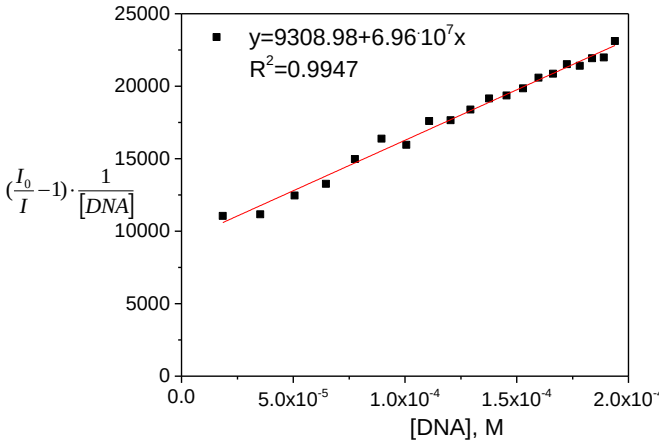

Fig. 6. The $(\frac{I_0}{I}-1)\cdot\frac{1}{[Q]}$ versus [Q] plot in cinoxacin - DNA system

The application of this first model did not allow determination of static and dynamic quenching constants since they exhibited the imaginar component. Nevertheless, the plot obtained allowed us to assume that the fluorescence quenching in cinoxacin - DNA system has occurred without involving ground-state complex formation.

Based on the assumptions from the sphere of action static quenching model, in the cinoxacin - DNA system, the linear plots (Figs. 7 and 8) with correlation coefficients nearly equal to unity were obtained.

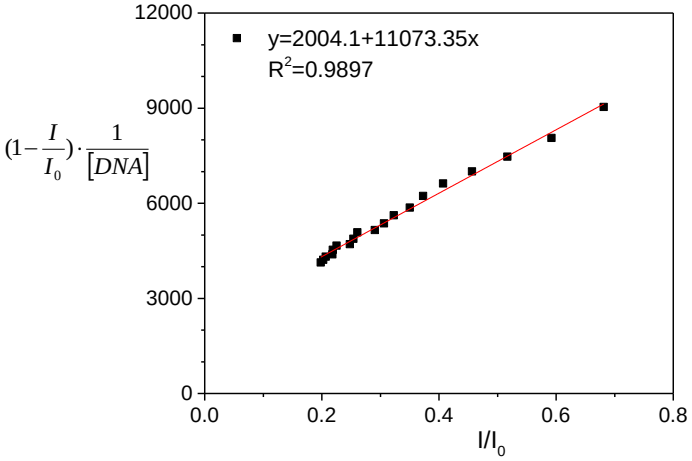

Fig. 7. The $(1-\frac{I}{I_0})\cdot\frac{1}{[Q]}$ versus I/I_0 plot in cinoxacin - DNA system

In the second model, a certain fraction (w) of the excited state is quenched by the dynamic (collisional) mechanism while the remaining fraction of the excited state (1–w) is deactivated almost instantaneously after the formation of the complex.

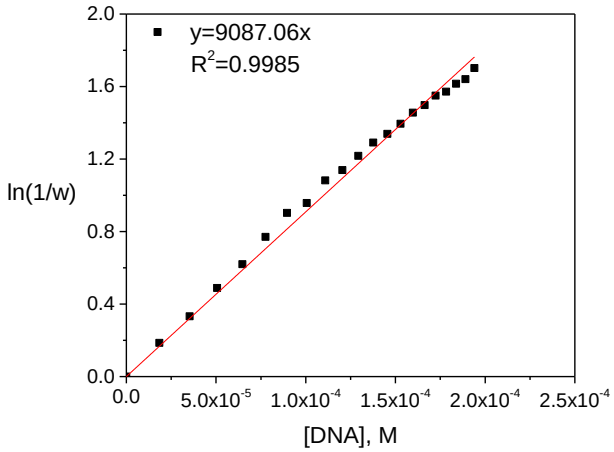

Fig. 8. The ln(1/w) versus *[Q]* plot in cinoxacin - DNA system

The value of K_a obtained in the sphere of action model was confirmed by the results obtained in the methods proposed by Benesi and Hildebrand (1949), Scatchard (1949) and Scott (1956) [15-17], supposing that one complex 1:1 between cinoxacin and salmon sperm DNA was formed. The Benesi and Hildebrand, Scatchard and Scott equations and the results obtained from these models are summarized in Table 1. It is observed that similar values for the association (static quenching) constant K_a were obtained for the four methods.

Table 1

The association constant in cinoxacin – DNA system

Methods	Equations	K_a, M^{-1}	R^2
sphere of action model	$(1-\frac{I}{I_0})\cdot\frac{1}{[DNA]}=K_a\cdot\frac{I}{I_0}+\frac{1-w}{[DNA]}$	9880	0.9897
Benesi-Hildebrand	$\frac{l}{\Delta I}=\frac{1}{C^0K_a\Delta\varepsilon[\mathrm{DNA}]}+\frac{1}{C^0\Delta\varepsilon}$	9900	0.9988
Scott	$\frac{l[DNA]}{\Delta I}=\frac{1}{C^0\Delta\varepsilon}[DNA]+\frac{1}{C^0K_a\Delta\varepsilon}$	9868	0.9991
Scatchard	$\frac{\Delta I}{l[DNA]}=-\frac{K_a}{l}\Delta I+C^0K_a\Delta\varepsilon$	9808	0.9795

where $\Delta\varepsilon=\varepsilon_B-\varepsilon_F$, ε_F and ε_B are the free and bound drug absorption coefficients,
l is path length, ΔI - the observed intensity of fluorescence change,
C^0 - the total concentration of drug, $[DNA]$ - DNA concentration, K_a - the association constant

To find out whether the reactions are diffusion limited one can use the finite sink approximation model [18-20]. Hence, the values of K_{SV} were determined at each quencher concentration and the Stern–Volmer plots of $\frac{1}{K_{SV}}$ versus $[Q]^{\frac{1}{3}}$ (Fig. 9) were realized, according to equation (4):

$$\frac{1}{K_{SV}}=\frac{1}{K_{SV}^0}-\frac{(2\pi N_A)^{\frac{1}{3}}}{4\pi N_A D\tau_0}\cdot[Q]^{\frac{1}{3}} \tag{4}$$

where K_{SV}° is the Stern-Volmer constant at [Q]=0 M and D is the mutual diffusion coefficient of the reactants ($D=D_{cinoxacin}+D_{DNA}$).

Based on the obtained values for the y-intercept and negative slope of the linear plot presented in Fig. 9, the values of D and K_{SV}° parameters were calculated. Moreover, the distance parameter R', the diffusion rate constant k_d and the activation energy controlled rate constant k_a were determined according to equations (5 - 7):

$$K_{SV}^0=\frac{4\pi N_A D\tau_0 Rk_a}{4\pi N_A DR+k_a}=4\pi N_A D\tau_0 R' \tag{5}$$

$$k_d=4\pi N_A DR' \tag{6}$$

$$k_a=\frac{4\pi N_A DRR'}{R-R'} \tag{7}$$

knowing the lifetime of cinoxacin (τ_0) and the Avogadro number (N_A).

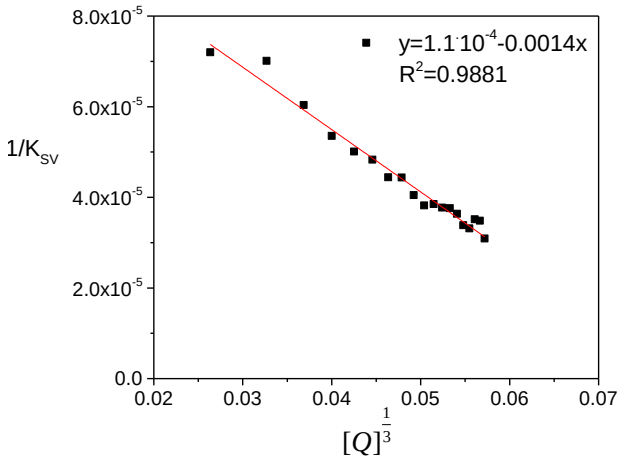

Fig. 9. The $\frac{1}{K_{SV}}$ versus $[Q]^{\frac{1}{3}}$ plot in cinoxacin - DNA system

The encounter distance *R* was calculated by adding the radii of cinoxacin and DNA molecules. The radius of cinoxacin molecule ($R_{cinoxacin}$) was determined by adding the atomic volumes of all the atoms constituting the molecule, as suggested by Edward, 1970 [21]. A value of 3.67 Å was calculated for the cinoxacin molecule radius and a value of 10 Å was taken as a reference for the radius of the DNA molecule [10] and, consequently, a value of 13.67 Å was assumed for the encounter distance *R*.

The obtained results for cinoxacin – DNA system in the assumptions from the finite sink approximation model are shown in Table 2.

Table 2

Parameters obtained by the finite sink approximation model for the cinoxacin – DNA system

***D*, dm^{2}s^{-1}**	***R'*, Å**	***k*$_d$, M^{-1}s^{-1}**	***k*$_a$, M^{-1}s^{-1}**	***k*$_q$, M^{-1}s^{-1}**
$1.15 \cdot 10^{-5}$	8.11	$6.99 \cdot 10^{12}$	$17.2 \cdot 10^{12}$	$9.8 \cdot 10^{12}$

Note: $R_{cinoxacin}$= 3.67 Å, R_{DNA}= 10 Å, R= 13.67 Å

It is observed that the value of the diffusion rate constant k_d ($6.99 \cdot 10^{12}$) is smaller than the values of the energy activation rate constant k_a ($17.2 \cdot 10^{12}$) and the bimolecular quenching constant k_q ($9.8 \cdot 10^{12}$) and, thereupon, the bimolecular quenching reaction in the cinoxacin - DNA system is considered to be controlled by diffusion. These results are in agreement with other results presented in literature [10, 22, 23].

4. Conclusions

The interaction of cinoxacin with salmon sperm DNA has been studied using UV-Vis absorption and fluorescence spectroscopy.

The viscosity measurements results indicated that the binding mode of cinoxacin to salmon sperm DNA is an intercalation binding. Determining the preferred binding site of cinoxacin in the DNA structure is of great importance in understanding the action mechanism of this antibiotic and, implicitly, of its antibacterial spectrum of action.

Nonlinear Stern-Volmer plots having an upward, positive curvature were obtained. This positive curvature of Stern-Volmer plots is a proof of the simultaneous presence of both static and dynamic quenching processes.

The analysis of the experimental data by the sphere of action and finite sink approximation models emphasizes the presence of a static quenching, but insignificant with respect to the dynamic one. This result is confirmed by the values obtained for the association (or static quenching) constant and the constant of the dynamic quenching. Also, it was found that the quenching reaction in the cinoxacin – DNA system is controlled by diffusion.

In conclusion, the analysis of absorption and emission spectra allowed the emphasis of a strong interaction between DNA and cinoxacin, manifested by a quench of fluorescence of the fluorophore studied with increasing molar DNA/ fluorophore ratio. We believe that our study leads to a better understanding of the role of the two compounds in the biological environment, the results being applicable in different fields, such as medicine, pharmacology, and biotechnology.

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