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Synthesis, Characterization and DNA Interaction Studies of (*N,N′*-Bis(5-phenylazosalicylaldehyde)-ethylenediamine) Cobalt(II) Complex

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In the present study, at first, *azo* Schiff base ligand of (*N,N′*-bis(5-phenylazosalicylaldehyde)-ethylenediamine) (H_2L) has been synthesized by condensation reaction of 5-phenylazosalicylaldehyde and ethylenediamine in 2:1 molar ratio, respectively. Then, its cobalt complex (CoL) was synthesized by reaction of $Co(OAc)_2{\cdot}4H_2O$ with ligand (H_2L) in 1:1 molar ratio in ethanol solvent. This ligand and its cobalt complex containing *azo* functional groups were characterized using elemental analysis, ^{1}H-NMR, UV-vis and IR spectroscopies. Subsequently, the interaction between native calf thymus deoxyribonucleic acid (ct-DNA) and CoL complex was investigated in 10 mM Tris/HCl buffer solution, pH = 7 using UV-vis absorption, thermal denaturation technique and viscosity measurements. From spectrophotometric titration experiments, the binding constant of CoL complex with ct-DNA was found to be $(2.4 \pm 0.2) \times 10^4$ M^{-1}. The thermodynamic parameters were calculated by van't Hoff equation.The enthalpy and entropy changes were 5753.94 ± 172.66 kcal/mol and 43.93 ± 1.18 cal/mol·K at 25 °C, respectively. Thermal denaturation experiments represent the increasing of melting temperature of ct-DNA (about 0.93 °C) due to binding of CoL complex. The results indicate that the process is entropy- driven and suggest that hydrophobic interactions are the main driving force for the complex formation.

Key Words : *Azo* schiff base, Calf thymus DNA, UV-vis spectroscopy, Viscosity, Thermal denaturation

Introduction

The studies on molecular interactions between drugs and DNA have great importance to study their biological activity and have become an active research area in recent years. DNA is vital for all living beings, even plants. It is important for genetic inheritance, coding for proteins and the genetic instruction guide for life and its processes. Interaction of DNA with small molecules, in general involve three types of binding modes: (i) electrostatic binding between the negatively charged DNA phosphate backbone that is along the external DNA double helix and the cationic or positive end of the polar molecule, (ii) groove binding involving hydrogen bond or Van der Waals interaction with the nucleic acid bases in the deep major groove or the shallow minor groove of the DNA helix and (iii) intercalative binding where the molecule interacts itself within the nucleic acid base pairs.[1-3] In recent years, metal complexes of Schiff bases have attracted considerable attention due to their remarkable antibacterial, antifungal and antitumor activities.[4-8] For example, Schiff bases complexes derived from 4-hydroxy salicylaldehyde and amines have strong anticancer activities.[9] Earlier work reported that some drugs showed increased activity, when administered as metal complexes rather than as organic compounds.[10,11] It has been suggested that the azomethine linkage is responsible for the biological activities of Schiff bases such as antitumor, antibacterial, antifungal and herbicidal activities.[12-17] In this respect, *azo* Schiff bases and their complexes with transition metal ions are also of importance due to their complexing, catalytical and biological properties.[18-20] They found to be important as biochemical, analytical and antimicrobial reagent.[21,22] Complexes of *azo* compounds also exhibit bacteriostatic and other biochemical activities due to the interesting liganting behavior of such system. In view of all of the above, it was thought worthwhile to study ct-DNA binding of a transition metal Schiff base complex with *azo* functional group namely (*N,N′*-bis(5-phenylazosalicylaldehyde)-ethylenediamine) Cobalt(II) complex (CoL). This complex can be synthesized in high yield using inexpensive starting materials. Its interaction with calf thymus DNA (ct-DNA) was investigated by electronic absorption, thermal denaturation and viscosity measurements.

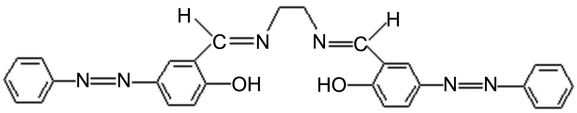
Experimental

Materials and Instruments. All reagents and solvents used were supplied by Merck chemical company and were used without further purification. Double strand Calf thymus DNA (ct-DNA) was purchased from sigma. Stock solution of CoL complex was prepared just prior to use by dissolving the solid in ethanol. The stock solutions of ct-DNA were prepared in 10 mM Tris/HCl buffer at pH = 7. The ct-DNA solutions gave a UV absorbance ratio (A_{260}/A_{280}) of about 1.9, indicating that the ct-DNA was sufficiently free from protein.[23] The concentration in base pairs of ct-DNA was determined using an extinction coefficient of 6600 $cm^{-1}M^{-1}$ at 260 nm.[24] Double distilled water was used to prepare all

stock solutions for ct-DNA binding studies. IR spectra were recorded, in KBr phase in a Perkin-Elmer FT IR-1605 spectrophotometer. ^{1}H-NMR spectra were measured with a Varian XL-400 MHz spectrometer with DMSO as a solvent at room temperature and tetramethylsilane (TMS) as the internal standard. C, H and N analysis data were obtained using a Perkin-Elmer 240B elemental analysis instrument.

Synthesis of (5-Phenylazosalicylaldehyde) as Precursor. This compound was prepared as described in the literature.[25] To a solution of an aniline derivative (10 mmol) in water (5 mL), concentrated hydrochloric acid (20 mL) was added slowly with stirring. The clear solution was poured into ice water mixture, diazotied with sodium nitrite (0.69 g, 10 mmol), dissolved in water (3.5 mL), during a period of 15 min at 0-5 ºC. The cold diazo solution was added dropwise to the solution of salicylaldehyde (1.05 mL, 10 mmol) in water (50 mL) containing sodium hydroxide (0.4 g) and sodium carbonate (7.3 g) during a period of 30 min at 0-5 ºC. The reaction mixture was stirred for 1 h in ice bath, allowed to warm slowly to room temperature and subsequently stirred for 4 h at this temperature. The product was collected by filtration and recrystallized from the mixture of EtOH and H_2O. Yellow powder; Yield 80%, IR (KBr, ν cm^{-1}): 3250 (O-H stretch), 3050 (aromatic C-H stretch), 1664 (aromatic aldehyde C=O stretch), 1600, 1568 (aromatic C=C stretch), 1481 (N=N stretch), 1105 (C-O stretch). Elemental analysis calcd. (%) for $C_{13}H_{10}N_2O_3$: C (68.78), H (4.40), N (12.42); found: C (68.65), H (4.10), N (11.85).

Synthesis of the Ligand (*N,N′*-Bis(5-phenylazosalicylaldehyde)-ethylenediamine) (H_2L). The ligand (H_2L) was prepared in a similar manner.[26] Firstly, ethylenediamine (0.013 mol) and 5-phenylazosalicylaldehyde (0.026 mol) was condensed by refluxing in absolute ethanol (100 mL) for 2 h. The solution was then left at room temperature, where upon the ligands were deposited as yellow microcrystals. The microcrystals were collected by filtration, washed with cold absolute ethanol (15 mL) and then recrystallized several times from ethanol. Orange; Yield, 87%, IR (KBr, ν cm^{-1}): 3450 (O-H stretch), 3050 (aromatic C-H stretch), 1637 (C=N stretch), 1582, 1550 (aromatic C=C stretch), 1501 (N=N stretch), 1285 (C-O stretch); ^{1}H NMR (400 MHz, DMSO-d_6) δ 10.7 (s, 2H), 7.2-8.2 (m, 2OH ArH), 8.8 (s, 2H imine), 3.4 (s, 4H CH_2); UV-vis (DMF): λ_{max} = 273.5 nm, 364.5 nm. The chemical structure of H_2L ligand was shown in Scheme 1.



Scheme 1. Structure of ligand (*N,N′*-Bis(5-phenylazosalicylaldehyde)-ethylenediamine) (H_2L).

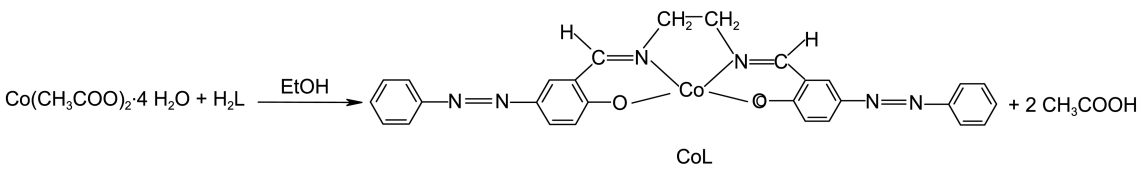
Table 1. The elemental analysis data of the azo Schiff base ligand (H_2L) and CoL complex

Name	Formula	Found (Calcd) %		
		C	H	N
H_2L	$C_{28}H_{24}N_6O_2$	70.57(70.45)	5.11 (5.06)	17.61 (17.65)
CoL	$C_{28}H_{22}N_6O_2Co$	62.97(63.04)	4.19 (4.14)	15.61 (15.65)

Synthesis of the (*N,N′*-Bis(5-phenylazosalicylaldehyde)-ethylenediamine)Cobalt(II). Cobalt(II) complex were prepared in a similar manner as previously described.[26] A solution of $Co(OAc)_2{\cdot}4H_2O$ (0.004 mol) in ethanol (10 mL) was added to a solution of the ligand (H_2L) (0.004 mol) in ethanol and the resulting mixture was refluxed for 2 h. The obtained solution was then left to stand at room temperature. The complex was obtained as dark red microcrystals. The microcrystals were collected by filtration, washed with absolute ethanol and then recrystallized from ethanol/chloroform (1:3, v/v). Red; Yield, 69%, IR (KBr, ν cm^{-1}): 1601 (C=N stretch), 1580, 1530 (aromatic C=C stretch), 1500 (N=N stretch), 1256 (C-O stretch) 534 (M-N), 415 (M-O); ^{1}H NMR (400 MHz, DMSO-d_6) δ 7.4-8.1 (m, 2OH ArH), 8.7 (s, 2H imine), 3.4 (s, 4H CH_2); UV-vis (DMF): λ_{max} = 266 nm, 403 nm. The elemental analysis data of the *azo* Schiff base ligand (H_2L) and CoL complex (given in Table 1) are consistent with the calculated results from the empirical formula of each compound. The synthetic pathway for CoL complex was shown in Scheme 2.

Absorption Spectral Studies. The absorbance measurements were carried out using UV-vis, Perkin Elmer Lambda 25 double beam Spectrophotometer, operating from 200 to 700 nm in 1.0 cm quartz cells. The absorbance titrations were performed at a fixed concentration of the CoL complex and varying the concentration of ct-DNA. In order to prevent interferences due to ct-DNA absorption, the data were obtained by keeping the same concentration of ct-DNA in the reference cuvette.

Viscosity Measurement. The viscosity of ct-DNA solutions was measured at 25 ± 0.1 ºC using an ostwald viscometer. Typically, 10 mM Tris/HCl buffer solution, pH 7 was transferred to the viscometer to obtain the reading of flow time. For determination of solution viscosity, 10 mL of buffered solution of ct-DNA (1.26×10^{-5} M) was taken to the viscometer and a flow time reading was obtained. An appropriate amount of CoL complex was then added to the



Scheme 2. Synthetic pathway for the CoL complex.

viscometer to give a certain R (R = [CoL]/[ct-DNA]) value while keeping the ct-DNA concentration constant and the flow time was read. The flow times of samples were measured after the achievement of thermal equilibrium (30 min). Each point measured was the average of at least five readings. The obtained data were presented as relative viscosity, $(\eta/\eta_o)^{1/3}$ *versus* R, where η is the reduced specific viscosity of ct-DNA in the presence of CoL complex and η_o is the reduced specific viscosity of ct-DNA alone.[27,28]

Melting Experiments. Melting curves were performed using an UV-vis Perkin Elmer Lambda 25 double beam spectrophotometer in conjuction with a thermostated cell compartment. The measurements were carried out in 10 mM Tris/HCl buffer, pH 7. The temperature inside the cuvette was determined with a platinum probe and was increased over the range 25-86 °C at a heating rate of 0.5 °C/min (Thermal software). The melting temperature, T_m was obtained from the mid-point of the hyperchromic transition. In all of the experiments, for the pH measurement, we used a potentiometer (Metrohm model, 744).

Results and Discussion

Electronic Absorption Study. Electronic absorption spectroscopy is usually utilized to determine the binding of complexes with the DNA helix. The absorption spectrum of the CoL complex in the absence and at various concentration of ct-DNA is shown in Figure 1. In the UV-vis region, the CoL complex exhibit two intense absorption bands: one at ~403 nm which is attributed to the metal-to-ligand charge transfer absorption (MLCT) and the other at *ca.* 266 nm which is assigned to the $\pi \rightarrow \pi^*$ transition of the aromatic chromophore.[26] A spectral change of the CoL complex due to addition of ct-DNA was shown in Figure 1. For obtaining these spectra, the fixed amount of CoL complex in Tris/HCl buffer solution, pH = 7 was titrated with a stock solution of ct-DNA. It exhibited the low hyperchromism in all spectral regions and negligible red shift due to the incremental addition of ct-DNA. Hypochromism and hyperchromism are

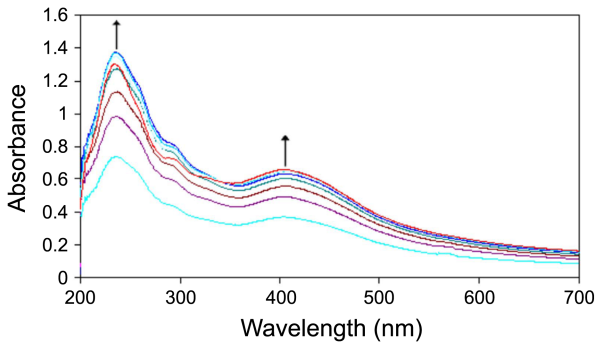


Figure 1. Electronic Absorption spectra of CoL complex [9.4×10^{-5} M] in the absence and in the presence of increasing amount of ct-DNA concentrations [0, 7.2, 3.6, 1.8 and 0.9 mM). Arrow shows the absorbance changes upon increasing ct-DNA concentrations. Also, the arrow shows the changes of absorbance intensities at specific wavelengths (266 nm and 403 nm).

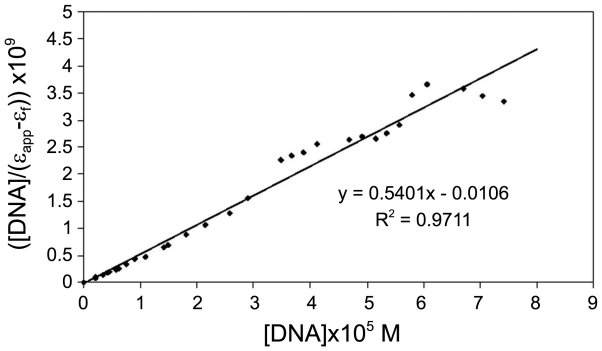


Figure 2. The plot of [ct-DNA]/(|$\varepsilon_{app} - \varepsilon_f$|) *versus* [ct-DNA].

both spectral feature of ct-DNA concerning changes in its double helix structure. Hypochromism happens when the DNA- binding mode of a complex has an electrostatic effect or an intercalation which stabilizes the DNA duplex.[29,30] While hyperchromism may probably be due to dissociation of aggregated ligand or external contact with DNA.[31,32] A similar hyperchromism has been observed for the soret bands of certain porphyrins when they interact with ct-DNA.[33] The apparent binding constant, K_{app}, for the interaction between the CoL complex and ct-DNA can be determined by analysis of absorption spectrophotometric titration data at room temperature using Eq. (1):

$$[DNA]_{total}/(|\varepsilon_{app} - \varepsilon_f|) = [DNA]_{total}/(|\varepsilon_b - \varepsilon_f|) + 1/K_{app}(|\varepsilon_b - \varepsilon_f|) \quad (1)$$

Where ε_{app}, ε_f and ε_b correspond to $A_{observed}$/[CoL], the extinction coefficient for the free CoL complex and the extinction coefficient for the CoL complex in the fully bound form, respectively. In the plot of $[DNA]_{total}/(|\varepsilon_{app} - \varepsilon_f|)$ *versus* $[DNA]_{total}$ that was shown in Figure 2, K_{app} is given by the ratio of the slope to the intercept.[34-36] The apparent binding constant of CoL complex was estimated and used for calculation of Gibbs free energy change of reaction at various temperatures.

Thermodynamics of ct-DNA-CoL Interaction. The energetics of DNA-CoL equilibrium can be conveniently characterized by three thermodynamic parameters, standard Gibbs free energy, $\Delta G°$, standard enthalpy, $\Delta H°$ and standard entropy changes, $\Delta S°$. $\Delta G°$ can be calculated from the equilibrium constant, K, of the reaction using the familiar relationship, $\Delta G° = -RT \ln K$, in which R and T refers to the gas constant, and the absolute temperature, respectively. Furthermore, K is the apparent equilibrium constant and consequently $\Delta G°$ is the apparent Gibbs free energy change. If heat capacity changes for the reaction are essentially zero, the van't Hoff equation (Eq. 2) gives a linear plot of ln$K$ *versus* 1/T (Fig. 3).[37,38]

$$d\ln K/d(1/T) = -\Delta H°/R \quad (2)$$

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 CoL complex with ct-

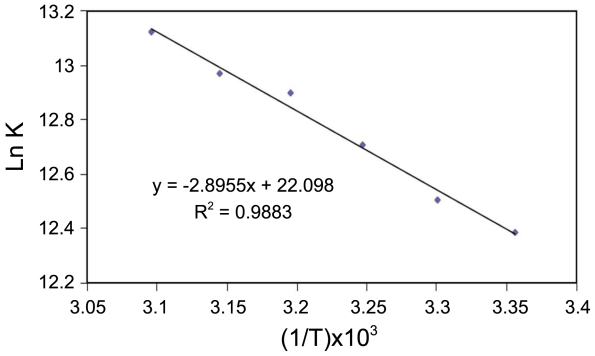


Figure 3. The Van't Hoff plot CoL complex binding to ct-NA.

Table 2. Thermodynamic parameters and binding constants for binding of CoL complex to ct-DNA in 10 mM Tris/HCl buffer, pH 7 at various temperatures

T(K)	Ln*K*	ΔG° (kcal/mol)	ΔH° (kcal/mol)	ΔS° (cal/mol·K)
298	12.39	−7336.23 ± 183.40	5753.94 ± 172.66	43.93 ± 1.18
303	12.51	−7530.24 ± 180.72	5753.94 ± 172.66	43.84 ± 1.14
308	12.71	−7777.32 ± 202.21	5753.94 ± 172.66	43.93 ± 1.10
313	12.90	−8023.23 ± 200.59	5753.94 ± 172.66	44.02 ± 1.05
318	12.97	−8197.10 ± 221.32	5753.94 ± 172.66	43.87 ± 1.14
323	13.12	−8422.80 ± 218.99	5753.94 ± 172.66	43.90 ± 1.10

DNA is shown in Figure 3 and the calculated thermodynamic parameters with their uncertainties are reported in Table 2. It has been revealed that the standard Gibbs free energy changes for ct-DNA-CoL interaction is negative, representing the relative affinity of the CoL complex to ct-DNA. It has also been indicated that the binding process is endothermic disfavored (ΔH° > 0) and entropy favored (ΔS° > 0). As proposed by Ross,[39] when ΔH° < 0 or ΔH° ≈ 0, ΔS° > 0, the mainly acting force is electrostatic; when ΔH° < 0, ΔS° < 0, the mainly acting force is van der Waals or hydrogen bonding and when ΔH° > 0, ΔS° > 0, the mainly force is hydrophobic. Therefore, in the cases of the present system, we presumed that hydrophobic interaction might be the main acting force in the binding of the CoL complex and ct-DNA. From the thermodynamic data, it was quite clear that the interaction processes were endothermic disfavored but entropy favored (ΔH° > 0, ΔS° > 0). The value of *K*, the interaction constants of ct-DNA-CoL, was ~10^4, which was at least 100 times smaller than reported examples of traditional intercalating mode, such as daunomycine,[40] cryptolepine,[41] and chlorobenzylidine.[42] These results furtherly illuminated that the interactions between ct-DNA and CoL complex did not follow the traditional intercalating mode, while the conformation changes of ct-DNA structure may be realized *via* entropy driven non-classical intercalation interaction. The mainly force is hydrophobic.

In order to examine the role of electrostatic interaction in the binding process, the effect of NaCl on the absorption spectrum of ct-DNA-CoL was studied. In this regard, the NaCl stock solution was added stepwise to the mixture of ct-DNA-CoL solution. The results are shown in Figure 4 and Table 3. The absorbance at 403 nm band of studied CoL complex has been decreased due to increasing of NaCl concentration. This hypochromicity is accompanying with blue shift at 403 nm band and confirms the thermodynamic results that correspond to the negligible role of electrostatic interaction.

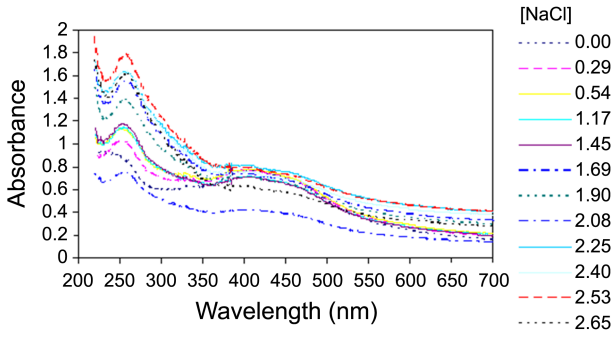


Figure 4. UV-vis spectra of ct-DNA-CoL solution (at a molar ratio of 40) in 10 mM Tris/HCl buffer, pH 7 in the absence and presence of varying concentrations of NaCl.

Table 3. Effect of addition of various concentrations of NaCl on the maximum of absorbance and wavelength of CoL complex in 10 mM Tris/HCl buffer, pH 7

NaCl	A_{max}	ΔA_{max}	λ_{max} (nm)
0	0.9083	0	404
0.54	0.9522	0.0439	404
0.97	0.96	0.0517	402
1.16	0.9225	0.0142	402
1.49	0.8946	−0.0137	401
1.89	0.9287	0.0204	401
2.08	0.9029	−0.0054	397
2.24	0.8154	−0.0929	397
2.39	0.7628	−0.1455	393

Viscosity Measurements. Photophysical spectroscopy measurements provide necessary, but not sufficient evidence to support the binding mode of metal complexes with ct-DNA. Hydrodynamic data provide perhaps the most critical

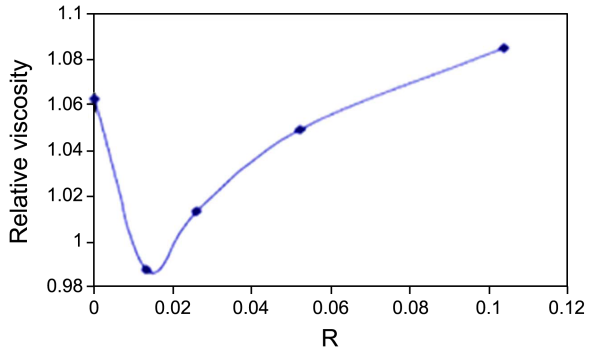


Figure 5. Relative viscosity of ct-DNA (9.4×10^{-5} M) in the presence of increasing amounts of [CoL] at stoichiometric ratios R = [CoL]/[ct-DNA] = 0.0-0.12, plotted as $(\eta/\eta_o)^{1/3}$ *vs.* R. Measurements were done in 10 mM Tris/HCl buffer, pH 7 and at 25 °C.

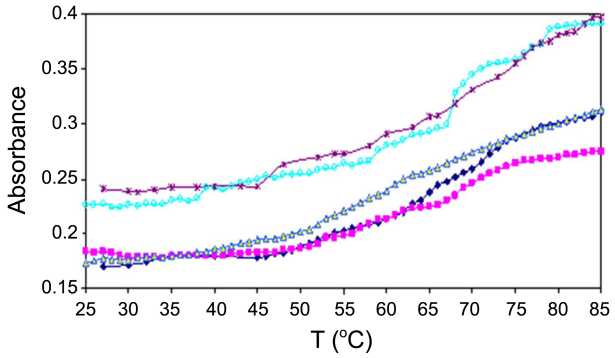


Figure 6. Melting profiles (λ = 260 nm) at various molar ratios (R = [CoL]/[ct-DNA]), R_1 = 0.0 (◆), R_2 = 0.013 (■), R_3 = 0.026 (△), R_4 = 0.052 (○) and R_5 = 0.104 (∗) in 10 mM Tris/HCl buffer, pH 7 and in range of temperature 25 °C-86 °C.

test for intercalative binding in the absence X-ray and NMR structural data.[43] A classical intercalation mode causes a significant increase in the viscosity of ct-DNA solution due to the increase in separation of the base pairs at intercalation sites and hence to an increase in overall DNA contours length.[44] A partial or non-classical intercalation mode of binding could bend or kink the DNA helix, which reduces its effective length and its viscosity.[45] The values of relative specific viscosity *vs* [ct-DNA]/[CoL] was plotted in the absence and presence of CoL complex in Tris/HCl buffer solution (Fig. 5). As it was observed from Figure 5, the relative specific viscosity of ct-DNA exhibited a dependence on the concentration of CoL complex, which decreased at low concentrations of CoL complex, indicating non-classical intercalation mode of binding that may be realized *via* hydrophobic interaction between the CoL complex and ct-DNA,[46] while at high concentrations of [CoL] complex, there was a slight increase in viscosity as shown in Figure 5. Since the change is far less than that observed for an intercalator such as ethidium bromide (EB), this observation leads us to support the above spectral studies which suggest that the complex interact with ct-DNA *via* non-classical intercalation.[43,47]

Thermal Denaturation Measurements. Other strong evidence for the binding mode between the metal complexes and ct-DNA was obtained from ct-DNA melting studies. The intercalation of small molecules into the double helix DNA is known to significantly increase the melting temperature of ct-DNA, at which the double helix denatures into single-stranded ct-DNA.[48] However, the T_m will lightly increase (< 0.6 °C) on the interaction of small molecules with ct-DNA through nonspecific electrostatic interactions with the phosphate backbone of ct-DNA.[49] The extinction coefficient of ct-DNA bases at 260 nm in the double helical form is much less than that in the single stranded form,[50] hence the melting of the helix leads to an increase in the absorption at this wavelength (Fig. 6). Thus, the helix to coil transition temperature can be determined by monitoring the absorbance of ct-DNA at 260 nm as a function of temperature (T_m). Obtained data show that interaction of the [CoL] complex with ct-DNA leads to relatively moderate stabilization of duplex structure (Table 4) Moreover, the increase in [CoL] to [ct-DNA] concentration ratio, R, in the range $0.013 \leq R \leq 0.026$, weakly affects T_m of the melting curve. At greater [CoL]/[ct-DNA] ratios, $0.052 \leq R \leq 0.104$, the T_m value increase with increasing concentration of [CoL] complex. At $R \geq 0.2$ aggregation effects are observed which hinder acquisition of the melting curve. The obtained results specify that at low [CoL]/[ct-DNA] ratios ($R \leq 0.026$) the external binding mode is more reasonable.[44,51,52] The results of thermal denaturation experiments presented are consistent with the absorption spectral profiles which demonstrate a non-intercalative mode. This indicates that CoL complex binds strongly to ct-DNA mostly in the outside-binding and hydrophobic interaction modes.

Table 4. Melting temperature of free ct-DNA in the absence and in the presence of various stoichiometric ratios (R=[CoL]/[ct-DNA])

$R = C_{CoL}/C_{DNA}$	T_m, °C
0	74.4
0.013	75.34
0.026	76.27
0.052	80.52
0.104	84.32

Conclusion

In this work, we present a comprehensive study of the interaction between ct-DNA and CoL complex containing *azo* Schiff base ligand. The CoL complex can be synthesized in high yield using inexpensive starting materials and characterized by elemental analysis and spectroscopic techniques. The mode of interaction of the CoL complex with ct-DNA has been elucidated by UV-vis, thermal denaturation and viscosity measurements. From spectrophotometric titration experiments, the binding constants of CoL complex with ct-DNA were found to be $(2.4 \pm 0.2) \times 10^4$ M^{-1}. The results show that CoL complex bind to ct-DNA by outside-binding and hydrophobic interaction modes. Our research should be valuable for seeking and designing new antitumor drugs, as well as for understanding the mode of the *azo* Schiff base metal complexes binding to ct-DNA and helical conformations of nucleic acids.

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