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Anthraquinones quinizarin and danthron unwind negatively supercoiled DNA and lengthen linear DNA

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ABSTRACT

The intercalating drugs possess a planar aromatic chromophore unit by which they insert between DNA bases causing the distortion of classical B-DNA form. The planar tricyclic structure of anthraquinones belongs to the group of chromophore units and enables anthraquinones to bind to DNA by intercalating mode. The interactions of simple derivatives of anthraquinone, quinizarin (1,4-dihydroxyanthraquinone) and danthron (1,8-dihydroxyanthraquinone), with negatively supercoiled and linear DNA were investigated using a combination of the electrophoretic methods, fluorescence spectrophotometry and single molecule technique an atomic force microscopy. The detection of the topological change of negatively supercoiled plasmid DNA, unwinding of negatively supercoiled DNA, corresponding to appearance of DNA topoisomers with the low superhelicity and an increase of the contour length of linear DNA in the presence of quinizarin and danthron indicate the binding of both anthraquinones to DNA by intercalating mode.

1. Introduction

DNA as a molecule of great biological significance that carries the genetic information is a major target of drugs which in turn interfere with many intracellular processes by binding to DNA. The drug-induced modifications in the mechanical or topological properties of DNA can have a profound impact on the metabolism of cells [1,2]. Therefore, the investigation of DNA–drug interactions is very important step for an understanding of the intracellular processes and plays a key role in the rational design of new potential drugs against diseases [3,4]. Indispensable to an understanding of DNA–drug interactions is a characterization of the binding modes. In addition to covalent binding there exist several classes of non-covalent binding of drugs to DNA including intercalation and groove binding [5].

The intercalating drugs represent a large group of compounds with various kinds of structures, but all of them possess a planar aromatic chromophore unit by which the intercalators insert between DNA bases causing the distortion of classical B-DNA form [6,7]. The planar tricyclic structure of anthraquinone belongs to the class of the chemical structures of common intercalating chromophore units [3,6]. The group of anthraquinones including quinizarin (1,4-dihydroxyanthraquinone) and danthron (1,8-dihydroxyanthraquinone) seems to be interesting from view of its pharmaceutical utilization. Quinizarin (Fig. 1A) is a part of the anticancer drugs such as doxorubicin, daunorubicin, and adriamycin which interact with DNA by intercalating mode. Danthron (Fig. 1B) is presented in other drugs as aclacinomycin, emodin, and hypericin. The interaction of both anthraquinones, quinizarin and danthron, with DNA has been already investigated using spectroscopic and electrochemical techniques revealing the different types of binding modes as intercalation [8,9], partial intercalation and hydrogen binding [10] or groove binding [11,12]. Moreover, it has been reported that the antraquinones just with one or two more OH groups compared to quinizarin and danthron interact with DNA by intercalating mode [13–15]. In order to enlarge the number of approaches investigating the interaction of quinizarin and danthron with DNA in more details, it was decided to investigate the binding mode of interaction of both anthraquinones with negatively supercoiled and linear DNA by using the electrophoretic methods such as agarose gel electrophoresis (AGE), temperature

Abbreviations: AGE, agarose gel electrophoresis; TGGE, temperature gradient gel electrophoresis; AFM, atomic force microscopy; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetic acid; APTES, 3-aminopropyltriethoxysilane; Tris, tris(hydroxymethyl)aminomethane; TBE, tris–borate–EDTA; EtBr, ethidium bromide.

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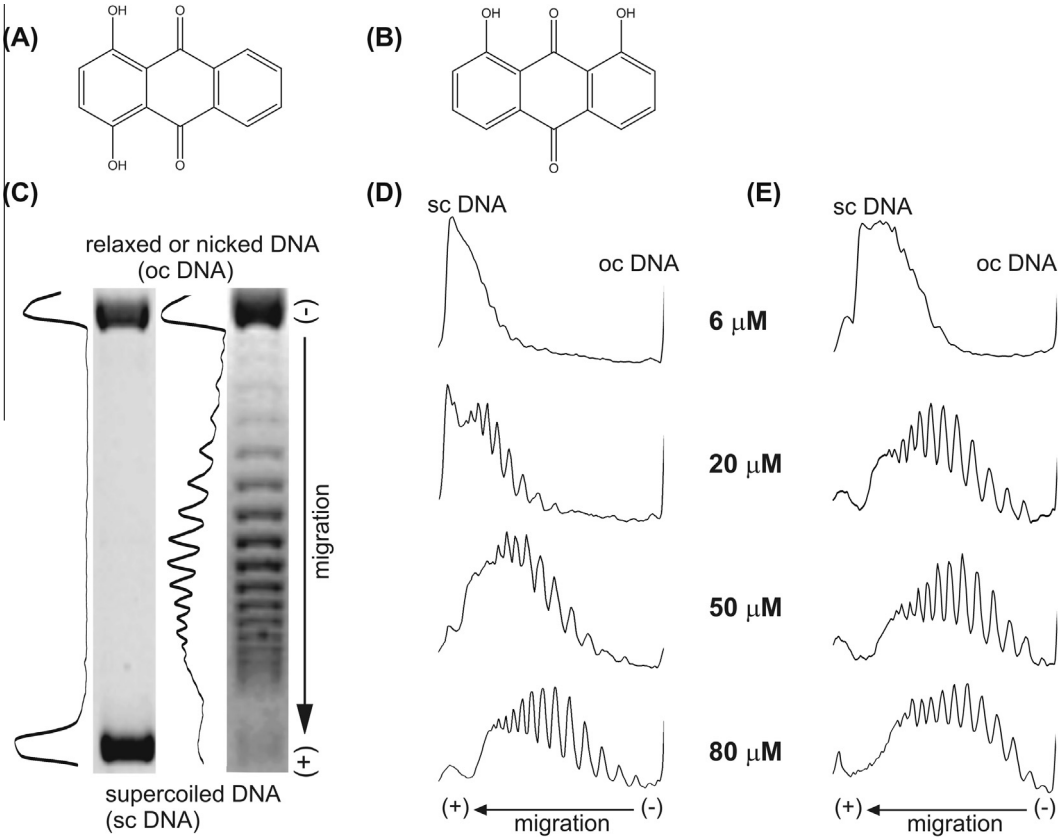

Fig. 1. Structure of anthraquinone (A) quinizarin and (B) danthron. (C) The electrophoregram of the agarose gel electrophoresis of negatively supercoiled plasmid pRES3 in the absence (left part) and in the presence of 8×10^{-5} M quinizarin (right part) with corresponding densitometric scans. The densitometric scans obtained from the electrophoregrams of agarose gel electrophoresis of negatively supercoiled plasmid pRES3 at given concentrations of (D) quinizarin and (E) danthron.

gradient gel electrophoresis (TGGE) combining with a single molecule technique atomic force microscopy (AFM). The fluorescence spectrophotometry was also used to estimate the binding characteristics of both anthraquinones. Obtained results, unwinding of negatively supercoiled DNA and lengthening of linear DNA, revealed the binding of quinizarin and danthron to DNA by intercalating mode.

2. Materials and methods

2.1. Materials

Quinizarin, Danthron, Dimethyl sulfoxide (DMSO), Ethylenediaminetetraacetic acid (EDTA), 3-aminopropyltriethoxysilane (APTES), agarose type II No. A-6877, Tris(hydroxymethyl)aminomethane (Tris), Boric acid, Ethidium bromide and calf thymus DNA were purchased from Sigma. The restriction enzyme BamHI was purchased from Fermentas. Stock solutions of quinizarin and danthron were prepared by dilution in DMSO. Maximum level of DMSO in the samples was less than 1% v/v in all experiments.

2.2. Supercoiled and linear plasmid DNA preparation

The negatively supercoiled plasmids pRES3 [16] and pUC19-ATGC14 [17] and linear plasmid pRES3 were isolated and purified as described previously [18]. The linear plasmid pRES3 was prepared by the digestion of supercoiled plasmid pRES3 with restriction enzyme BamHI.

2.3. Agarose gel electrophoresis

Negatively supercoiled plasmid pRES3 was separated by electrophoresis on a 0.8% agarose gels buffered with 0.5 × Tris–Borate–EDTA (TBE) buffer (40 mM Tris, 2 mM EDTA, 90 mM boric acid, pH 8.3) in the presence of various concentrations of anthraquinones for 16 h at 60 V/16 mA or 85 V/20 mA. The electrophoretic gel was stained with the fluorescent intercalating dye ethidium bromide (EtBr) (0.5 μg/ml) per 1 h and DNA in the gel was visualized using a UV transilluminator and photographed with a Camera 3030 digital apparatus.

2.4. Temperature gradient gel electrophoresis

TGGE experiments were performed using the same equipment and conditions as previously described [19]. Briefly, the electrophoretic separation of negatively supercoiled plasmid pUC19-ATGC14 in the absence or in the presence of anthraquinones was performed using 1% agarose gel in 0.5 × TBE buffer at 8.5 V/cm for 4 h. Visualization of DNA was performed by the same procedure as at AGE experiments.

2.5. Atomic force microscopy

Negatively supercoiled and linear plasmid pRES3 in the absence or in the presence of anthraquinones were adsorbed on mica substrate chemically modified with APTES (called as AP-mica) following the protocol described by Adamcik et al. [20]. A final concentration of DNA in all solutions was 10 ng/μl. A 10 μl aliquot

of the DNA solution was deposited onto AP-mica and incubated for 30 s at room temperature. The sample was then rinsed with nanopure water and dried by air. Images were collected using a Multimode 8 (Bruker) operated in tapping mode under ambient conditions. Images were simply flattened using the Nanoscope 8.1 software and no further image processing was carried out. The contour length of linear DNA was estimated by the software Ellipse [21].

2.6. Fluorescence spectrophotometry

Fluorescence binding experiments were performed on a Shimadzu RF-5301 PC spectrofluorophotometer using a 1-cm light-path quartz cell with 1 ml volume in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.0). The fluorescence spectra of quinizarin (spectral region 490–750 nm) and danthron (spectral region 420–750 nm) were obtained upon excitation at 475 and 410 nm, respectively. The emitted fluorescence was analyzed at 540 nm for quinizarin and 550 nm for danthron. The various anthraquinone/DNA complexes were researched by changing the concentration of calf thymus DNA in range 10^{-7}–10^{-3} M at constant concentration (10^{-6} M) of anthraquinones.

3. Results and discussion

The supercoiled plasmid DNA represents the covalently closed circular molecules which occur in nature in the negatively supercoiled conformation with the superhelicity about −0.05 and with the Gaussian distribution of DNA topoisomers. Agarose gel electrophoresis (AGE) is one of the methods used to analyze the supercoiled DNA according to the different values of superhelicity [22]. The observation of individual topoisomers of negatively supercoiled DNA isolated from bacteria is not possible detect by AGE and DNA topoisomers migrate as one intensive band as a consequence of the high superhelicity. However, the negatively supercoiled DNA in the presence of intercalators undergoes the topological change, called as unwinding of negatively supercoiled DNA, when the high superhelicity decreases and the distribution of individual DNA topoisomers with the lower values of superhelicity is very well detected by electrophoresis.

Fig. 1C (left part) displays the electrophoregram with corresponding densitometric scan of negatively supercoiled plasmid pRES3 isolated from bacteria. Only two intensive bands corresponding to negatively supercoiled DNA and nicked or relaxed DNA were observed. Fig. 1C (right part) shows the electrophoregram with corresponding densitometric scan of negatively supercoiled plasmid pRES3 in the presence of 8×10^{-5} M quinizarin. In this case the distribution of DNA topoisomers with the lower superhelicity and with the reduced electrophoretic mobility was very well detected while the intensive band corresponding to negatively supercoiled DNA with the high superhelicity was diminished. In order to study the effect of different concentrations of anthraquinones on a behavior of negatively supercoiled DNA the series of AGE experiments were performed where the electrophoretic mobility of negatively supercoiled plasmid pRES3 was analyzed in the presence of quinizarin and danthron in the concentration range of 6×10^{-6}–2×10^{-4} M. The densitometric scans of the electrophoregrams of negatively supercoiled plasmid pRES3 at different concentrations of quinizarin and danthron are displayed in Fig. 1D and Fig. 1E, respectively. At low concentration of anthraquinones (6×10^{-6} M) the individual DNA topoisomers were still not detected. With an increase of anthraquinone concentration (5×10^{-5} M) the appearance of distribution of DNA topoisomers with the low superhelicity was observed and the electrophoretic mobility of individual DNA topoisomers was detected. The observed topological change of negatively supercoiled plasmid pRES3 in the presence of quinizarin and danthron allows to suggest that the anthraquinones interact with DNA by intercalating mode. Thus, the anthraquinones quinizarin and danthron unwind negatively supercoiled DNA.

AGE gives the information about the topology of negatively supercoiled DNA in the presence of intercalating drugs at constant temperature. It has been shown that TGGE can also be used to study DNA–drug interaction [23,24]. TGGE detects over a wide range of temperature the conformational changes of DNA occurring in different topological states in the presence of drugs. In addition, this technique has been also applied to study the extrusion of cruciform structure in negatively supercoiled DNA containing the palindromic sequences in the presence of well-known intercalating drug used in molecular biology chloroquine [17].

Fig. 2 displays the electrophoregrams of negatively supercoiled plasmid pUC-ATGC14 in the absence of anthraquinones (Fig. 2A) and in the presence of 25×10^{-6} M quinizarin (Fig. 2B) and 25×10^{-6} M danthron (Fig. 2C). The bands represent the mobility of individual DNA topoisomers at different temperatures. In the absence of anthraquinones (Fig. 2A) the most abundant negatively supercoiled DNA migrates as a single unresolved band at lower temperatures (similar electrophoretic mobility of DNA topoisomers as in the case of AGE experiments), but at temperatures above 45 °C different DNA topoisomers were distinguishable. In the presence of quinizarin (Fig. 2B) and danthron (Fig. 2C) the population of DNA topoisomers with lower superhelicity was detected and the migration of individual DNA topoisomers over the whole

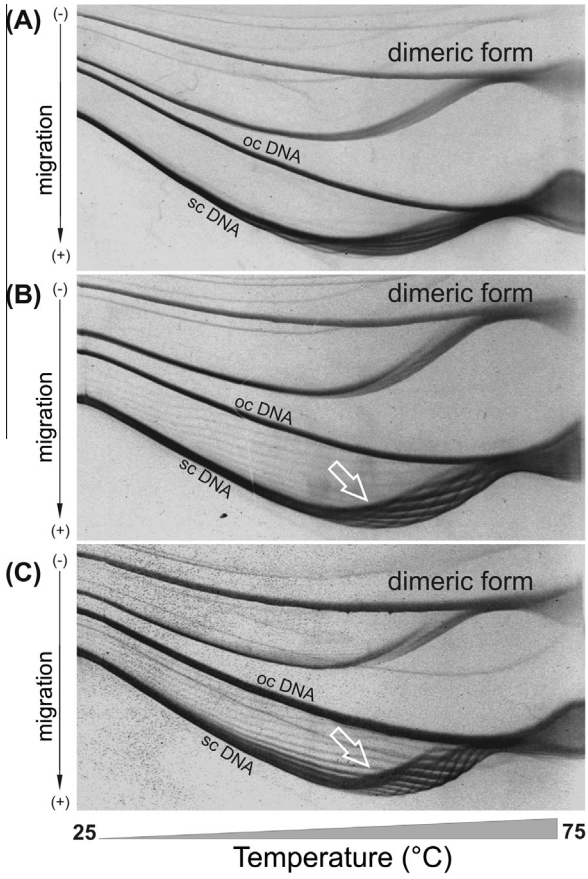

Fig. 2. TGGE electrophoregram of negatively supercoiled plasmid pUC19-ATGC14 (A) in the absence of anthraquinones, (B) in the presence of 25×10^{-6} M quinizarin and (C) in the presence of 25×10^{-6} M danthron. The arrows indicate the extrusion of cruciform.

temperature range was observed. In addition, due to the presence of anthraquinones the extrusion of cruciform (indicated by arrows) was also observed similar to previously reported results [17]. These results also allow suggesting that quinizarin and danthron interact with negatively supercoiled DNA by intercalating mode.

Previously, it has been shown that by the interaction of typical intercalating agent such as EtBr or anticancer drug such as doxorubicin (quinizarin is a part of doxorubicin) with plasmid DNA the negatively supercoiled DNA convert to the positively supercoiled DNA due to the strong intercalating effect [23]. Moreover, TGGE experiments could also help to describe the reversibility/irreversibility of binding of intercalating agents to DNA [24]. Since the presence of positively supercoiled DNA was not detected the lower binding affinity of quinizarin and danthron to DNA in comparison with the typical intercalating ligand could be expected.

AFM was also applied to investigate the binding of quinizarin and danthron to negatively supercoiled and linear plasmid pRRES3. In comparison with electrophoretic methods as bulk techniques, AFM allows us to observe the behavior of DNA molecules in the presence of drugs at single molecule level [25]. Electrophoresis gives the information about the shape of DNA topoisomers in the presence of intercalating agents based on the electrophoretic mobility in gel and AFM provides the information about the structural change of DNA topoisomers [23]. The topological conformation of negatively supercoiled DNA at different concentrations of intercalating agents has been evaluated by AFM showing the transformation of the negatively supercoiled DNA in plectonemic conformation to the positively supercoiled DNA in toroidal [26] or in highly tight plectomenic conformation [27]. In the absence of intercalators at low ionic strength the negatively supercoiled DNA breaths and small single stranded bubbles have been detected [28]. To observe negatively supercoiled DNA in the presence of quinizarin and danthron the same concentrations of anthraquinones as in electrophoretic experiments were used.

Fig. 3A shows negatively supercoiled plasmid pRES3 in the absence of anthraquinones and DNA molecules possess many crossings typical for the negatively supercoiled DNA. As negatively supercoiled plasmid pRES3 was in TBE buffer any single stranded bubbles were not observed. In the presence of 10^{-3} M quinizarin (Fig. 3B) and 10^{-3} M danthron (Fig. 3C) DNA molecules were more relaxed just with few crossings. These results also confirm the unwinding of negatively supercoiled DNA by anthraquinones, thus, they bind to DNA by intercalating mode.

The intercalators by insertion between DNA bases lengthen DNA molecules and AFM is a technique which is able to provide data to estimate the contour length of DNA. It has been reported that the investigation of the contour length of DNA in the presence of binding ligand provides very important information about the binding mode [29,30]. Fig. 3D displays AFM image of linear plasmid pRES2 in the absence of anthraquinones while Fig. 3E and F displays AFM image of linear plasmid pRES3 in the presence of 10^{-3} M quinizarin and 10^{-3} M danthron, respectively. The contour length of linear plasmid pRES3 in the absence of anthraquinones was estimated to be 1489 ± 20 nm while in the presence of quinizarin the contour length was 1647 ± 35 nm and in the presence of danthron it was 1673 ± 30 nm. This change corresponds approximately to a 10% increase in the contour length of DNA molecules which is smaller value compared to 35% in the case of EtBr [30]. Thus, in the presence of anthraquinones the lengthening of linear plasmid pRES3 was observed which again suggested the binding of quinizarin and dantron to DNA by intercalating mode.

Fluorescence spectrophotometry is an effective method to examine the binding mode of different ligands including determination of their binding characteristics. In order to perform fluorescence titration to examine the association of the anthraquinones with DNA the linear calf thymus DNA and similar concentrations of anthraquinones as in electrophoretic and AFM experiments were used (Fig. 4).

An increase of fluorescence intensity connected with a very small red shift were recorded when concentration DNA in complex was lower than anthraquinone concentration. This indicates an electrostatic or H-binding interaction mode between DNA and anthraquinones [31]. On the other hand, the fluorescence quenching of anthraquinones with blue shift of the emission peaks due to an increasing concentration of DNA was observed. Yang et al. [32] have found that fluorescence intensity of fluorescence probe

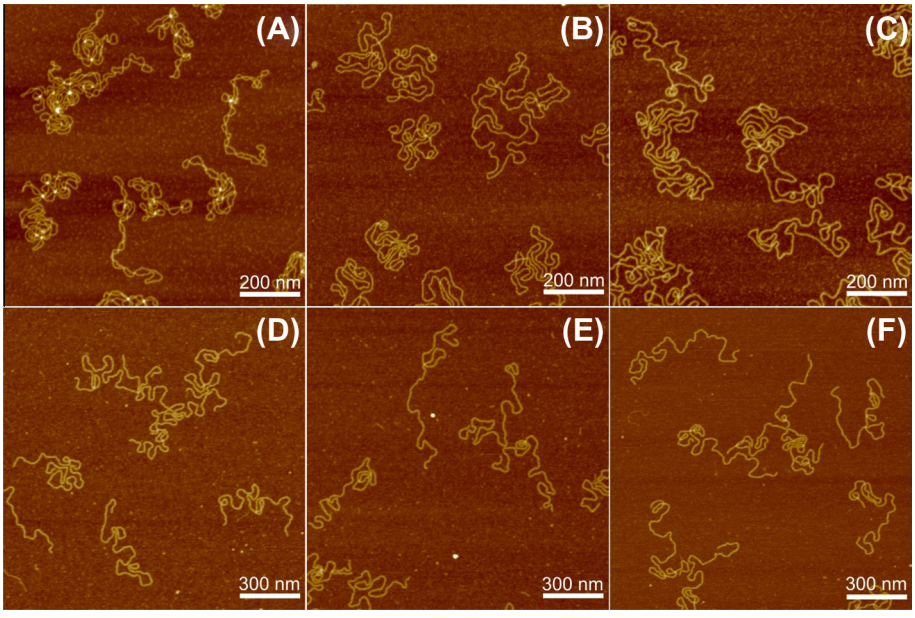

Fig. 3. AFM height image of negatively supercoiled plasmid pRES3 deposited on chemically modified mica by APTES (AP-mica) from 0.5 × TBE buffer (A) in the absence of anthraquinones, (B) in the presence of 10^{-3} M quinizarin and (C) in the presence of 10^{-3} M danthron. AFM height image of linearized pRES3 plasmid DNA deposited on AP-mica from 0.5 × TBE buffer (D) in the absence of anthraquinones, (E) in the presence of 10^{-3} M quinizarin and (F) in the presence of 10^{-3} M danthron. *Z* scale for all images is 4 nm.

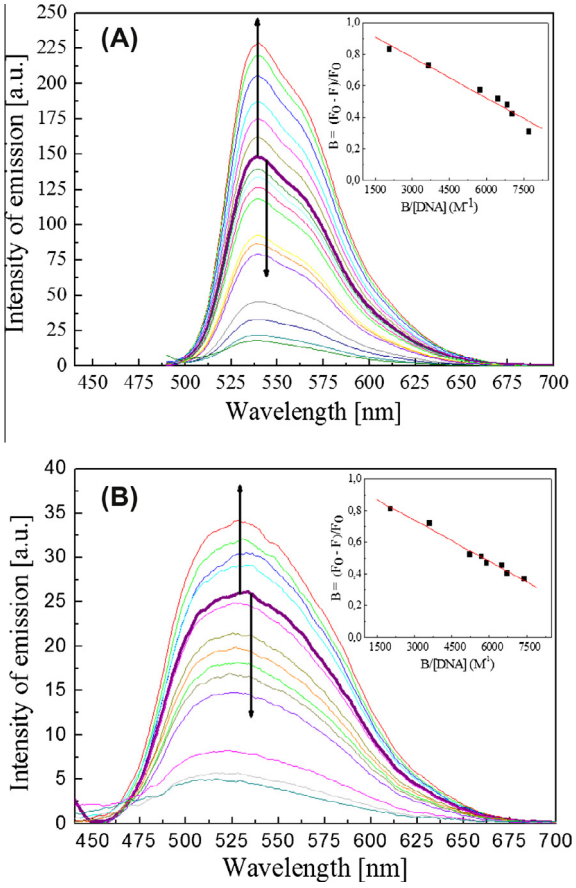

Fig. 4. (A) Dependence of quinizarin fluorescence on concentration of calf thymus DNA. Emission spectra were obtained upon excitation at 475 nm. (B) Dependence of danthron fluorescence on concentration of calf thymus DNA. Emission spectra were obtained upon excitation at 410 nm. The various anthraquinone/DNA complexes were researched at constant concentration of anthraquinones (10^{-6} M) and the concentrations of DNA in the range of 10^{-7}–10^{-3} M. Inset: Scatchard plot for calculation of the binding characteristics.

Table 1
Binding characteristics of DNA – quinizarin/danthron complex.

Ligand	K_d (10^{-5} M)	n	K_b (10^4 M^{-1})
Quinizarin	8.61 ± 0.82	1.04 ± 0.05	1.16
Danthron	8.64 ± 0.45	0.99 ± 0.03	1.15

3-methoxybenzanthrone increases with increasing solvent polarity. Plus the increasing solvent polarity causes bathochromic shift in fluorescence spectra [32,33]. Considering these findings the fluorescence intensity decrease and small blue shift as a consequence of anthraquinones interaction with DNA would tend to indicating that they enter DNA-stacking region with lower polarity rather than the bulk solution of the DNA. The observed phenomena (the fluorescence quenching and small blue shift) in our fluorescence spectra (Fig. 4) enable us to declare that the mode of interaction of anthraquinones with DNA is intercalation [34]. Moreover, it was found that pigment base stacking propensity and intercalation site specificity depend upon electrostatic interactions [35]. Electrostatic complementation, which is represented by fluorescence intensity increase, is an important factor in π – stacking interactions [36]. These findings could correspond to our results about intercalation demonstrated by fluorescence quenching and simultaneous existence of electrostatic interactions.

Fluorescence titration data were used to determine the binding constants (K_b) and the binding stoichiometry (n) for the anthraquinone/DNA complex formation [37]. Eq. (1) was used to estimate K_b and n:

$$(F_0 - F)/F_0 = -K_d[(F_0 - F)/F_0[DNA]] + n \quad (1)$$

where F_0 and F are the fluorescence intensities of anthraquinones in the absence and presence of DNA, respectively. Then a slope in Scatchard plot (Fig. 4 inset) represents dissociation constant $-K_d$ and n expressing the number of specific binding sites can be obtained from the value of y intercept. Fitting by linear function (1) gives results summarized in Table 1.

Inverse relationship between equilibrium association and dissociation constants provides the binding constant for quinizarin: $1.16 \times 10^4\ M^{-1}$ and for danthron: $1.15 \times 10^4\ M^{-1}$. The values of K_b indicate middle affinity of both anthraquinones to DNA and they are approximately equal which is not surprising with regard to their similar structure.

In conclusion, the interaction of quinizarin and danthron, two simple derivatives of anthraquinone, with negatively supercoiled and linear DNA was investigated by means of electrophoretic methods, fluorescence spectrophotometry and AFM. The presented results such as the unwinding of negatively supercoiled DNA and lengthening of linear DNA allow to conclude that both quinizarin and danthron bind to DNA by intercalating mode.

Acknowledgments

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