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A Study on the Aggregation and Calf Thymus DNA Binding Characteristics of Anionic Cobalt(II) Tetrasulfonated Phthalocyanine

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The aggregation behavior of anionic Cobalt(II) 4,4′,4″,4‴-tetrasulfonated phthalocyanine, [Co(TSPc)$^{4-}$] was studied at its various concentrations and different ionic strengths using optical absorption and resonance light scattering (RLS) spectroscopies in 5 mM phosphate buffer, pH 7.0 at 25 °C. The results show no aggregation behavior at concentration range of 5.1×10^{-6}-7.5×10^{-5} M. Also, the interaction of [Co(TSPc)$^{4-}$] with calf thymus DNA (ct-DNA) was studied by UV-Vis absorption, fluorescence spectroscopies and thermal denaturation measurement. The binding constants were obtained at various ct-DNA concentrations using SQUAD software. The thermodynamic parameters were calculated by the van't Hoff equation. The results show that the process is entropy-driven. The fluorescence study represents the quenching effect of [Co(TSPc)$^{4-}$] on bound ethidium bromide (EtBr) to ct-DNA.

Keywords: DNA, Phthalocyanine, SQUAD, Thermodynamic, Fluorescence

INTRODUCTION

The main objective of the most anti-cancer chemotherapy drugs is DNA molecule. So, studying the chemical interactions between small molecules and DNA is important. Many small molecules such as drug molecules are able to bind to single-or double-stranded nucleic acids and cause structural changes in DNA molecule [1-4]. Different types of interactions between small molecules and double helix structures of DNA include covalent binding, major or minor groove binding and intercalation [5]. Phthalocyanine are heterocyclic aromatic flat compounds which have 18 electrons. Large structure of Phthalocyanines prevent them from dissolving in common solvents. This causes problems in their separation, identification and medical applications such as photosensitizers in the photodynamic therapy (PDT) of cancer. The presence of amino, carboxylic and sulfonic acid groups in the phthalocyanine structure appears to enrich it with water solubility. Aggregation of molecules is a well-known phenomenon in phthalocyanine compounds. The presence of bulky substituents on the peripheral sites can reduce interactions between phthalocyanine molecules, their aggregation and self-oxidation [6-10]. Phthalocyanines have various applications, for example as catalyst, pigments, electrodes and sensitizers in photodynamic therapy (PDT) [11-13]. Biological effects of porphyrins and phthalocyanines depend on their physico-chemical properties. Aggregation phenomenon causes changes in the absorption spectra of phthalocyanine [14], fluorescence intensity [15], lifetime and quantum yield of produced singlet molecular oxygen [16,17]. The aggregate phenomenon will depend on the structure of phthalocyanine molecules such as the existence of central metal atom and the type of metal atom, the type of substituents and environmental factors such as solvent polarity, pH, hydrogen bonding, ionic strength and [DNA]/[phthalocyanine] mole ratio [14,15]. In solution, the microheterogenous system can induce aggregation of certain heterocyclic compounds [18]. So, binding to macromolecules such as DNA induces aggregation of some water soluble phthalocyanines in solution [19,20].

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Phthalocyanines with positive charge showed photosensitizing properties for the photodynamic therapy of cancer and their photodynamic activity are higher than the commonly used hematoporphyrin [21]. The scientific research has shown that positively charged sensitizers show higher activity than neutral or negatively charged ones [22]. The interaction between the anionic molecules with DNA molecule has not been well studied [23]. So, in this paper we have studied the interaction of an anionic phthalocyanine with calf thymus DNA. We have used here the anionic form to examine whether this negatively charged phthalocyanine could form phthalocyanine-ct-DNA complex which is necessary for designing anti-cancer drugs. In this research, the aggregation treatment of Cobalt (II) 4,4′,4″,4‴-tetrasulfonated phthalocyanine, $[Co(TSPc)^{4-}]$ (Fig. 1) has been investigated at different experimental conditions such as ionic strength and concentration of $[Co(TSPc)^{4-}]$. Then, the binding properties of $[Co(TSPc)^{4-}]$ as an anionic phthalocyanine with ct-DNA have been considered. The interaction process has been followed by UV-Vis optical absorption and fluorescence spectroscopy in parallel with ct-DNA thermal melting studies. Finally, the spectral data has been analyzed to gain the mode of binding and binding constant using SQUAD software. The mode, strength and nature of binding were determined according to the collected data.

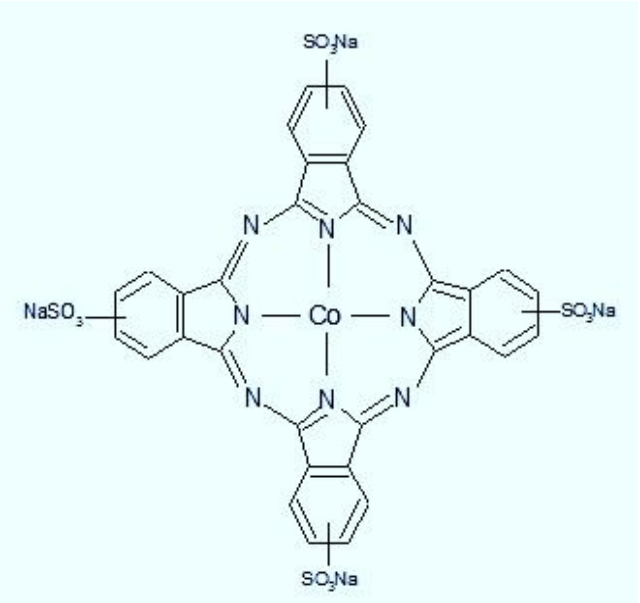

Fig. 1. Molecular structure of $[Co(TSPc)^{4-}]$.

EXPERIMENTAL

Materials and Instruments

Calf thymus DNA was purchased from Sigma Chemical Co. and used without further purification. The anionic phthalocyanine $[Co(TSPc)^{4-}]$ was obtained from Fluka and used as received. All of the other chemicals were analytical grades. All experiments were performed in 5 mM phosphate buffer at pH 7.0 at 25 °C. The buffer solution includes (1.53 mM KH_2PO_4 and 0.97 mM K_2HPO_4) dissolved in the double distilled water. Also, the stock solution of this anionic phthalocyanine $[Co\ (TSPc)^{4-}]$ was prepared by dissolving its crystals in water and stored in dark at 4 °C. The stock solution of $[Co(TSPc)^{4-}]$ in the appropriate buffer was prepared before using. The ct-DNA concentrations in terms of base pair l^{-1} and in terms of nucleotide l^{-1} were determined spectrophotometrically by employing an extinction coefficient of 13,200 $M^{-1}\ cm^{-1}$ (base pair)$^{-1}$ and 6600 $M^{-1}\ cm^{-1}$ (nucleotide) at 260 nm, respectively [24]. The absorbance monitoring was performed on a GBC UV-Vis Cintra 6 Spectrophotometer equipped with thermostat cell compartment and UV-Lite software. The resonance light scattering and fluorescence measurements were carried out on a Shimadzu model RF-5000 spectrofluorimeter. The scattered light intensity was monitored using the right angle in the synchronous scanning monochromators in the region of 300-600 nm. The thermal melting curves of both free ct-DNA and $[Co(TSPc)^{4-}]$-ct-DNA in phosphate buffer were obtained by measuring the hyperchromicity of ct-DNA absorbance at 260 nm *vs.* the temperature. Melting temperatures were measured in phosphate buffer solutions containing 1.5×10^{-5} M of ct-DNA at pH 7.0 and scanned from 26 to 85 °C.

RESULTS AND DISCUSSION

Solution Properties of $[Co(TSPc)^{4-}]$

The solution properties of this phthalocyanine were determined by UV-Vis and RLS spectroscopies. The optical absorption spectrum of $[Co(TSPc)^{4-}]$ includes a Q-band and

a Soret band. These bands are intense Q-band near 663 nm and less intense B-band (also called the Soret band) with peaks at about 318 nm. Furthermore, a weak satellite band is observed near 628 nm. This weak band is related to higher vibrational levels of the relevant electronic state. The molar extinction coefficient of the Q-band of $[Co(TSPc)^{4-}]$ in our experimental conditions was 2.97×10^4 M^{-1} cm^{-1} at 25 °C. The Lambert-Beer law was obeyed at the studied concentration range (5.1×10^{-6}-7.5×10^{-5} M) for $[Co(TSPc)^{4-}]$. Aggregation phenomenon induces changes in the spectral specifications of the Q-band and B-band and show deviation from Beer' s law [25]. This results suggest that this anionic phthalocyanine $[Co(TSPc)^{4-}]$ does not aggregate in the experimental concentration range.

Effect of the Ionic Strength

The effect of NaCl on the UV-Vis spectrum of $[Co(TSPc)^{4-}]$ (2×10^{-5} M) in water is shown in Fig. 2 and corresponding data are presented in Table 1. The results showed the hypochromicity without significant red or blue shifts. As shown in Fig. 2, with increasing the concentration of NaCl, decreasing of Q band intensity is accompanied by reinforcement of band intensity at 628 nm that appears as a new band. Also, the band at 318 nm appears as a shoulder at high NaCl concentration. These results show the aggregation of $[Co(TSPc)^{4-}]$ [26] and also represent the strong electrolyte effect on aggregation behavior of $[Co(TSPc)^{4-}]$. For further support, we have used RLS as a powerful technique to identify aggregation accompanied by addition of NaCl solution to the $[Co(TSPc)^{4-}]$ solution. Figure 3 illustrates, the RLS spectrum of $[Co(TSPc)^{4-}]$ at different NaCl concentrations in 5 mM phosphate buffer pH 7.0 at 25 °C. According to the theory of resonance light scattering, the scattered light intensity (SLI) of solution in the absence of optical absorption follows Rayleigh light law ($SLI \alpha 1/\lambda^4$). In the absence of phthalocyanine, water and NaCl solutions do not absorb in the studied spectral region, hence, scattered light spectra (SL) of the $[Co(TSPc)^{4-}]$ solutions at different NaCl concentrations obeyed the Rayleigh law. The SLI increases with increasing the NaCl concentration. In the optical absorption spectral range, an increased SLI can be observed as a result of resonance light scattering effect, in which showed the increasing in the refractive index of the scattering medium at the studied range [27,28]. At first, the RLS increases significantly due to the increased NaCl (the RLS effect) and then decreases upon further increase in NaCl. We can attribute this result to the formation of aggregates during the phthalocyanine

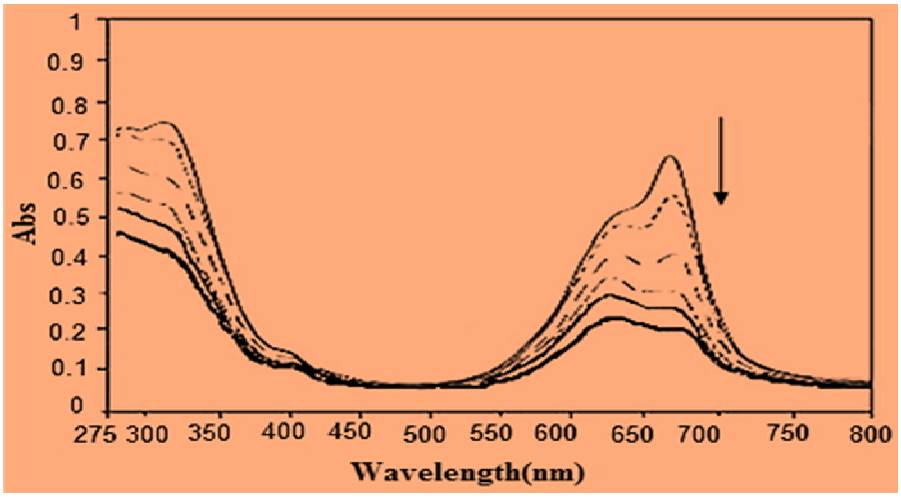

Fig. 2. Absorption spectra of $[Co(TSPc)^{4-}]$ solution (2×10^{-5} M) upon addition of NaCl in 5 mM phosphate buffer, pH 7.0 at 25 °C, NaCl concentrations are: 0; 0.24 M; 1.0 M; 1.55 M; 1.97 M; 2.5 M. Arrows indicate the change in absorbance upon increasing the NaCl concentration.

Table 1. UV-Vis Spectra of $[Co(TSPc)^{4-}]$ (2×10^{-5} M) upon Increasing NaCl

[NaCl]	A_{max}	λ_{max} (nm)	$W_{1/2}$ (nm)
0.00	0.653	664.3	90.0
0.45	0.555	665.2	95.0
1.00	0.396	664.3	100.0
1.67	0.305	663.5	105.0
2.14	0.260	663.5	105.0
2.50	0.207	663.5	105.0

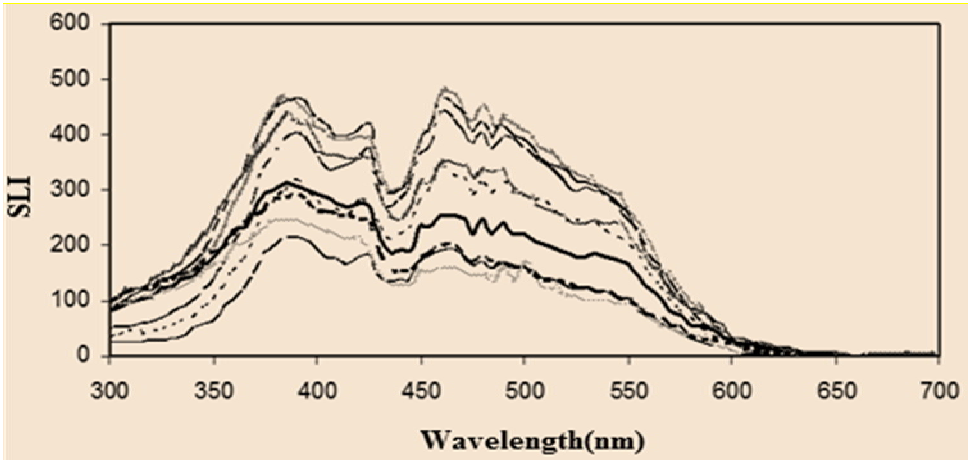

Fig. 3. Spectra of resonance light scattered of $[Co(TSPc)^{4-}]$ solution (2×10^{-5} M) upon addition of NaCl in 5 mM phosphate buffer, pH 7 at 25 °C, NaCl concentrations are: 0.45 M (– – –); 0.83 M (·····); 1.05 M (– · – · –); 1.43 M (·····); 1.67 M (— · · — · ·); 1.87 M (~~~~~); 2.06 M (——); 2.22 M (·····); 2.37 M (▪ ▪ ▪).

binding to NaCl at low concentrations of salt. As a result of increasing the NaCl concentration, the aggregates decompose in favor of the phthalocyanine bound dimers [29].

Binding of $[Co(TSPc)^{4-}]$ to ct-DNA

In order to investigate the binding of phthalocyanine to ct-DNA, absorption titration experiments were performed. We have performed the titration of $[Co(TSPc)^{4-}]$ solution at

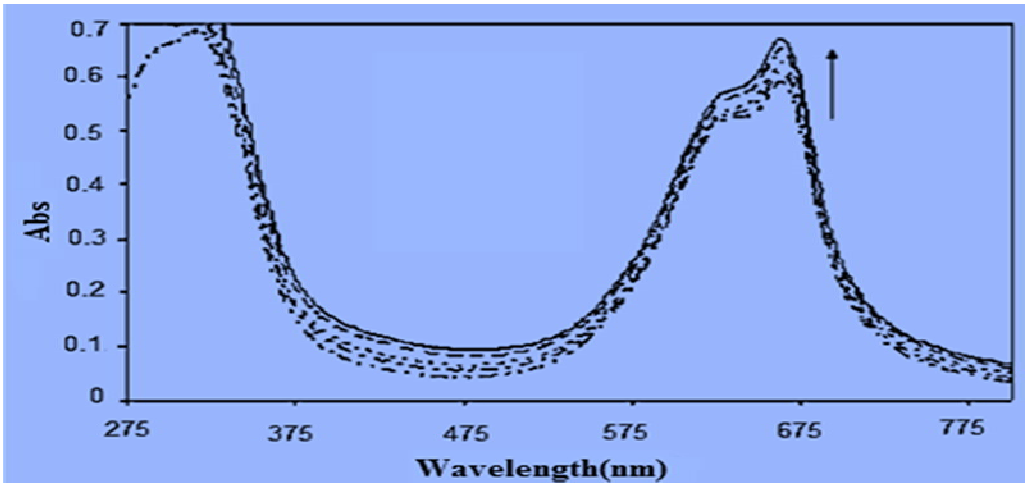

Fig. 4. Absorption spectra of $[Co(TSPc)^{4-}]$ (2×10^{-5} M) upon titration with stock solution of ct-DNA in 5 mM phosphate buffer, pH 7 and at 25 °C, DNA concentrations are: 0; 6.09×10^{-5} M; 10.33×10^{-5} M; 13.29×10^{-5} M; 15.04×10^{-5} M. Arrows indicate the changes in absorbance upon increasing the ct-DNA concentration.

Table 2. Thermodynamic Parameters of First Equilibrium of Interaction of $[Co(TSPc)^{4-}]$ with ct- DNA in 5 mM Phosphate Buffer, pH 7.0 at Various Temperatures

T (K)	logK_1	ΔG_{b1} (kJ mol^{-1})	$H_{b1}\Delta$ (kJ mol^{-1})	$S_{b1}\Delta$ (J mol^{-1} K^{-1})
299.15	0.16 ± 4.65	-26.63 ± 0.92	69.56 ± 4.32	321.54 ± 17.52
309.15	4.98 ± 0.18	-29.47 ± 1.06	69.56 ± 4.32	320.33 ± 17.40
319.15	5.32 ± 0.20	-32.50 ± 1.22	69.56 ± 4.32	319.79 ± 17.36
329.15	5.77 ± 0.23	-36.36 ± 1.45	69.56 ± 4.32	321.80 ± 17.53

a invariable concentration of $[Co(TSPc)^{4-}]$ (2×10^{-5} M) and different concentrations of ct-DNA (6.09×10^{-5}, 10.33×10^{-5}, 13.29×10^{-5}, 15.04×10^{-5} M) in 5 mM phosphate buffer pH 7.0 at 25 °C (Fig. 4). The magnitude of hypochromism indicates the strength of intercalation mode [30]. Often, the hypochromicity characteristic of intercalation is due to the interaction between the electronic states of the complex and the DNA bases [31]. The hyperchromism effect has been observed for the interaction of many drugs with DNA [32]. The hyperchromic effect might be attributed to external contact (electrostatic binding [33]) or to partial uncoiling of the double helix structure of DNA [34]. The extent of the hyperchromism is indicative of the partial or non-intercalative binding modes such as electrostatic forces, Van

Table 3. Thermodynamic Parameters of Second Equilibrium of Interaction of $[Co(TSPc)^{4-}]$ with ct- DNA in 5 mM Phosphate Buffer, pH 7.0 at Various Temperatures

T (K)	logK_2	ΔG_{b2} (kJ mol^{-1})	ΔH_{b2} (kJ mol^{-1})	ΔS_{b2} (J mol^{-1} K^{-1})
299.15	0.37 ± 2.63	-15.06 ± 2.12	106.27 ±6.20	405.58 ± 27.81
309.15	3.43 ± 0.40	-20.30 ± 2.37	106.27 ± 6.20	409.41 ± 27.72
319.15	4.00 ± 0.43	-24.44 ± 1.22	106.27 ± 6.20	409.56 ± 27.67
329.15	4.31 ± 0.47	-27.16 ± 2.96	106.27 ± 6.20	405.38 ± 27.83

der Waals interaction, hydrogen bonds and hydrophobic interaction [35]. In the presence of increasing amount of ct-DNA concentration, phthalocyanine $[Co(TSPc)^{4-}]$ exhibited hyperchromism with nearly no shift at λ_{max} of the Q-band. In this case, the percentage of hyperchromism $[Co(TSPc)^{4-}]$ Q-band due to binding to ct-DNA was found to be 7.6%. These results suggest that the interaction of $[Co(TSPc)^{4-}]$ with ct-DNA are partial or non-intercalative binding modes which would possibly by hydrophobic forces or hydrogen bonds of the oxygen atoms on the phthalocyanine with DNA nucleobases [36,37]. The binding constant at any particular temperature was determined by the concentration dependence of UV-Vis absorption data, using the SQUAD program. The SQUAD program has been developed to calculate the best set of binding constants of the proposed equilibrium model by using a nonlinear least-squares route [38,39]. The input data consists of (a) the absorbance amounts (b), the total DNA and phthalocyanine concentrations. The Gauss-Newton nonlinear least-squares algorithm is used for minimizing the residual sum of squares, *U*, using the following Eq. (1):

$$U_{obs} = \sum_{i=1}^{I} \sum_{K=1}^{NW} [(A_{i,k}^{cal} - A_{i,k}^{obs})]^2 \quad (1)$$

where $A_{i,k}^{obs}$ is the absorbance value of the *i*th solution and NW is the total of wavelengths. The output data are the logarithm of the binding constant (K_{ij}) for formation of D_iP_j, where D is DNA and P is phthalocyanine corresponding to the following equilibrium:

$$i\ D + j\ P \leftrightarrow D_i\ P_j \quad (2)$$

The values of *U* and percentage of uncertainty for logK_{ij} are calculated by the program. The absorption data were investigated by assuming 1:1 or 2:1 and/or simultaneous 1:1 and 2:1 molar ratios of phthalocyanine to DNA. Fitting of the experimental data (15 points) to the proposed stoichiometric models was estimated by the sum of squares of the calculated points by this model. The results show that the best fitting corresponds to 1:1 and 2:1 combining models at the studied temperatures [40,41]. The calculated binding constants are given in Tables 2 and 3. As shown in these tables, with increasing temperature, the binding constants rise. This result can be ascribed to an increase of complex stability in higher values of the binding constants.

Thermodynamics Studies

In order to obtain a deeper vision into the molecular basis of interaction between phthalocyanine and DNA in the presence of DNA, we determined the energetics governing on the complex formation. The energy of $[Co(TSPc)^{4-}]$-ct-DNA complex can be calculated by three thermodynamic parameters, standard Gibbs free energy changes, $\Delta G°$, standard enthalpy changes, $\Delta H°$ and standard entropy

changes, $\Delta S°$. $\Delta G°$ can be obtained from the equilibrium constant, K, of the reaction using the relationship, $\Delta G° = -RT \ln K$, in which R and T refer to the gas constant, and the absolute temperature, respectively. Furthermore, K is the apparent equilibrium constant and $\Delta G°$ is the apparent Gibbs free energy change. If heat capacity changes for the reaction are essentially zero, the van't Hoff equation (Eq. (3)) gives a linear plot of $\ln K$ *vs.* $1/T$ (Figs. 5 and 6) [42].

$$d\ln K/d(1/T) = -\Delta H°/R \quad (3)$$

The apparent standard enthalpy change $\Delta H°$ can be calculated from the slope of the straight line, $-\Delta H°/R$ and the apparent standard entropy change from its intercept, $\Delta S°/R$. The van't Hoff plots for interaction of $[Co(TSPc)^{4-}]$ with ct-DNA are shown in Figs. 5 and 6. The calculated thermodynamic parameters for binding of $[Co(TSPc)^{4-}]$ to ct-DNA are listed in Tables 2 and 3. It has been expressed that the standard Gibbs free energy changes for interaction of $[Co(TSPc)^{4-}]$ with ct-DNA is negative, indicating the relative affinity of the $[Co(TSPc)^{4-}]$ complex to ct-DNA. Also, It has been implied that the binding process is endothermic-disfavored ($\Delta H° > 0$) and entropy-favored ($\Delta S° > 0$). As proposed by Ross [43], when $\Delta H° < 0$ or $\Delta H° \approx 0$, $\Delta S° > 0$, the most influential force is electrostatic, when $\Delta H° < 0$, $\Delta S° < 0$, the most influential force is van der Waals or hydrogen bonding and when $\Delta H° > 0$, $\Delta S° > 0$, the effective force is hydrophobic. In the present study, we assumed that hydrophobic interaction might be the major acting force in binding of $[Co(TSPc)^{4-}]$ to ct-DNA. These results showed that the interactions between ct-DNA and $[Co(TSPc)^{4-}]$ are not of intercalating type.

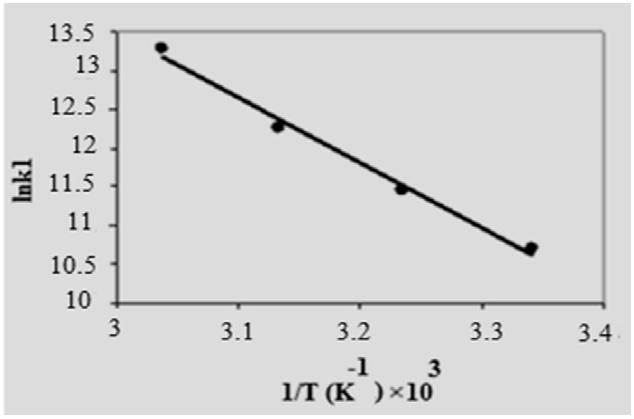

Fig. 5. The van't Hoff plot of $[Co(TSPc)^{4-}]$ binding to ct-DNA.

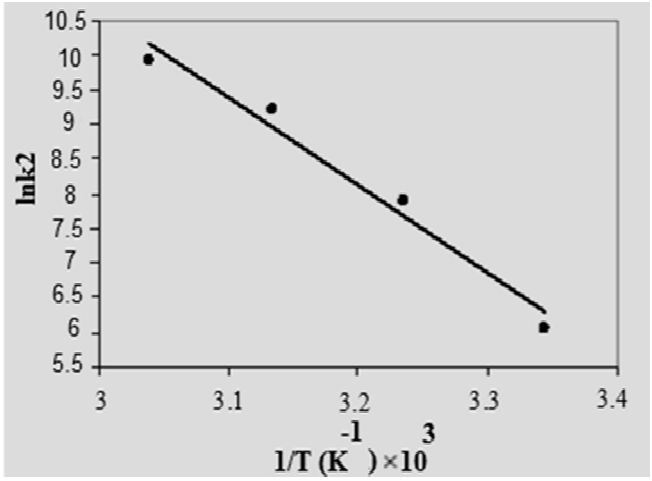

Fig. 6. The van't Hoff plot of $[Co(TSPc)^{4-}]$ binding to ct-DNA.

Fluorescence Spectroscopic Studies

In aqueous solution, no luminescence was observed for $[Co(TSPc)^{4-}]$ in the presence of ct- DNA. So, the binding of $[Co(TSPc)^{4-}]$ to ct-DNA cannot be directly presented in the emission spectra. Therefore, we studied the fluorescence spectra of the interaction between $[Co(TSPc)^{4-}]$ with ct-DNA in the presence of ethidium bromide (EtBr). Ethidium bromide (EtBr) was used as a sensitive fluorescence probe whose properties are derived from its strong intercalation between the adjacent DNA base pair. The extent of fluorescence quenching of EtBr binding to DNA is used to determine the extent of binding between the second molecule and DNA. Adding complexes to DNA pre-treated with EtBr causes significant reduction in the emission intensity, indicating the replacement or electron transfer of EtBr by the complexes and decreasing the binding constant of EtBr to DNA [44,45]. Excitation and emission wavelengths were 530 and 600 nm. Addition of $[Co(TSPc)^{4-}]$ (1.7 M in each addition from 1.36×10^{-5}-8.36×10^{-5} M) to

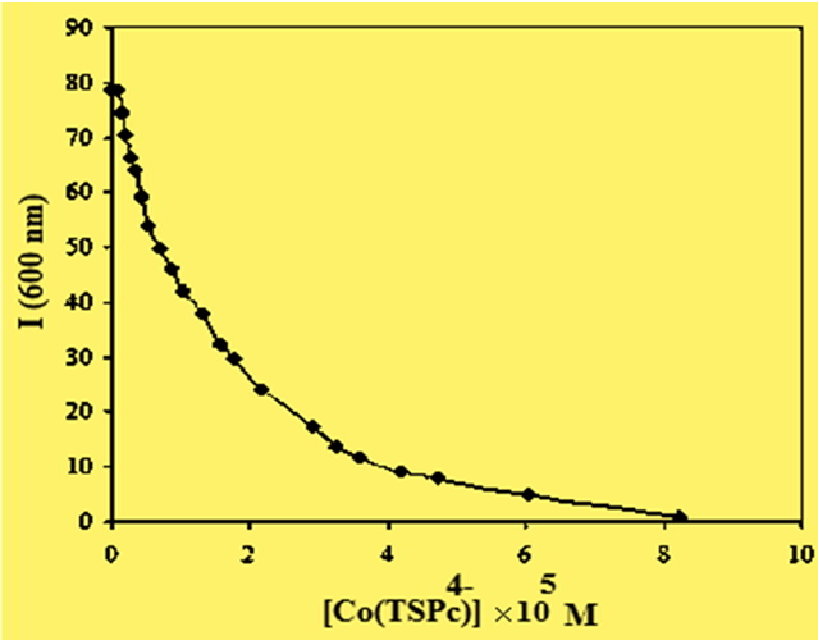

Fig. 7. Fluorescence quenching of ct-DNA-EtBr ([EtBr] = 3.6×10^{-4}M; [ct-DNA] = 7.5×10^{-7} M) at various concentrations of $[Co(TSPc)^{4-}]$, $[Co(TSPc)^{4-}]$ concentrations are: 0; 1.36×10^{-5} M; 2.36×10^{-5} M; 3.36×10^{-5} M; 4.36×10^{-5} M; 5.36×10^{-5} M; 6.36×10^{-5} M; 7.36×10^{-5} M; 8.36×10^{-5} M.

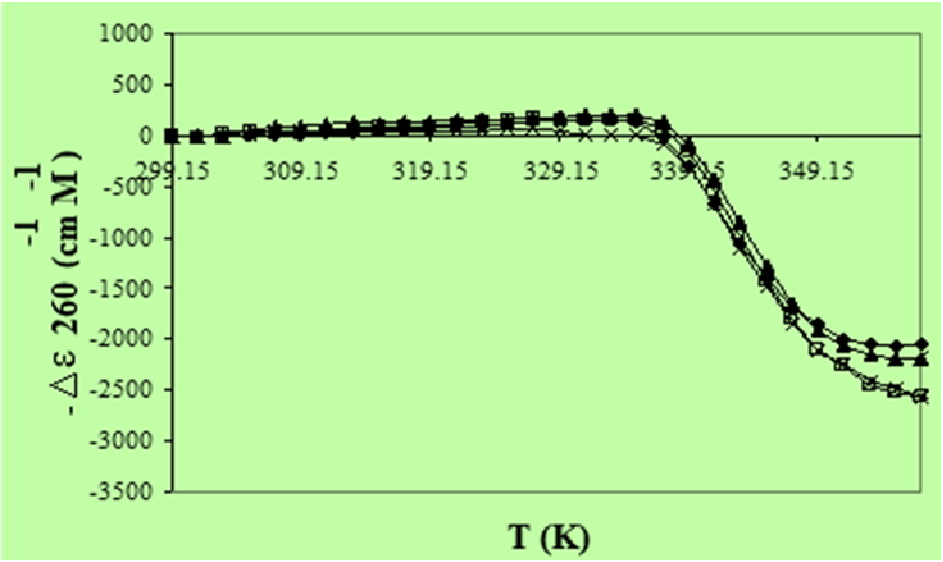

Fig. 8. Melting profiles ($\lambda_{detection}$ = 260 nm) for the free ct-DNA (2×10^{-5} M) in the absence and presence of different molar ratios of $[Co(TSPc)^{4-}]$ to ct-DNA, $[Co(TSPc)^{4-}]$/ct-DNA ratios are: 0; 0.035; 0.0697; 0.1349.

Table 4. CT-DNA Melting Temperature Changes upon Increasing the Molar Ratio of $Co(TSPc)^{4-}$] to ct-DNA

$[Co(TSPc)^{4-}]$ / [ct-DNA]	0.000	0.035	0.0697	0.1349
T_m (K)	343.0	342.9	343.4	343.2

the EtBr (4.2×10^{-5} M)-ct-DNA (2.8×10^{-5} M) complex causes significant reduction in the emission intensity (Fig. 7). Upon increasing concentration of EtBr, ct-DNA shows strong fluorescence emission at 600 nm. The three effective fluorescence quenching paths for binding of EtBr to DNA are: (i) replacement by a quencher and quenching induced by solvent (ii) transferring energy from EtBr molecule to the quencher (Föster energy transfer); (iii) electron transfer from excited EtBr to an acceptor mediated by DNA [46]. The obtained results from fluorescence and UV-Vis spectroscopy suggested that in the presence of $[Co(TSPc)^{4-}]$, fluorescence quenching of EtBr-DNA complex was due to the replacement or electron transfer of EtBr by $[Co(TSPc)^{4-}]$ and finally decreasing the binding constant of EtBr to ct-DNA.

Thermal Melting of ct-DNA

The thermal melting temperature (T_m), show the stability of DNA double helix versus temperature. The change in the thermal melting temperature (T_m) represents an interaction between DNA and $[Co(TSPc)^{4-}]$ [47]. Also, it is possible to obtain information about the strength of the interaction. To further support the interaction between $[Co(TSPc)^{4-}]$ and ct-DNA, the thermal melting temperature experiments (T_m) were performed as the mid-point of hyperchromic transition. The thermal melting temperature (T_m) showed the stability of DNA double helix and the interaction of the molecules with DNA may change the T_m by stabilizing or destabilizing complex [48]. In this study, the thermal melting temperature (T_m) of ct-DNA and $[Co(TSPc)^{4-}]$-ct-DNA complex were measured in phosphate buffer solution containing ct-DNA with concentration of 1.5×10^{-5} M at pH = 7.0. The temperature was scanned from 26-85 ºC at the speed of 0.4 °C min^{-1}. The melting curves of ct-DNA and $[Co(TSPc)^{4-}]$-ct-DNA complex are shown in Fig. 8. The T_m values of ct-DNA in the absence and presence of $[Co(TSPc)^{4-}]$ were measured to be 343.0 °C and 342.9°C, respectively (Table 4). In general, groove binding or electrostatic binding along the phosphate backbone of DNA gives rise to small change in thermal melting temperature, while intercalation between DNA base-pairs leads to a significant increase in thermal melting temperature of DNA molecule. These results showed the non-intercalative binding between ct-DNA and $[Co(TSPc)^{4-}]$ [49-51].

CONCLUSIONS

In this work, we show a perfect study of the interaction between ct-DNA and an anionic phthalocyanine $[Co(TSPc)^{4-}]$. The interactions of anionic phthalocyanine $[Co(TSPc)^{4-}]$ with ct-DNA have been investigated using UV-Vis and fluorescence spectroscopy, RLS and ct-DNA thermal melting measurements. Also, the binding constants and thermodynamic parameters were calculated. From the fluorescence spectra, the replacement or electron transfer of EtBr by $[Co(TSPc)^{4-}]$ decreased the binding constant of EtBr to ct-DNA. The thermal denaturation studies suggested that the interaction of the anionic phthalocyanine $[Co(TSPc)^{4-}]$ with ct-DNA leads to moderate stabilization of ct-DNA structure. Moreover, increasing the concentration of phthalocyanine to ct-DNA poorly affected the thermal melting curve of ct-DNA. All obtained results showed an outside binding mode. This study could help further understanding of binding mechanism of $[Co(TSPc)^{4-}]$ with ct-DNA and structural designing of an effective drug molecule that targets ct-DNA.

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