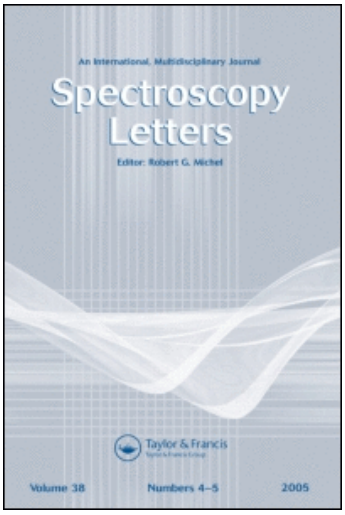

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SPECTRAL STUDIES ON THE BINDING OF A BISACRIDINIUM DERIVATIVE LUCIGENIN WITH DOUBLE HELIX DNA

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ABSTRACT

In this paper, the noncovalent binding of the cationic reagent lucigenin (LC) to DNA was investigated using spectroscopic methods. The results from absorption, circular dichroism and fluorescence studies demonstrated that LC could intercalate into the helix of DNA. Polarization and melting studies further supported the intercalation binding of LC with DNA. The binding constant was obtained by varying the DNA concentration, while keeping the concentration of LC constant. It was of the order of $10^4\,mol^{-1}\,L$ in DNA base pairs. The experiment also showed that electrostatic interaction played a significant role in the intercalation of LC with DNA.

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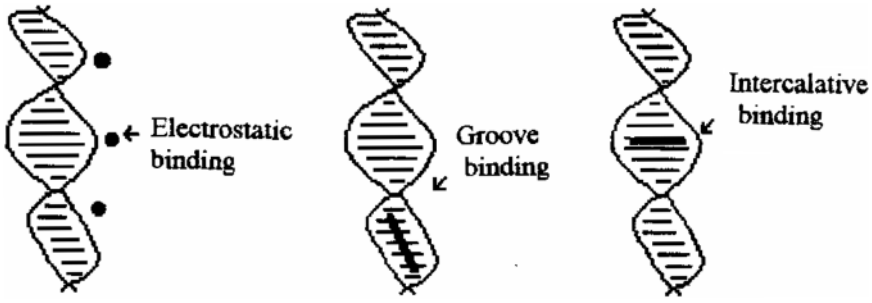
It is supposed to be because of being attracted first by anionic DNA that LC can be intercalated into the interior of the DNA double helix. This research offers a new intercalation functional group to DNA-targeted drug design.

Key Words: Lucigenin; DNA; Intercalation binding; Spectroscopic method

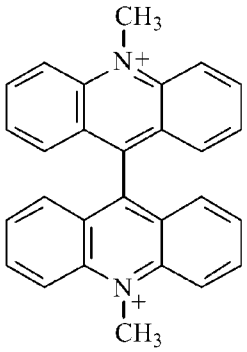
INTRODUCTION

The study of the interaction of small molecules with DNA has been the focus of some recent research works.[1–5] This is partly because that, Chemical carcinogens, radiation and many chemical antitumor agents share a common property in that they exert their biological effects through mechanisms involving DNA damage. On the one hand, certain DNA-bound forms of chemotherapeutic agents are responsible for arresting tumor cell growth. On the other hand, some of the damages induced by carcinogenic chemicals are the mediators of mutational change, which, in turn, are likely to be necessary prerequisites for carcinogenic transformation. The studies of the molecular details of these binding interactions have been crucial in rational design of new antitumor agents as well as developing sensitive probes of local nucleic acid structure.

Small molecules bind to the double helix by three dominant modes as Sch. 1: electrostatic binding, groove binding and intercalation binding.[6] The steric structure of small molecule determines the binding properties such as affinity and binding site. In general, planarity moiety is suggested to be one of the important features needed for efficient intercalators.[7]



Scheme 1. Three binding modes of small molecules with DNA.



Scheme 2. Structure of lucigenin (LC).

Non-negative charge on the small molecules is considered another crucial feature.[8]

In this connection, this paper reports on the DNA binding properties of a bisacridinium derivative, lucigenin (LC), which features two planar acridine rings and two positive charges (Sch. 2). The planar hydrophobic acridine moieties of LC with positive charges are expected to facilitate intercalation of LC into the relatively nonpolar interior of the DNA helix. The strong absorption and fluorescence characteristics of LC provide a sensitive spectroscopic handle to study its interaction with DNA. The changes in spectra can be used to decipher the nature and the strength of the stacking interaction between the chromophore and the DNA base pairs.

Lucigenin is one of the chemiluminescence substances. As a chemiluminigenic probe, it has a long-standing history.[9,10] More recently, LC has become increasing important in detecting reactive oxygen production by various celluar systems.[11] That we report here on the interaction of LC with DNA may broaden the understanding of bioactivity of bisacridinium derivatives at the biomolecular level. To date, most intercalators studied featured acridinyl, thiazinyl and phenazinyl moieties and, hence, the findings of intercalation binding of LC with DNA base pairs are likely to provide the insight necessary to design new analogs of the compound.

EXPERIMENT

Materials

Lucigenin and ethidium bromide (EB) were purchased from Aldrich Chemical Company and Sino-American Biotechnology Company, respectively.

Sodium dodecyl sulfonate (SDS) and sodium laury sulfate (SLS) were obtained from Fluka.

Calf thymus DNA (ctDNA) (Sino-American Biotechnology Company) was directly dissolved in water to prepare stock solution and stored at 4°C. The DNA concentration per nucleotide (DNA-P) was determined spectrophotometrically[12] at 260 nm by using the extinction coefficient as 6600 cm^{-1} mol^{-1} L.

pH7.6 tris-HCl buffer was used to control the solution acidity. All other chemicals were of analytical reagent grade or better and deionized distilled water was used throughout.

Spectral Measurements

The absorption spectra recordings were conducted on a Shimadzu UV-240 Ultraviolet-Visible Spectrophotometer and the fluorescence spectra were taken with a Shimadzu RF-540 Spectrofluorometer. The absorption and fluorescence titrations were performed by keeping the concentration of LC constant, and varying the DNA concentration. Data from the fluorescence titration were used to determine the binding constant of LC with the DNA as well as the quenching constants by other quenchers according to the Stern-Volmer equation:

$$F_0/F = 1 + K_{SV}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence and in the presence of DNA, respectively. K_{SV} is the Stern-Volmer fluorescence quenching constant, which is a measurement of the efficiency of quenching by quencher. [Q] is the concentration of quencher. In fluorescence measurements, the samples were excited at 370 nm, and the fluorescence intensity was monitored at 495 nm.

The fluorescence polarization measurements were made on a Shimadzu RF-540 Spectrofluorometer with a pair of polarizers. The solutions were excited at 370 nm and the fluorescence signal was monitored at 495 nm, through crossed polarizers.

The DNA melting experiments were carried out on a Shimadzu UV-365 UV-VIS-NIR Spectrophotometer with a temperature controller SPR-8.

Circular dichroic (CD) spectra were measured by using a Jasco J-715 Spectropolarimeter with the following settings: Response, 4 sec; Sensitivity, 20 mdeg; Speed, 100 nm/min; Resolution, 0.5 nm; Band width, 1.0 nm.

RESULTS AND DISCUSSION

Absorption Studies

The electronic absorption spectra of LC in the presence of DNA were illustrated in Fig. 1. Distinct decreases in the intensity of the 366 nm peak were found when various amounts of DNA were added to LC solution. The absorption spectra clearly showed red shifts in the presence of DNA. The absorption maximum was shifted from 366 nm for the free LC to 369 nm for the bound LC with DNA. Accompanying the binding process, two isosbestic points appeared in the family of absorption curves, one at 370 nm and the other at 394 nm. Hypochromism is suggested to be due to strong interactions between the electronic states of the intercalating chromophore and that of the DNA base pairs.[13,14] These spectral changes, such as hypochromity, red shift and isobestic point, are consistent with the intercalation of the chromophore into the DNA base pairs. From the observations detailed above, we surmised that intercalation occurred upon binding of LC to DNA.

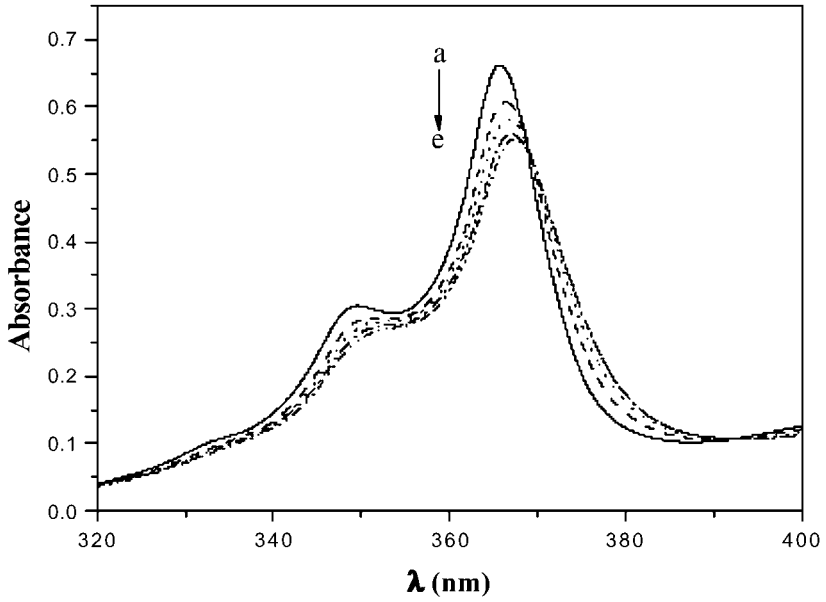


Figure 1. Absorption spectra of LC in the presence of different amounts of ctDNA. LC, 2.0×10^{-5} mol L^{-1}; ctDNA ($\times 10^{-4}$ mol L^{-1}): a, 0.0; b, 1.1; c, 2.2; d, 4.5; e, 6.7.

Circular Dichroism Studies

Circular dichroism spectra provided direct evidence of the strong interaction of LC with the asymmetric environment of DNA helix. CD spectra were recorded in both the 220–300 nm and 300–400 nm regions at various values of R (the ratio of LC to DNA-P concentration).

The well-known CD spectrum of DNA is a conservative pattern with a positive band at approximately 276 nm and negative band at approximately 246 nm. The major effect of LC on the CD spectrum of the DNA is to alter both the amplitudes and positions of the peaks for the positive and negative band (Fig. 2). Increasing amount of LC not only results in an increase in the negative or the positive band, but also leads to an evident red shift (ca. 4 nm) in the negative band and a slightly more evident blue shift (ca. 8 nm) accompanied profile narrowing in the positive band. The spectra change suggests that a structural/conformational change has taken place. Free LC does not exhibit a detectable signal in either the ultraviolet or visible region. Therefore it must also be

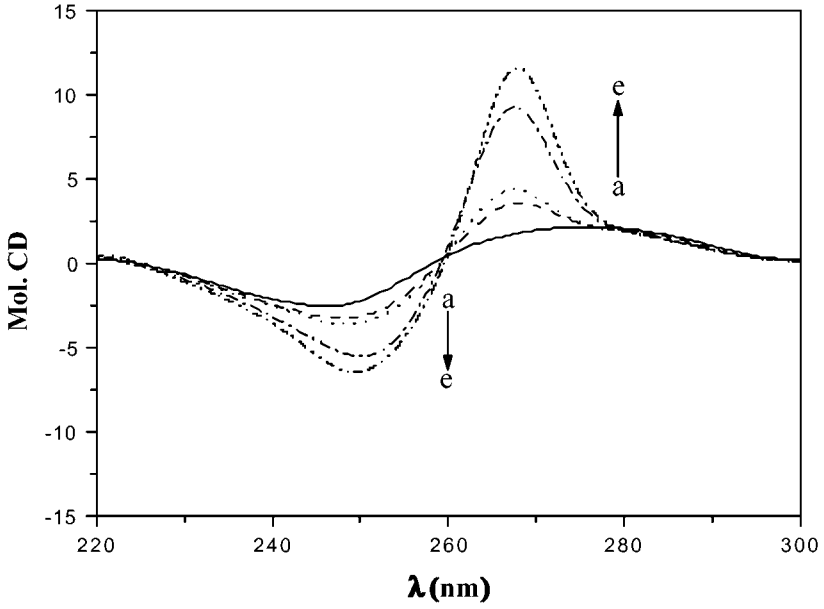


Figure 2. Circular dichroism spectra of ctDNA in the presence of different amounts of LC. ctDNA, 1.2×10^{-4} mol L^{-1}. LC ($\times 10^{-5}$ mol L^{-1}.): a, 0.0; b, 0.25; c, 0.5; d, 1.0; e, 1.5.

considered that the change is the result of an induced CD signal of DNA bound LC.[15]

In general, intercalators bound to the helix exhibit induced CD spectra due to their asymmetric environment. The signal and magnitude of the induced CD band can be used to deduce the relative orientation of the chromophore with respect to the base pair.[16] Intercalation of LC is expected to result in CD bands in the longer wavelength region of the spectrum, where the bound probe absorbs. In accordance to our expectation CD activity of LC is firmly induced in the 300–400 nm region in the presence of DNA. This is showed in Fig. 3. Both free LC and DNA do not exhibit detectable signal in 300–400 nm region. However, upon adding DNA to LC solution, a strongly positive band appears at 333 nm and a slightly stronger negative band appears at 376 nm. It is clear from Fig. 4, the amplitude of each band is inversely proportional to R. Therefore the two bands must result primarily from a LC-base interaction that strongly increases as the concentration of DNA increases, which indicates a close proximity of the LC chromophore to the DNA base pairs. It is noteworthy that the strength of the electronic interaction is expected to decrease as the

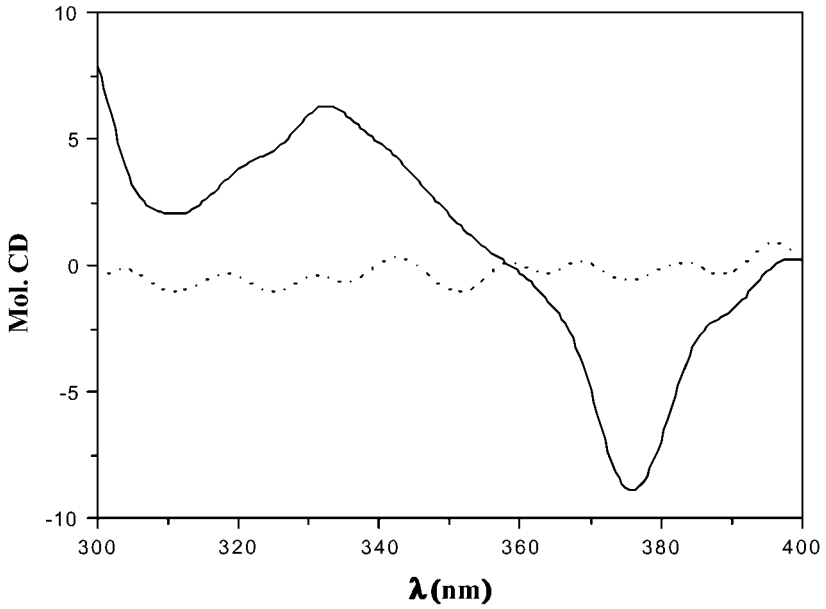


Figure 3. Circular dichroism spectra of LC (- - -), ctDNA (- - -) and LC-ctDNA (—). LC, 1.0×10^{-5} mol L^{-1}; ctDNA, 7.7×10^{-4} mol L^{-1}.

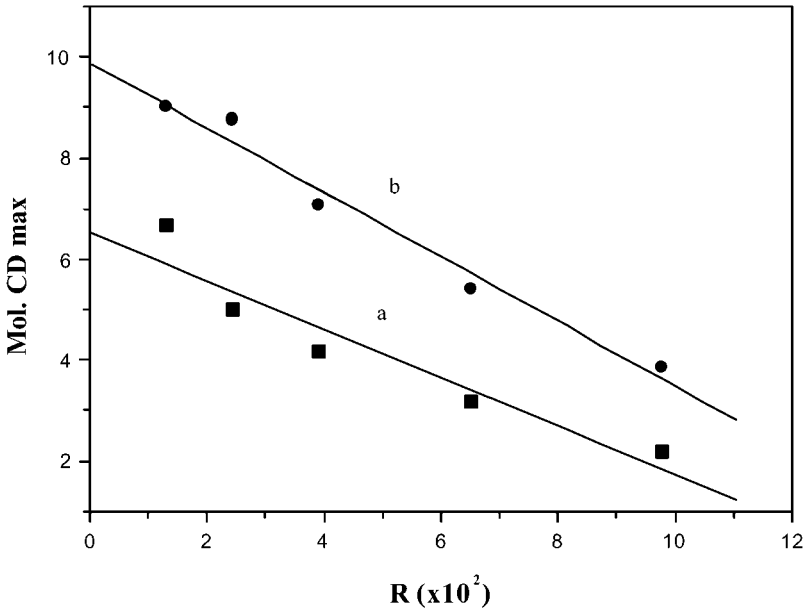


Figure 4. Change in Mol.CD max for the positive (a) and negative (b) CD bands as a function of R. LC, 1.0×10^{-5} mol L^{-1}.

cube of the distance of separation between the chromophore and the DNA bases.[17] Accordingly, the induced CD spectra are supposed to be characteristic of LC intercalatively bound to DNA.

Fluorescence Studies

The fluorescence spectra were employed to characterize binding characteristic of chromophore (Fig. 5). Excitation maximum at 370 nm and emission maximum at 495 nm are seen. The excitation spectrum is consistent with the absorption spectrum for free LC seen in Fig. 1. Once binding to the DNA helix, the intensities of spectra from LC are efficiently minimized, with no detectable shift in the maximums of emission. However, when LC binds to SLS or SDS micelles, its fluorescence is not quenched. In contrast, a result with increase of emission is observed upon binding to these anionic media. So quenching is not due to counterion condensation or self-quenching at the negatively charger surfaces of the DNA helix and is perhaps due to the intercalation of LC into the DNA base pairs.

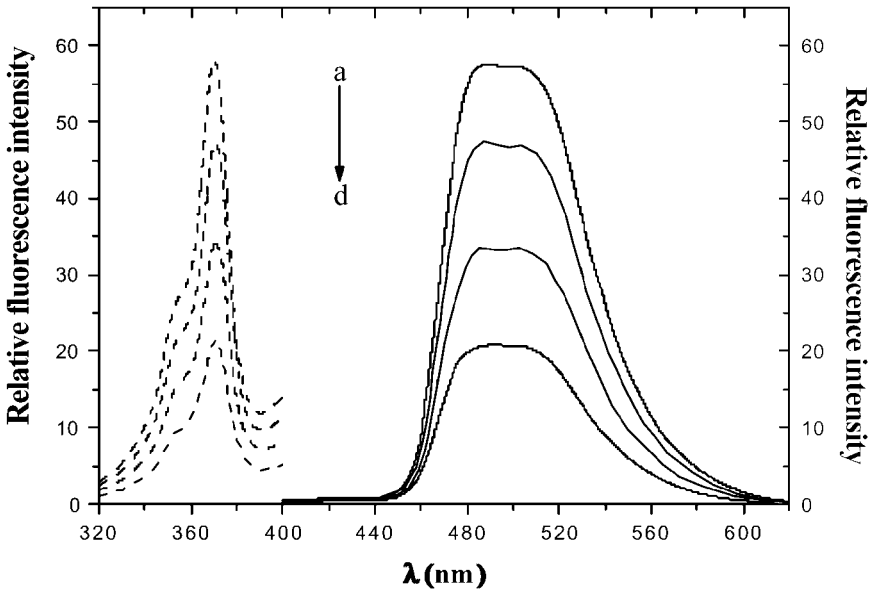


Figure 5. Fluorescence excitation (- - -) and emission (—) spectra of LC/DNA system. LC, 1.2×10^{-6} mol L^{-1}. DNA ($\times 10^{-5}$ mol L^{-1}): (a) 0.0; (b) 3.2; (c) 5.3; (d) 10.7.

Using the Stern-Volmer equation, the fluorescence quenching constant evaluated is 1.5×10^4 mol^{-1} L of DNA phosphates which is comparable to the value 1.0×10^4 mol^{-1} L with intercalator (9-anthrylmethyl) ammonium chloride.[18] The large quenching constant observed is indicative of the high affinity of LC chromophore for the DNA base pairs and strengthens the possibility for intercalation binding of LC.

Fluorescence Polarization Studies

We followed fluorescence polarization experiment by a Shimadzu RF-540 Spectrofluorometer. In the absence of DNA, the fluorescence of LC was weakly polarized due to the rapid tumbling motion of the LC molecule in aqueous media. However, upon binding LC to DNA the fluorescence was significantly polarized (Fig. 6). It should be noted that an intercalatively bound form, held more rigidly in the helix with long residence times on the time scale of the emission lifetimes, should yield finite polarization, while a more flexible surface binding along the groove would not contribute

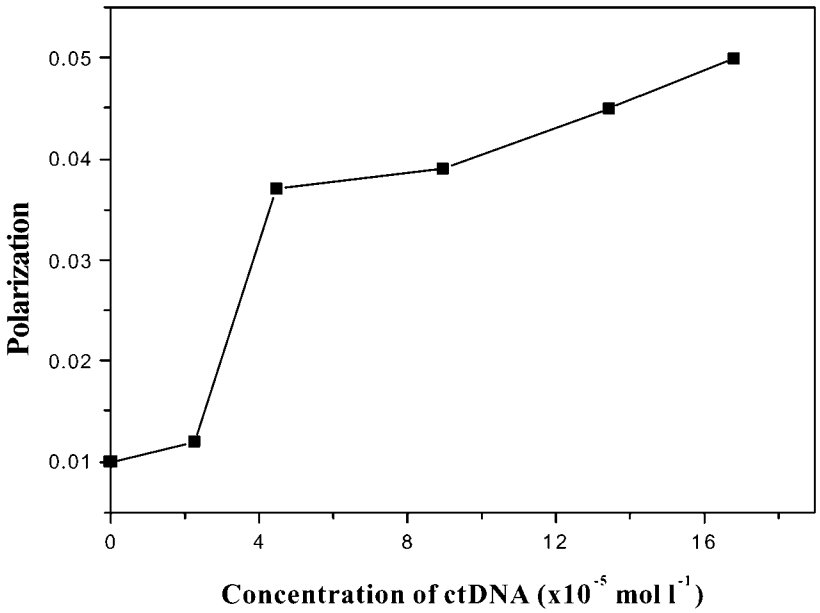


Figure 6. Influence of ctDNA on fluorescence polarization of LC. LC, 1.2×10^{-6} mol L^{-1}. $\lambda ex = 370$ nm, $\lambda em = 495$ nm.

significantly to the polarization.[12,18] Accordingly, the experiment results exhibited in Fig. 6 suggest intercalation of LC into the DNA helix.

Competitive Binding Studies

EB, a well-known intercalator, is often used as spectral probe to establish the binding mode of small molecules to the target molecule, double-helical DNA. Figure 7 showed the effect of increasing LC concentration on the fluorescence intensity of EB bound to DNA. Given that, like EB, LC intercalates into the helix of DNA, it would compete with EB for the intercalation sites in DNA, and lead to a fast decrease in fluorescence intensity of EB-DNA complex system. In fact, as expected for a displacement effect, with increasing the concentration of LC the fluorescence intensity of EB-DNA complex system strongly decreased first, and then slight weakly change. The fluorescence intensity was decreased by about 50% when the concentration rate of LC to DNA (R) rose to 0.47. Clearly, the significant decrease in intensity provides another criterion to establish

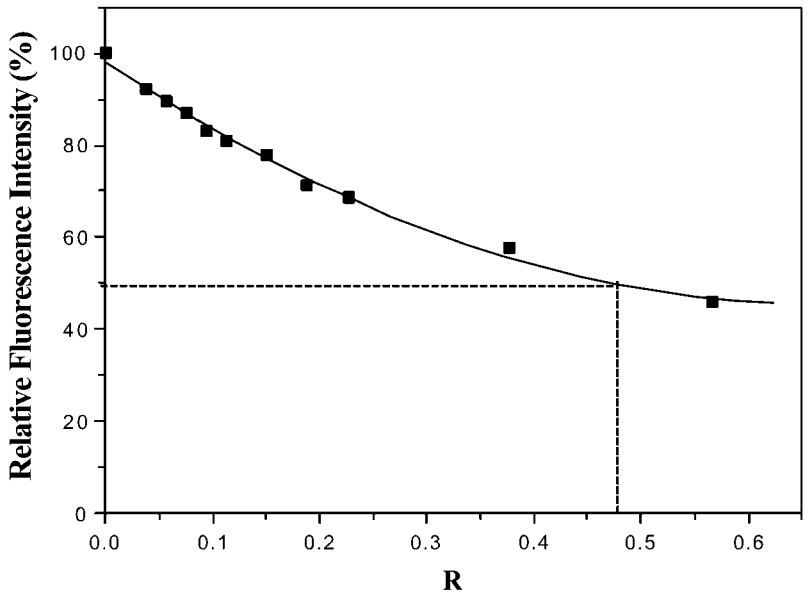


Figure 7. Competitive binding between LC and EB for ctDNA. ctDNA, 5.3×10^{-5} mol L^{-1}; EB, 2.5×10^{-5} mol L^{-1}. $\lambda ex = 540$ nm, $\lambda em = 590$ nm.

intercalation,[19,20] and hence, lends strong support for the intercalation of LC into the helix.

Quenching Studies

Other evidence in support of the intercalation mechanism comes from determining the relative accessibility of the free and bound LC to anionic quencher potassium iodide as well as potassium ferrocyanide.

If LC is intercalated into the helix stack, it should be protected from the anionic quencher, owing to the base pairs above and below the intercalator. Whereas groove binding exposes the bound molecules to the solvent surrounding the helix much more than does the intercalated species, it should provide much less protection for the chromophore.[21] Consistent with our expectation for intercalation effect, passing on to solution with anionic quencher potassium iodide or potassium ferrocyanide, fluorescence quenching was found to be much smaller in the presence of DNA than in the absence of DNA (Fig. 8). The quenching curves showed simple linear behavior, indicating the single accessibility of bound LC to the quencher as

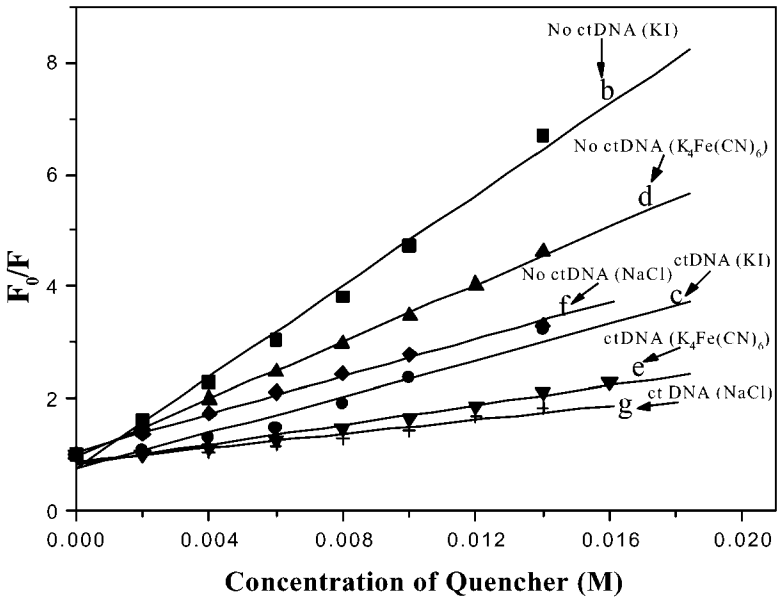


Figure 8. Quenching of LC fluorescence by KI without (b) and with (c) ctDNA, $K_4Fe(CN)_6$ without (d) and with (e) ctDNA, and NaCl without (f) and with (g) ctDNA. LC, 1.2×10^{-6} mol L^{-1}; ctDNA, 5.3×10^{-5} mol L^{-1}. F_0: the fluorescence intensity of the reagent blank; F: the fluorescence intensity of the mixed solution. $\lambda ex = 370$ nm, $\lambda em = 495$ nm.

would be expected for only one major binding mode as a result of single-component donor-quencher system. The quenching constants of KI were 406 and 162 mol^{-1} L without and with DNA, respectively, i.e., the quenching of LC fluorescence was decreased by a factor 2.5 upon binding LC to the DNA. For quencher potassium ferrocyanide, the evaluated quenching constants were 256 and 88 mol^{-1} L for free and bound LC with DNA, respectively, i.e., a threefold reduction in the quenching constant was obtained. We further tested the influence of strong electrolyte NaCl on LC fluorescence yield. As compared to potassium iodide or potassium ferrocyanide, similar but less efficiently quenching was observed because influence of NaCl on the LC fluoresence intensities comes from the ionic strength alone (Fig. 8).[22] The quenching constants for NaCl were 167 and 62 mol^{-1} L without and with DNA, respectively. In a word, anions were employed above to quench the LC emission from the free form at much faster rate than that of bound form. These results clearly demonstrated that LC chromophore intercalated into the DNA base pairs.

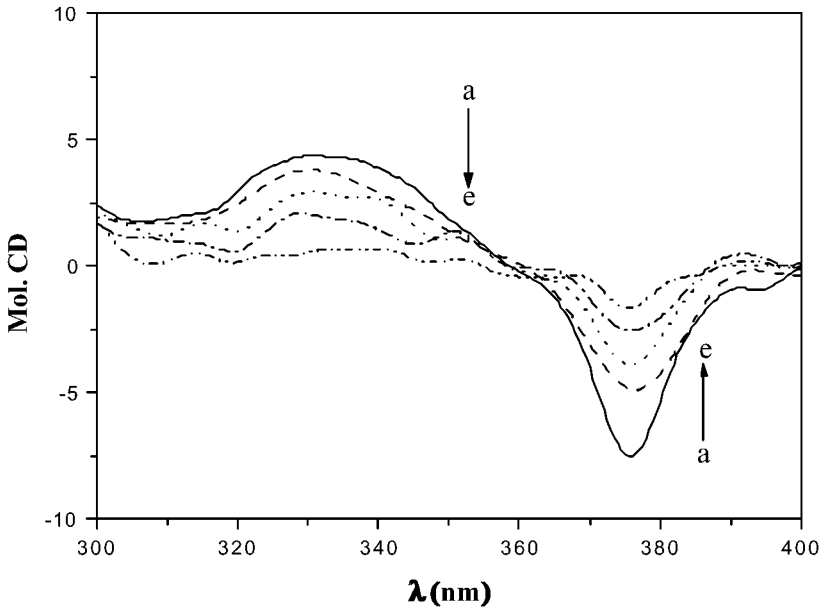


Figure 9. NaCl effect on the induced CD spectrum of LC. LC, 1.0×10^{-5} mol L^{-1}; ctDNA, 2.5×10^{-4} mol L^{-1}. NaCl ($\times 10^{-3}$ mol L^{-1}.): 0 (solid line); 2 (dash line); 6 (dot line); 12 (dash dot line); 18 (dash dot dot line).

Alternatively, the induced CD spectra also provided additional evidence of the effect of ionic strength on the interaction of LC with DNA (Fig. 9). Passing on to the mixture solution of LC and DNA with NaCl, both the positive and negative band apparently decreased with increasing of NaCl concentration. When NaCl concentration was higher than 0.02 mol L^{-1}, the two bands were absent due to the high concentration of counterion. Combined with the ability of LC to intercalate DNA bases, as previously noted, we inferred the probable intercalation process was that LC was attracted to the periphery of DNA by electrostatic attraction, and then intercalated into the stack base pairs.

DNA Melting Study

In order to gain further insight into the intercalation of LC into the helix, the DNA melting studies were monitored by following the UV absorbance of the DNA at 260 nm as a function of temperature in the absence or in the presence of LC. The DNA melting curves were presented

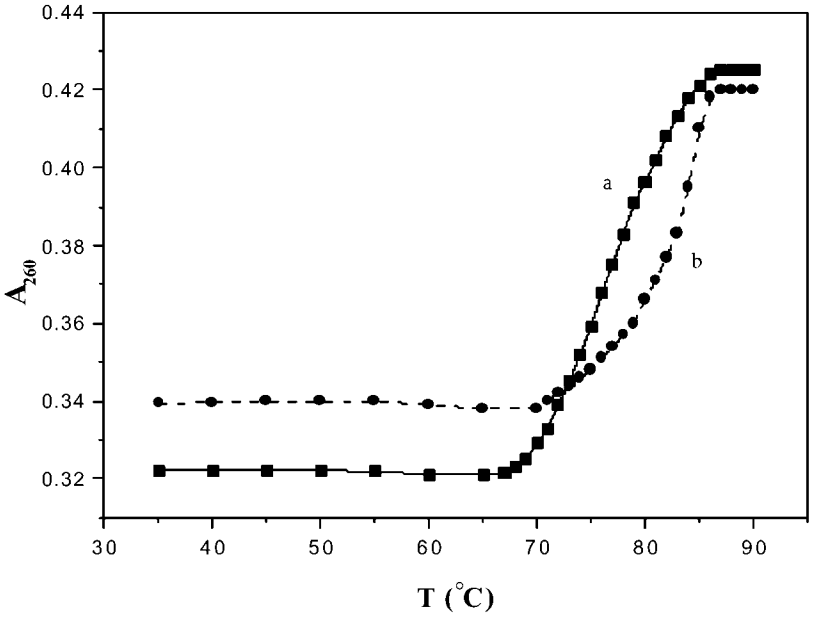


Figure 10. Hyperchromicity–temperature profile at 260 nm for DNA without LC (a) and with LC (b). ctDNA, 5.6×10^{-5} mol L^{-1}; LC, 1.2×10^{-6} mol L^{-1}.

in Fig. 10. Owing to the increased stability of the helix in the presence of an intercalator, intercalation into the helix increases the temperature at which the helix denatures by as much as 5–8°C.[23,24] Such large increase in the melting temperature is not observed if the probe binds in the groove.[21] Our experiment data in DNA melting studies reveal that LC stabilizes DNA secondary structure and the melting temperature (Tm) of DNA is increased from 75°C (in the absence of LC) to 80.2°C in the presence of LC (1:47 ratio of LC to DNA-P concentration). We consider that the major increase in the melting temperature originates from intercalation of LC.

CONCLUSIONS

The interaction between LC and DNA was studied using spectroscopic methods. In accordance to the observations of intercalation binding, titrating solution of LC with calf thymus DNA caused a distinct reduction in absorption intensity and a clear shift of maximum absorption to longer wavelength along with appearance of isosbestic points. From a variety of

fluorescence studies, including the studies of fluorescence spectra, fluorescence polarization, competitive binding and fluorescence quenching, it was concluded that the LC chromophore intercalated into the helix. This conclusion was further reinforced by the observation that the amplitudes of the induced CD bands strongly depended upon the ratio of LC to DNA-P concentration. Alternatively, the DNA helix melting experiment showed large increase in the melting temperature when LC binds to DNA, consistent with the results obtained when intercalators bind to the helix. Additional evidences from circular dichroism and fluorescence quenching studies indicated that electrostatic interaction between cationic LC and polyanions DNA played an important role in the intercalation process of LC with DNA. In conclusion, LC binds DNA by intercalation binding, and the electrostatic attraction between the LC probe and the DNA phosphates seems to be essential for the intercalation process. The intercalation of such a large LC molecule induces local conformational changes in DNA, which might disturb further biological processes. Current studies not only could provide us a possibility to design molecular systems based on the bisacridinium chromophore that bind to DNA avidly, but also could lead us to extend this work to a study of possible antitumoral or carcinogenic activity of LC.

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