

Multi-spectroscopic DNA interaction studies of sunset yellow food additive

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Abstract The use of food dyes is at least controversial due to their essential role. Synthetic color food additives occupy an important place in the food industry. Moreover many of them have been related to health problems mainly in children that are considered the most vulnerable group. The purpose of this work is to present spectrophotometric methods to analyze the interaction of native calf thymus DNA (CT-DNA) with sunset yellow (SY) at physiological pH. Considerable hyperchromism and no red shift with an intrinsic binding constant of $7 \times 10^4 \text{ M}^{-1}$ were observed in UV absorption band of SY. Binding constants of DNA with complex were calculated at different temperatures. Slow increase in specific viscosity of DNA, induced circular dichroism spectral changes, and no significant changes in the fluorescence of neutral red-DNA solutions in the presence of SY suggest that this molecule interacts with CT-DNA via groove binding mode. Furthermore, the enthalpy and entropy of the reaction between SY and CT-DNA showed that the reaction is exothermic and enthalpy

favorable ($\Delta H = -58.19 \text{ kJ mol}^{-1}$; $\Delta S = -274.36 \text{ kJ mol}^{-1}$) which are other evidences to indicate that van der Waals interactions and hydrogen bonding are the main running forces in the binding of the mentioned molecule and mode of interaction with DNA.

Keywords DNA · Sunset yellow · Groove binding

Introduction

Food dyes are the most interesting group of food additives. Frequently color of a product determines its attractiveness for consumer [1]. Materials of natural origin have been used to provide color in foods, drugs and cosmetics for the thousands of years. Ash from fires, mineral compounds and plants were probably among the first materials used for cosmetic purposes. Later, it was discovered that certain materials, mostly plant derived, could be used to enhance the appearance of foods and thus turmeric, paprika and saffron were used for more than just their flavoring properties [2]. However natural dyes are unstable and easily undergo degradation during the food processing. In the nineteenth century, synthetic organic dyes were developed to provide a more economical and extensive array of colorants. Synthetic dyes show several advantages compared with natural dyes such as high stability to light, oxygen and pH, color uniformity, low microbiological contamination and relatively lower production costs. However, many of them may exhibit adverse health effects (allergy, respiratory problems, thyroid tumours, chromosomal damage, urticaria, hyperactivity, abdominal pain, etc.) [3]. Red (E-129), sunset yellow (E-110), and tartrazine (E-102) are three highly used synthetic dyes which are added to many food products [4]. The color additive sunset

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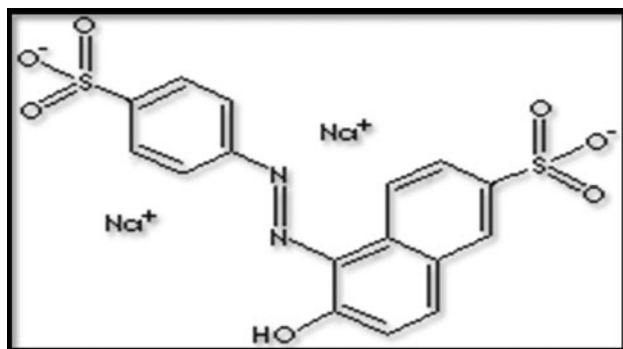


Fig. 1 Structure of disodium salt of 1-(4 sulphophenyl azo)-2 naphthol-6 sulphonic acid SY

yellow (SY) (Fig. 1) is principally the disodium salt of 1-4-sulphophenyl azo -2 naphthol-6 sulphonic acid. So SY belongs to “azo” family. Azo compounds are formed from arenediazonium ions reacting with highly reactive aromatic compounds, in what is called a diazo coupling reaction. Azo compounds are generally deeply colored because the azo linkage brings the two aromatic rings into conjugation. In addition to possessing extended conjugation, many azo dyes are also ring substituted with sulfonic acid substituents, which significantly increase polarity and water solubility and decrease absorption *in vivo*.

Many agents used in food colors have been shown to be mutagenic in albino rats and thus may have the potential to increase genetic damage in future human population. Due to mutagenicity of some of the agents present in color additive SY, use in food products has become a matter of great concern for human and animal health [5]. This dye gives a reddish-yellow color to foods and drugs. The maximum daily intake established by the FDA is 225 mg for a 60-kg person [6]. Children usually eat and drink food containing both food preservative (NaNO_3) and food colorants at the same time. Ingestion of this mixture (NaNO_3 and SY) significantly decreased rat body weight, RBC and WBC counts, Hb%, Hct%, Serum inorganic phosphorus, serum protein and serum albumin, and significant increases were observed in serum glucose, T_3 , T_4 , calcium, and cholesterol [7]. SY administration in rats produce liver necrosis and steatosis and it can be inferred that the change in lipid metabolism and increase in serum cholesterol. This increase may be indication of liver injury due to toxic effects of SO_2 [8].

Small molecules can react with DNA via covalent or non covalent interactions, with interest generally focusing on the latter. There are several sites in the DNA molecule where such binding can occur: (i) between two base pairs (full intercalation), (ii) in the minor groove, (iii) in the major groove, (iv) on the outside of the helix and (v) electrostatics binding [9]. In the study presented here, we investigated the interaction of native calf thymus DNA

(CT-DNA) with SY in 10 mM of Tris-HCl aqueous solution at neutral pH 7.4.

Experimental

Chemical and materials

The highly polymerized CT-DNA and Tris-HCl were purchased from Sigma Co. SY was purchased from Sigma-Aldrich. All solutions were prepared using double distilled water. Tris-HCl buffer solution was prepared from (Tris-(hydroxymethyl)-amino-methane-hydrogen chloride) and pH was adjusted to 7.4. SY stock solution (10^{-3} M) was prepared by dissolving an appropriate amount of the compound in Tris-HCl buffer. Solutions of CT-DNA gave an UV absorbance ratio (260 over 280 nm) of more than 1.8, indicating that the DNA was sufficiently free of protein [10]. The DNA concentration per nucleotide was determined by absorption spectroscopy using the molar absorption coefficient of ($\epsilon = 6,600 \text{ M}^{-1} \text{ cm}^{-1}$ at 260 nm) [11].

Instrumentation

UV-Vis absorption spectra

The UV-Vis spectra for DNA-SY interactions were obtained using a Cary (UV 100 Bio) spectrophotometer. Absorption titration experiments were conducted by keeping the concentration of SY constant (5×10^{-5} M) while varying the DNA concentration from 0 to 2×10^{-4} M ($r_i = [\text{DNA}]/[\text{SY}] = 0.0, 0.5, 1.0, 2.0, 3.0,$ and 4.0). It should be added, a blank cuvette was used to omit the DNA concentration each time, so no changes were observed for DNA band. Also Absorption titration experiments were conducted by keeping the concentration of SY constant (3×10^{-5} M) while varying the DNA concentration from 0 to 1.5×10^{-4} M at three different (281, 290, 300) temperatures (K). Solutions of DNA and SY were scanned in a 0.5 cm (1 ml) quartz cuvette.

SsDNA solution was prepared by heating native dsDNA solution in a boiling water bath for 8 min and then rapidly cooling in an ice-water bath. Gradually up to 298 K, dsDNA turns to be ssDNA [12]. Similar to UV titration at above experiment, here 2 ml solutions of SY (5×10^{-5} M) were transferred into cuvettes. Absorbance titration was recorded after adding solutions of native CT-DNA (2×10^{-4} M) and denatured CT-DNA directly to each cuvette.

Viscosity measurements

For viscosity measurements a viscometer (SCHOT AVS 450) was used, which thermo stated at 25 °C by a constant

temperature bath. Flow time was measured with a digital stopwatch; the mean values of two replicated measurements were used to evaluate the viscosity (η) of the samples. The data were reported as $(\eta/\eta_0)^{1/3}$ versus the [SY]/[DNA] ratio, where (η_0) is the viscosity of the DNA solution alone. Viscosity values were calculated from the observed flow time of CT-DNA containing solutions (t) and corrected from the flow time of buffer alone (t_0), $\eta = (t - t_0)/t_0$ [13].

CD spectra

Circular dichroism (CD) measurements were recorded on a JASCO (J-810) spectropolarimeter by keeping the concentration of DNA constant (8×10^{-5} M) while varying the SY concentration from 0 to 5.6×10^{-5} M ($r_i = [\text{SY}]/[\text{DNA}] = 0.0, 0.1, 0.2, 0.7$).

Fluorescence quenching

All fluorescence measurements were carried out using a Cary spectrofluorometer. The excitation wavelength 530 nm was used for the fluorescence measurements and the emission spectra were recorded between 560 and 700 nm. The excitation and emission slits were both 5 nm. In the competitive binding studies, concentrations of DNA and neutral red (NR) were kept constant (7×10^{-5} and 10^{-5} M, respectively), while varying the SY concentration from 0 to 1.5×10^{-5} M.

Results and discussion

Electronic spectral studies

Effect of DNA on the sunset yellow spectra

Complex binding with DNA via intercalation generally results in hypochromism and a red shift (bathochromism) of the absorption band, the hypochromicity characteristic of intercalation has usually been attributed to the interaction between the electronic states of the compound and those of the DNA bases, while the red shift has been associated with the decrease in the energy gap between HOMO and LUMO molecular orbitals after binding of the ligand to DNA. Hyperchromism has been observed for the interaction of many drugs with DNA. The hyperchromic effect might be ascribed to external contact or partial uncoiling of the helix structure of DNA, exposing more bases of the DNA [14]. The absorption intensity of SY is increased (hyperchromism) upon increasing the concentration of CT-DNA (Fig. 2). This hyperchromism can be attributed to external contact (surface binding) with the

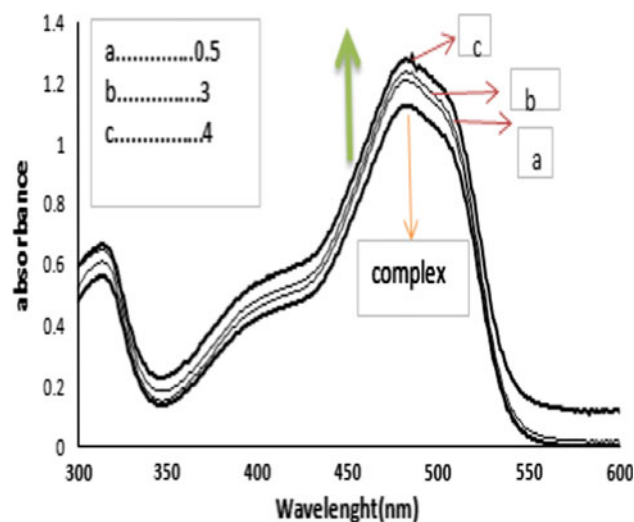


Fig. 2 UV-Vis spectra of SY (5.0×10^{-5} M) with DNA in 0.01 M Tris-HCl buffer (pH 7.4) with $r_i = [\text{DNA}]/[\text{SY}] = [0, 0, 0.5, 3, 4]$

duplex. The extent of the hyperchromism is indicative of non-intercalative binding modes, such as electrostatic forces, van der Waals interactions, dative bonds, hydrogen bonds and hydrophobic interactions. In order to further illustrate the bonding strength of the SY with CT-DNA, the intrinsic binding constant K_b was determined from the spectral titration data using the following equation:

$$\frac{[\text{DNA}]}{(\varepsilon_a - \varepsilon_f)} = \frac{[\text{DNA}]}{(\varepsilon_b - \varepsilon_f)} + \frac{1}{K_b(\varepsilon_b - \varepsilon_f)} \quad (1)$$

where [DNA] is the concentration of DNA in nucleotides, the apparent absorption coefficients ε_a , ε_f and ε_b correspond to $A_{\text{obsd}}/[\text{M}]$, the extinction coefficients of the free compound and that of the compound when fully bound to DNA, respectively. In plots of $[\text{DNA}]/(\varepsilon_a - \varepsilon_f)$ versus [DNA], K_b is given by the ratio of slope to the intercept (Fig. 3). The intrinsic binding constant, K_b of SY was calculated to be $7 \times 10^4 \text{ M}^{-1}$. The K_b value obtained is lower than that of classical intercalators, whose binding constants have been found to be in the order of 10^6 – 10^7 M^{-1} [15]. In comparing the intrinsic binding constant

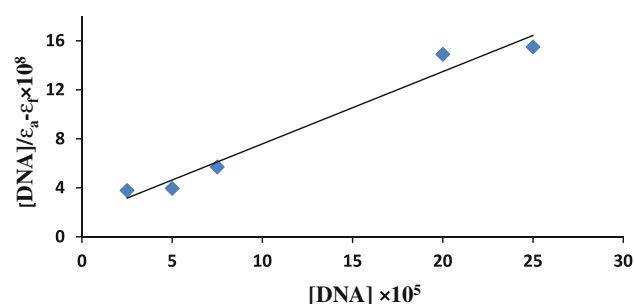


Fig. 3 The plot of $[\text{DNA}]/(\varepsilon_a - \varepsilon_f)$ versus [DNA]

(K_b) of the SY with some DNA groove binders as observed in the literature [16–18], we may assume that this molecule binds to CT-DNA via groove binding mode.

Thermodynamics of the sunset yellow (SY)–DNA complex interactions

In order to have a better understanding of thermodynamic of the complexation reactions between SY and DNA, contributions of enthalpy and entropy in the reaction should be determined. In this study, binding constants (K_b) were measured at three different temperatures using UV–Vis. Experiment was performed by monitoring the spectroscopic changes in the UV–Vis (especially followed by measuring the absorbance at 482 nm), by adding increasing amounts of DNA from 1.5×10^{-5} to 1.5×10^{-4} to a fixed concentration of SY (3×10^{-5} M), the intrinsic binding constant K_b was determined from the spectral titration data using Eq. 1. Thermodynamic parameters describing the binding reactions can be divided into three contributions. The first contributions are due to hydrogen bonding and hydrophobic interactions between the SY and DNA binding sites. The next contribution is from the conformational changes in either the nucleic acid or the SY upon binding. Finally, there are contributions from coupled processes like ion release, proton transfer, or changes in the hydration water [19]. Evaluation of the formation constant for the SY–DNA complex at 3 different temperatures (281, 290 and 300 K) allows thermodynamic parameters ΔH , ΔS and ΔG of SY–DNA formation via Van't Hoff Eq. 2 by plotting $\ln K_b$ versus $1/T$ (Fig. 4) to be determined (Table 1).

$$\ln K_b = -\Delta H/RT + \Delta S/R \quad (2)$$

Ross et al. [20] reported that when $\Delta H < 0$ or $\Delta H \approx 0$, $\Delta S > 0$, the main force acting is electrostatic; when $\Delta H < 0$, $\Delta S < 0$, the main forces acting are van der Waals or hydrogen bonds and when $\Delta H > 0$ and $\Delta S > 0$, the main force acting is hydrophobic interaction. It can be seen from the results obtained, that for the binding system

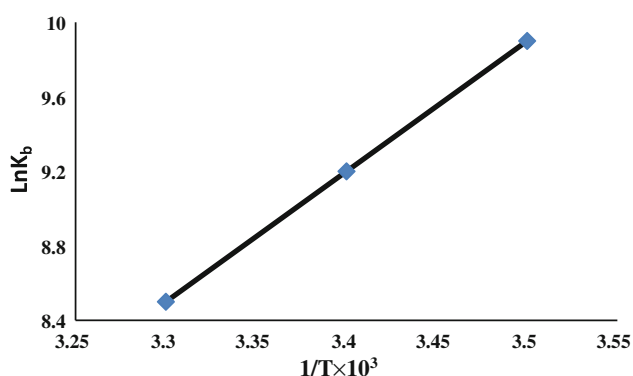


Fig. 4 Van't Hoff plot for SY–DNA complex

Table 1 Thermodynamic parameters and binding constants for binding of SY to CT-DNA in 10 mM Tris–HCl buffer (pH 7.4)

T (K)	K_b	ΔG° (kJ mol ⁻¹)	ΔH° (kJ mol ⁻¹)	ΔS° (J mol ⁻¹ k ⁻¹)
281	2×10^4	-23.12	-58.198	-274.36
290	10^4	-22.18	-58.198	-274.36
300	0.5×10^4	-21.12	-58.198	-274.36

of SY and CT-DNA $\Delta H < 0$, $\Delta S < 0$. Therefore, the van der Waals and/or hydrogen bonding are the main running forces acting during the binding of SY to CT-DNA [21].

Comparison of the effects of ssDNA with dsDNA

Both dsDNA and ssDNA can increase the absorbance of SY, and ssDNA has a stronger effect (Fig. 5), which suggests that sunset yellow reacts with the base pairs of DNA duplex in grooves through hydrogen bonding and van der Waals forces. If the binding mode was intercalation, the absorbance effect of ssDNA would be weaker than that of dsDNA. Moreover, the maximum absorbance wavelength of SY does not change with addition of CT-DNA, which also suggests that the interaction is a groove binding rather than to be an intercalative binding mode [22, 23].

Circular dichroism spectroscopy

To establish in more detail whether binding of the dye brings about any significant conformational changes of the DNA double helix, CD spectra of CT-DNA were recorded at increasing dye/CT-DNA ratios. The observed CD spectrum of natural CT-DNA consists of a positive band at 275 nm due to base stacking and a negative band at 245 nm due to helicity, which is characteristic of DNA in right-handed B form [24]. In the presence of the SY

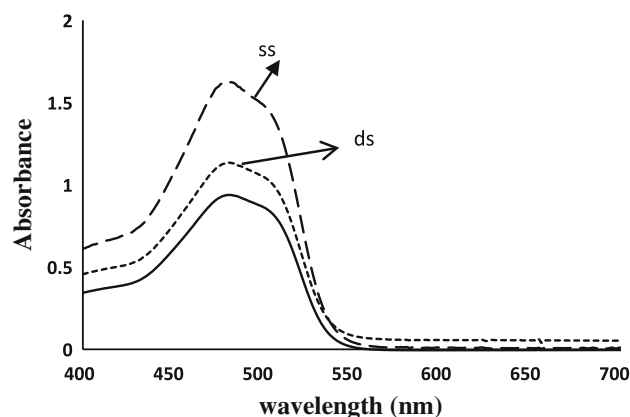


Fig. 5 UV–Vis spectra of SY (5.0×10^{-5} M) with native (ds) and denatured DNA (ss) in 0.01 M Tris–HCl buffer (pH 7.4) with $r_1 = [\text{DNA}]/[\text{SY}] = 4$

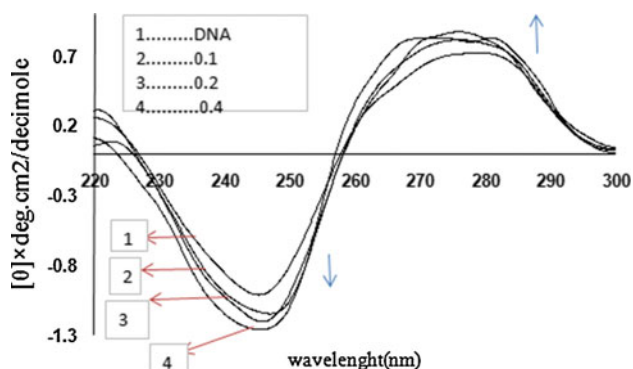


Fig. 6 CD spectra of DNA (8.0×10^{-5} M) in 0.01 M Tris-HCl buffer $r_i = [\text{SY}]/[\text{DNA}] = [0.0, 0.1, 0.2, 0.4]$

complex, both the positive and negative peak intensities of the CD spectra of DNA were increased (Fig. 6). The changes in the CD spectra in the presence of SY show stabilization of the right-handed B form of CT-DNA, may be due to fused aromatic rings in the food colorant structure [14]. It should be added these CD spectral data indicate no apparent changes in the conformation of CT-DNA; similarly to other groove binders the perturbation in CD bands is less than that of intercalators. It is notable the probability of intercalation is mainly reduced due to the presence of SO_3 groups in the SY structure.

Viscosity study

Optical photophysical probes generally provide necessary, but not sufficient clues to support a binding model. Measurement of DNA viscosity which is sensitive to DNA length is regarded as the least ambiguous and the most critical test of binding in solution in the absence of crystallographic structural data. Intercalating agents are expected to elongate the double helix to accommodate the

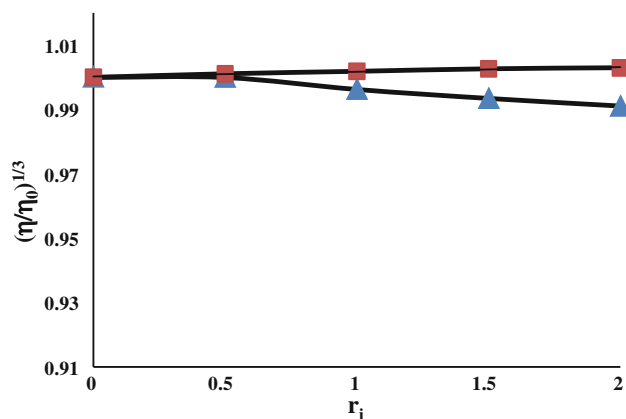


Fig. 7 Effect of increasing amounts of SY (square) and Hoechst 33258 (triangle) on the viscosity of CT-DNA (5×10^{-5} M) in 0.01 M Tris-HCl buffer (pH 7.4), ($r_i = 0.0, 0.3, 0.5, 0.7, 1.0$)

ligands in between the base pairs, leading to an increase in the viscosity of DNA. In contrast, molecules which bind exclusively in the DNA grooves by partial and/or non-classical intercalation, under the same conditions, typically cause less pronounced (positive or negative) or no change in the DNA solution viscosity [25]. Figure 7 shows the relative viscosity of DNA in the presence of varying amounts of the SY and Hoechst 33258. As expected the groove binder Hoechst 33258 in contrast to intercalators does not lengthen the DNA helix, and does not increase the viscosity of DNA solution [26]. No significant changes in specific viscosity of DNA in comparison with Hoechst 33258 indicating that it can bind to DNA via groove and/or surface binding mode nature [27].

Competitive binding studies

It is well known that the fluorescence intensity of DNA itself is very weak, and no fluorescence was observed for the SY. Therefore, direct use of its fluorescence emission properties to monitor the interaction of this dye with DNA is not possible. The standard method for fluorescence enhancement of DNA is based on ethidium bromide (EB) usage. EB displays a dramatic enhancement of DNA fluorescence efficiency when intercalated into DNA [28]. Recently, due to carcinogenic properties of EB, small molecules were replaced and have become safe reagents in nucleic acid chemistry. In general, when small molecules bind to DNA changes to fluorescence and absorption are noted in the respective spectra relative to what can be observed for solutions without DNA. An early example was the NR which showed enhanced fluorescence when intercalated into the DNA structure. However, when a second ligand, which competed for the DNA binding sites, was added, fluorescence quenching was observed [29]. It should be noted, in the case of the highest concentration of SY, there is no significant decrease in the fluorescence intensity of the NR-DNA complex (Fig. 8). The emission

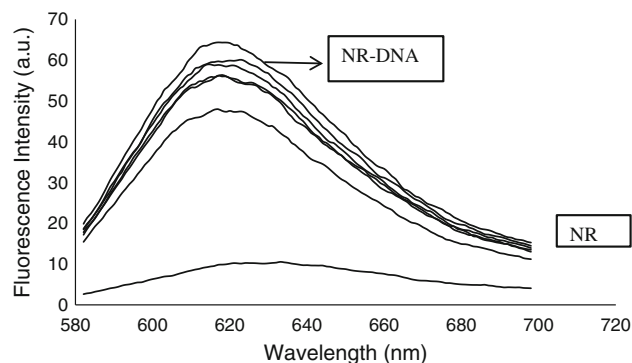


Fig. 8 Emission spectra of the NR-DNA complex (SY concentration from 0 to 1.5×10^{-5} M)

spectra of the NR–DNA complexes in the presence of the increasing amounts of SY concentration clearly reveal its non-intercalative binding nature.

Effect of the ionic strength on the binding properties

CT-DNA is an anionic polyelectrolyte due to possessing phosphate groups. Monitoring the spectral changes at a high ionic strength is an efficient method to distinguish the binding mode between the ligand and CT-DNA. NaCl is used to control the ionic strength of the solutions. Due to the competition for phosphate groups, the addition of Na⁺ would weaken the electrostatic interaction between CT-DNA and molecules [30]. In the Tris–HCl buffer with pH 7.4, the K_b of SY–DNA is $7 \times 10^4 \text{ M}^{-1}$. Due to high dielectric constant of water, Na⁺ ions are covered by H₂O molecules, SO₃[−] groups have repulsive interaction with oxygens in phosphate groups of DNA. Therefore, electrostatic binding mode would be excluded and one can conclude at physiological ionic strength the only mode of interaction is groove binding. By increasing the concentration of NaCl up to 30 mM the K_b will increase ($1 \times 10^5 \text{ M}^{-1}$), because of the probable existence of electrostatic binding mode in addition to groove binding in the presence of high concentration of salt.

Conclusion

According to the results arise from, UV spectroscopy, viscosity and spectrofluorimetric studies we conclude that, SY noncovalently bind to CT-DNA via non-intercalative mode. It should be noted, these noncovalent bindings have an important effect on life phenomena at the molecular level [12]. In addition, DNA binding agents (i.e., groove binders and intercalators) are termed as topoisomerase poisons because they exhibit the ability to convert the enzyme into a cellular toxin. The formation of topoisomerase-binding agent–DNA ternary complexes is implicated in DNA scission and subsequent cell death [31].

The intrinsic binding constant ($K_b = 7 \times 10^4 \text{ M}^{-1}$) and viscosity measurements results are similar to groove binders. The thermodynamic parameters ($\Delta H < 0$ and $\Delta S < 0$) and considering the differences between the affinities of native and denatured DNA towards SY show that van der Waals interactions and hydrogen bonding are the main running forces in the binding of the mentioned molecule with DNA. Our results indicate that this food colorant has a toxic potential to CT-DNA in vitro and it seems that it binds directly to DNA.

Combining our result and that of the other researchers, we conclude that, more caring must be done to prevent our children from eating or drinking large amounts of food

containing this colorant. It should be noted that, SY concentration which was used in this study (2.5×10^{-5}) is much less than that of currently being used as a food additive in food industry.

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