Osmolyte Changes the Binding Affinity and Mode of Interaction of Minor Groove Binder Hoechst 33258 with Calf Thymus DNA

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The aim of this work was to investigate the effect of altered water activity on Hoechst 33258–calf thymus DNA (CtDNA) interaction by using osmotic stress approach. Water activity was changed by using osmolytes *viz.*, sucrose and triethylene glycol (TEG). We have reported the results of thermal denaturation, absorption and fluorescence spectroscopy and binding affinity measurements as a function of osmolytes concentration. TEG dramatically lowered the thermal stability of CtDNA, $\Delta T_m = -16$ °C whereas sucrose induced very little decrease. Hoechst 33258 increases the stability of CtDNA, but in the presence of TEG, the ΔT_m was −37 °C and a marginal decrease was observed with sucrose. Binding affinity of Hoechst 33258 with CtDNA was found to be reduced from 4.75×10^7 to 0.16×10^7 M^{-1} in TEG and this was accompanied with the increased uptake of 74±2 water molecules. In the presence of sucrose this uptake of water molecules was found to be 30±1. Method of continuous variation suggests that the osmolytes lowered the stoichiometry of Hoechst 33258–CtDNA complex. On the contrary, van't Hoff plot revealed the hydrophobic interaction ($\Delta S = 130.66$ J mol^{-1} K^{-1}) between the Hoechst 33258 and CtDNA. The detailed absorption and fluorescence spectral measurements including the fluorescence lifetime and anisotropy indicated bound state of Hoechst 33258 in osmotic stress condition. Fluorescence lifetime measurement revealed that the contribution from the planar conformer of Hoechst 33258 dominated the binding interaction with CtDNA in presence of TEG. These results can be useful for understanding of interaction of Hoechst 33258 with genomic DNA in a complex environment having altered water activity.

Key words DNA; Hoechst 33258; hydration; osmolyte; energetic

The bis-benzimidazole molecule Hoechst 33258 (Fig. 1) is an important DNA minor groove binding ligand.[1)] Interaction of Hoechst 33258 has been extensively studied using oligonucleotides.[2—5)] Different aspects of this interaction *viz.*, physicochemical, thermodynamics, NMR, X-ray crystallography, *in silico* simulation are now well established.[3,6—11)] From the classical observation on the ability of Hoechst 33258 binding in the AT rich region of minor groove of DNA. Synthetic chemists have designed various DNA minor groove binders for possible applications such as anticancer, antiviral, and other diseases.[12,13)] Studies at cellular level have demonstrated the desired biological effects.[14—16)] New applications are emerging from studies related to radiosensitization and radioprotection in relation to modification of radiation response by Hoechst derivatives.[17—21)] Attempts have also been made to correlate these observations with different Hoechst analogues.

Physicochemical and thermodynamic studies have revealed important details on the mechanism of Hoechst 33258–DNA interaction.[12,22—24)] Role of water surrounding DNA has emerged as an important entity in the interaction processes.[12,22,25)] The network of newly formed hydrogen bonds between the ligand and DNA replaces previously existing direct solute-solvent (water) hydrogen bonds in the minor groove. Hydration changes strongly influences the thermodynamics of ligand–DNA interaction. The change in hydration after the complex formation appeared to be governed by the structure of the ligand.[3,26—29)] Most of the studies and hence our understandings are still based on oligonucleotides.[5,27,30)]

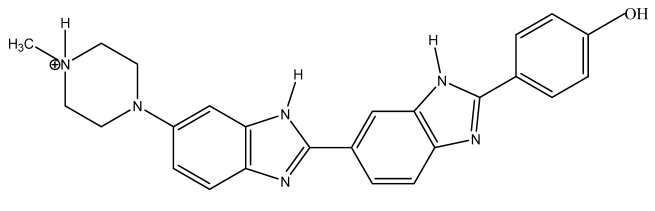

Fig. 1. Structure of Hoechst 33258

Water molecules located close to the binding surfaces are more structured than those of bulk solvent. These locally structured water molecules are retained or released during a binding process with different thermodynamic consequences. Various techniques are available to study hydration changes accompanying ligand–DNA interaction[31,32)] and osmotic stress approach has gained importance due to its ability to specifically alter water activity of the surrounding solution; a fact that has been harnessed to measure forces between molecules.[31,32)] It is relatively a simple technique to study the hydration changes upon ligand–DNA interaction and gives stoichiometric amount of water release or uptake upon biomolecular complexation.[27)] The principle of osmotic stress method is based on Gibbs–Duhem equation.[34,35)] The neutral solutes are added directly to the solution containing the ligand and macromolecule causes changes in the water activity of the solution. Osmotic stress method has been employed for studying the hydration changes for minor groove binder as well as for intercalators.[5,26,27,36—38)]

In living system, small molecules interact with DNA in a very crowded environment. The presence of co-solutes in the *in-vivo* conditions affects the interaction process therefore it is important to study the interaction of Hoechst 33258 with genomic DNA in a very complex environment. In the present study the objective was to elucidate the changes in hydration for Hoechst 33258–calf thymus DNA (CtDNA) interaction. We have used CtDNA thermal denaturation, UV–Vis absorp-

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tion, fluorescence spectroscopy, to obtain information about additional molecular properties associated with changes in hydration. Sucrose and triethylene glycol (TEG) were used as osmolyte in solutions of Hoechst 33258–CtDNA complexes. The results from this study are expected to be beneficial in understanding the role of hydration and physicochemical environment in CtDNA and Hoechst 33258 interactions.

Experimental

Materials All measurements were performed in 1 mM NaH_2PO_4/Na_2HPO_4 buffer with 20 mM NaCl. Calf thymus DNA (CtDNA) (E. Merck, Germany) and Hoechst 33258(2′-[4-hydroxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5′-bi-1*H*-benzimidazole) was purchased from Sigma Chemical Co., U.S.A. and used without further purification. All buffer components and osmolytes *viz.* sucrose and triethylene glycol (TEG) (Sigma Chemicals) were also used as received. All sample preparations were carried out in Milli Q grade water obtained from Elix 3 Water Purification system (Millipore Corp., U.S.A.).

The stock solution of CtDNA was prepared in the phosphate buffer by adding the required amount of CtDNA in buffer and the solution was slowly stirred overnight at room temperature. The stock solution of Hoechst 33258 was prepared in Milli Q water. The concentration of CtDNA was determined spectrophotometrically using molar extinction coefficient of 13200 $cm^{-1}\,M^{-1}$ (bp) at 260 nm.[39] The concentration of Hoechst 33258 was determined using the extinction coefficient 42000 $cm^{-1}\,M^{-1}$ at 340 nm.[40] The osmolality of solutions were measured on freezing point depression based osmometer. The data reported in the result section is mean of two independent experiments.

DNA Melting and Absorption Spectroscopic Measurements CtDNA thermal denaturation and absorption spectral studies of CtDNA, and Hoechst 33258–CtDNA complex were carried out using double beam UV–Visible Spectrophotometer (Cary Bio 100, Varian, Australia) equipped with a 6×6 Peltier temperature controlled sample chamber. Thermal denaturation measurement of CtDNA (100 μM) and Hoechst 33258 (1, 10 μM)–CtDNA (100 μM) complex were carried out in the presence of osmolytes. The solutions were kept in cuvettes and heated from 45 to 90 °C at 0.5 °C/min heating rate and from the plot of optical density (OD) at 260 nm against temperature T_m was calculated. Osmolytes at the desired concentration were added directly to buffer and then required concentration of CtDNA and Hoechst 33258 was prepared and measurements were carried out after 30 min. The absorption spectrum of Hoechst 33258–CtDNA complex in presence of osmolyte was recorded in the spectral range from 500 to 200 nm.

Fluorescence Spectroscopy Fluorescence spectra, anisotropy and excited state lifetime were measured by using an integrated steady state and the time resolved spectrofluorimeter (Model FS900/FL900CDT, Edinburgh Instruments, U.K.). Fluorescence anisotropy measurements were carried out using a pair of Glan–Thompson prisms fitted in the excitation and emission light path. The intensity in parallel and perpendicular directions, *viz.* I_{VV}, I_{VH}, I_{HV}, and I_{HH} were recorded from the respective spectra and were used for the anisotropy plot against wavelength. The equation used for the anisotropy calculation from these intensity files is as given below:

$$r=(GI_{VV}-I_{VH})/(GI_{VV}+2I_{VH}) \quad (1)$$

Where r is the steady state fluorescence anisotropy, I_{VV} corresponds to vertically polarized excitation and vertically polarized emission, I_{VH} corresponds to vertically polarized excitation and horizontally polarized emission, G is the correction factor which is the ratio of sensitivities of the detection system for vertically and horizontally polarized light and obtained from the anisotropy software provided in the instrument. The anisotropy value was calculated at spectral position corresponding to the emission maximum of the respective solutions.

Excited state fluorescence lifetime measurements were carried out using time correlated single photon counting (TCSPC) technique. The excitation source was hydrogen gas filled at low pressure (0.4 bar) flash lamp operating at 40 kHz. The excitation wavelength was selected at 354 nm for Hoechst 33258–CtDNA complexes. The intensity decay curves were fitted as sum of exponentials as

$$I_t=I_o\sum A_i\exp(-t/\tau_i) \quad (2)$$

where, τ_i and A_i are fluorescence lifetime and pre-exponential factor for ith decay component. The excited state lifetime values were calculated by deconvoluting the intensity decay profiles. More precisely, multiexponential fittings are applied along with the instrument response function of the excitation wavelength in the deconvolution process. The instrument response was obtained with Ludox as scattering medium. The analysis of the fitted data was tested using statistical parameters provided in the analysis software.

The emission spectra of the Hoechst 33258–CtDNA complexes was measured with excitation wavelength at 354nm and scanned from 375 to 650 nm. The measuring conditions for emission spectrum were: excitation and emission monochromator slits 1 mm; dwell time 0.2 ns and wavelength step 0.5 nm. For anisotropy measurement the polarizer was moved into the excitation and emission light paths in the sample compartment and emission spectra was recorded from 375 to 650 nm range in all the four directions. From the emission anisotropy spectra the value of anisotropy was calculated at corresponding spectral maximum position. Excited state fluorescence lifetime measurement of Hoechst 33258–CtDNA was carried out at 354 nm excitation and the intensity decay file at 480 nm was acquired.

Determination of Binding Constant. Effect of Osmolyte and Temperature The binding constant of Hoechst 33258 with CtDNA was determined by fluorescence titration method. Fixed ligand concentration (2 ml) was titrated with increasing concentration of CtDNA in phosphate buffer. Data were transformed in the form of a Scatchard plot of r/C_f *versus* r, where r is the ratio of bound ligand to the total DNA (base pair) concentration and C_f is the concentration of free ligand. The total fluorescence intensity (I_t) was assumed to be sum of the contributions from both free and bound Hoechst 33258 as

$$I_t=I_o(C_t-C_b)+I_bC_b \quad (3)$$

where I_o and I_b are the fluorescence intensities of free and fully bound Hoechst 33258 and C_t and C_b are the corresponding total and bound concentrations. From the slope of the plot, the binding constant K_o is determined. Similar titration was carried out in presence of different concentrations of osmolytes, *viz.* sucrose (0—3 Osm) and TEG (0—2.5 Osm). This binding titration was also carried out at different temperature to measure the binding forces from van't Hoff plot. The temperature range from 298 to 318 K. Using van't Hoff equation

$$\log K_o=-\Delta H/2.303RT+\Delta S/2.303R \quad (4)$$

When the measured $\log K_o$ is plotted with respect to $1/T$, where T is temperature in Kelvin, the thermodynamic parameters namely, the enthalpy change (ΔH) and the entropy change (ΔS) can be calculated from slope and y-intercept of the linear van't Hoff plot based on $\log K_o$ *versus* $1/T$. In the equation, R denotes molar gas constant. The free energy change (ΔG) is estimated from the following relationship:

$$\Delta G=\Delta H-T\Delta S \quad (5)$$

The concentration of Hoechst 33258 was taken as 1 μM and that of CtDNA was 50 μM. The solutions of CtDNA and Hoechst 33258 were prepared in phosphate buffer. The effect of osmolyte was investigated on the binding affinity of Hoechst 33258. For understanding the effect of osmolyte on Hoechst 33258–CtDNA interaction, osmolytes were added to buffer and then the required concentration of CtDNA and Hoechst 33258 was prepared in osmolyte containing buffer.

Effect of Osmolyte on Thermodynamics of Hoechst 33258–CtDNA Interaction The fluorescent binding titration of Hoechst 33258 with CtDNA at five different temperatures (298 to 318 K) at two different concentration of sucrose and TEG was carried out to study the effect of these osmolytes on thermodynamics of Hoechst 33258–CtDNA interaction. The concentration of Hoechst 33258 was 1 μM and of CtDNA was 50 μM. The osmolyte concentration chosen was 0.5 and 1 Osm. The osmolytes were added to the buffer and the required concentration of CtDNA and Hoechst 33258 was prepared in osmolyte containing buffer and binding affinity was calculated from the slope of Scatchard plot at five different temperatures.

Method of Continuous Variation The stoichiometry for the binding of Hoechst 33258 to CtDNA was obtained in phosphate buffer using the method of continuous variation.[41,42] The concentrations of Hoechst 33258 and CtDNA both were varied, while the sum of the concentrations was kept constant at 2 μM. Varying volumes of equally concentrated stock solutions of the Hoechst 33258 and CtDNA were mixed to give a mole fraction of ligands ranging from 0 to 0.97. Fluorescence intensity of the complex is plotted against the input mole fraction of the dye (χ). Break point in the resulting plot corresponds to the mole fraction of the ligand in the complex. The stoichiometry is obtained in terms of DNA–ligand $[(1-\chi_{ligand})/\chi_{ligand}]$, where

χ_{ligand} denotes mole fraction of ligand.

Results

CtDNA Melting Study The UV thermal denaturation measurements of CtDNA and Hoechst 33258–CtDNA complex were carried out to measure melting temperature, T_m in the presence of sucrose and TEG. The melting curve showed a decrease in T_m of CtDNA from 75 to 68.8 °C and 59 °C in the presence of 1 and 3 Osm of TEG respectively (Fig. 2A). Sucrose caused relatively lesser extent of lowering of T_m to 73.5 and 70.5 °C at 1 and 3 Osm respectively. The results obtained from the thermal denaturation of CtDNA in presence of osmolytes are summarized in Table 1.

Hoechst 33258 increases T_m of CtDNA in concentration dependent manner.[43,44] Osmolytes appear to lower T_m of CtDNA. Therefore the effect of two different concentration of Hoechst 33258 (1, 10 μM) in phosphate buffer in Hoechst 33258–CtDNA complex studied using melting temperature measurements. The measured T_m values depicted the expected trend *viz.*, 79.6 and 83.5 °C (*i.e.*, Hoechst 33258 concentration dependent increase). But in the presence of osmolyte the stability of Hoechst 33258–CtDNA complex decreased. For example, 3 Osm of TEG lowered the T_m of Hoechst 33258–CtDNA complex to 42.3 °C from 79.6 °C at 1 μM of Hoechst 33258. At increasing concentration of Hoechst 33258 (10 μM), although the initial increase of T_m was observed at 83.5 °C but this value lowered to 46.8 °C. The relative changes (ΔT_m) for both the concentrations of Hoechst 33258 are in same range *viz.*, 37 °C. Sucrose caused only marginal changes ΔT_m=6.2—4.9 °C depending upon the concentration of Hoechst. The melting profiles of Hoechst 33258–CtDNA at higher concentration of Hoechst 33258 in presence of TEG are shown in Fig. 2B and the values of T_m are shown in Table 2.

Binding Affinity of Hoechst 33258 with CtDNA and Change in Hydration The decrease in T_m is likely to have consequence on the binding affinity of Hoechst 33258. Sucrose and TEG significantly decreased the binding affinity (K_o) of Hoechst 33258 with CtDNA. In particular, the binding constant has decreased gradually from 4.75×10^7 to 0.93×10^7 M^{-1} with increasing concentration of sucrose whereas TEG further lowered the binding affinity of Hoechst 33258 (Table 3). This difference in binding affinity of Hoechst 33258 is evident from the slope of plot of [Osm] against $\ln K_s/K_o$ (K_s is binding affinity of Hoechst 33258 in presence of the osmolyte) (Fig. 3). The slope derived from this plot was used in Eq. 6,[37] to derive the changes in hydration of Hoechst 33258–CtDNA complex in presence of osmolyte

$$\partial \ln(K_s/K_o)/\partial[\mathrm{Osm}] = -\Delta n_w/55.5 \quad (6)$$

Where $\ln(K_s/K_o)$ is the change in binding free energy, Osm is

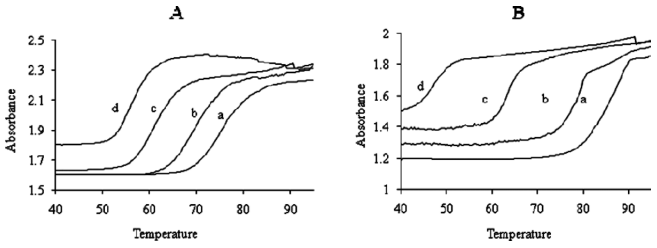

Fig. 2. (A) UV Melting Curves for CtDNA at Different Concentration of TEG, (a) 0 Osm, (b) 1 Osm (c) 2 Osm and (d) 3 Osm and (B) UV Melting Curves of Hoechst 33258 (10 μM)–CtDNA Complex in the Absence (a) and in the Presence of (b) 1 Osm, (c) 2 Osm and (d) 3 Osm TEG

Concentration of CtDNA was 100 μM (bp). All measurements were carried out in phosphate buffer consisting of 1 mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 20 mM NaCl.

Table 1. UV Melting Temperature (T_m in °C) of CtDNA with and without Sucrose and TEG

Osmolyte (mol/kg)	T_m (°C)	
	Sucrose	TEG
0	75.0 (±0.08)	75.0 (±0.08)
1	73.5 (±0.7)	68.8 (±1.02)
2	72.5 (±0.7)	61.4 (±0.3)
3	70.5 (±0.7)	59.0 (±0.07)

Table 2. UV Melting Temperature (T_m in °C) of Hoechst 33258–CtDNA Complex with and without Sucrose and TEG

Hoechst (mM)	Osmolyte (mol/kg)	T_m (°C)	
		Sucrose	TEG
1	0	79.6 (±1.1)	79.6 (±1.1)
	1	75.2 (±1.2)	73.4 (±0.5)
	2	74.2 (±0.7)	54.5 (±2.8)
	3	73.4 (±0.6)	42.3 (±1.4)
10	0	83.5 (±2.1)	83.5 (±2.1)
	1	80.5 (±0.7)	78.5 (±0.0)
	2	79.6 (±0.6)	65.0 (±2.0)
	3	78.6 (±0.9)	46.8 (±1.3)

Table 3. Comparison of Binding Constant (K_s) for Minor Groove Binder Hoechst 33258 with CtDNA in Presence of Different Concentration of Sucrose and TEG

Osmolyte conc. (mol/kg)	K_s (10^7 M^{-1})	
	Sucrose	TEG
0.5	3.75 (±0.07)	2.6 (±0.14)
1	3.12 (±0.28)	1.3 (±0.14)
1.5	2.0 (±0.14)	0.55 (±0.035)
2	1.47 (±0.098)	0.37 (±0.02)
2.5	1.3 (±0.28)	0.16 (±0.014)
3	0.93 (±0.035)	—

The concentration of Hoechst 33258 and CtDNA were 1 μM and 50 μM respectively. K_o is the binding constant in absence of osmolyte and it was $4.75(\pm0.07)\times10^7$ M^{-1}.

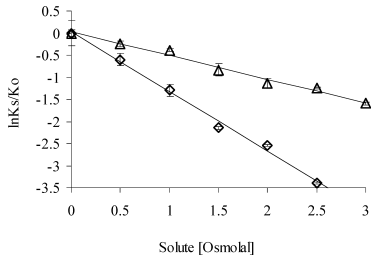

Fig. 3. Plot of Natural Logarithm of the Ratio of the Binding Affinity (K_s) at a Given Concentration of Osmolyte to the Binding Affinity (K_o) without Osmolyte

The osmolyte sucrose (△) and TEG (◇) concentration is represented on the abscissa. Linear least square fit using Eq. 6 gives changes in number of water molecule accompanying Hoechst 33258–CtDNA complexation Δn_w 30±1 for sucrose and 74±2 for TEG.

the osmolality of the solution and Δn_w is the number of exchanged water or difference in number of bound water molecules between the complex and the free reactants. A positive sign for Δn_w signifies the uptake of water upon complexation and the calculated values are 74 ± 2 and 30 ± 1 in the presence of TEG and sucrose respectively.

Binding Forces between Hoechst 33258 and CtDNA The interaction forces between a DNA minor groove binding molecule Hoechst 33258 and DNA include hydrophobic force, electrostatic interactions, vander Waals interactions, hydrogen bonds, their relative contribution are likely to vary. Due to a possible dependence of association binding constant on temperature, a thermodynamic process was considered to be responsible for the formation of the complex. If the enthalpy change (ΔH) does not vary significantly over the temperature range studied, then its value and entropy change (ΔS) can be determined using the van't Hoff equation (Eq. 4). The temperatures used in the study were 298 K, 303 K, 308 K, 313 K and 318 K. The change in enthalpy (ΔH) and the change in entropy (ΔS) are calculated from the slope and intercept of the plot $\log K$ *versus* $1/T$, respectively. The free energy change (ΔG) is determined from the Eq. 5. The thermodynamic parameters ΔH, ΔS and ΔG values so calculated were found to be $-4.88\,\mathrm{kJ\,mol^{-1}}$, $+130.66\,\mathrm{J\,mol^{-1}\,K^{-1}}$ and $-43.8\,\mathrm{kJ\,mol^{-1}}$ respectively (Table 4A).

Binding Forces between Hoechst 33258 and CtDNA in Presence of Osmolyte The effect of osmolyte *viz.* TEG (0.5, 1 Osm) and sucrose (0.5, 1 Osm) was studied on thermodynamics of Hoechst 33258–CtDNA interaction. The temperatures used in the study were 298 K, 303 K, 308 K, 313 K and 318 K. The binding affinity of Hoechst 33258 with CtDNA in presence of osmolyte decreases with the increase in temperature. The change in enthalpy and entropy was calculated from van't Hoff plot and change in free energy was calculated from Eq. 5. Thermodynamic data in the presence of 0.5 Osm TEG (Table 4B) clearly reveals as $\Delta H=-18.8\,\mathrm{kJ\,mol^{-1}}$, $\Delta S=+79.3\,\mathrm{J\,mol^{-1}\,K^{-1}}$ and $\Delta G=-42.3\,\mathrm{kJ\,mol^{-1}}$ respectively while in presence of 0.5 Osm sucrose the ΔH, ΔS and ΔG values so calculated were found to be $-29.8\,\mathrm{kJ\,mol^{-1}}$, $+45.2\,\mathrm{J\,mol^{-1}\,K^{-1}}$ and $-43.2\,\mathrm{kJ\,mol^{-1}}$ respectively. The effect of osmolyte on thermodynamics of Hoechst 33258–CtDNA interaction is summarized in Table 4B.

Table 4. Binding Constants and Thermodynamic Parameters of the Interaction Hoechst 33258 with CtDNA at Different Temperatures

(A)

T (K)	K_a ($10^7\,\mathrm{M^{-1}}$)	Thermodynamic parameters		
		ΔS ($\mathrm{J\,mol^{-1}\,K^{-1}}$)	ΔG ($\mathrm{kJ\,mol^{-1}}$)	ΔH ($\mathrm{kJ\,mol^{-1}}$)
298	4.75		−43.8	
303	4.75	130.66 (±3.53)	−44.5	−4.88 (±1.01)
308	4.45		−45.1	
313	4.3		−45.8	
318	4.2		−46.4	

(B)

Thermodynamics parameter (at 298 K)	Osmolytes			
	TEG		Sucrose	
	0.5 Osm	1 Osm	0.5 Osm	1 Osm
ΔH ($\mathrm{kJ\,mol^{-1}}$)	−18.8	−27.5	−29.8	−29.4
ΔS ($\mathrm{J\,mol^{-1}\,K^{-1}}$)	+79.3	+43.8	+45.2	+43.6
ΔG ($\mathrm{kJ\,mol^{-1}}$)	−42.3	−40.5	−43.2	−42.4

(A) Without osmolyte and (B) with 0.5 Osm and 1 Osm TEG and sucrose.

Stoichiometry of the Hoechst 33258–CtDNA Interaction The method of continuous variation was used to obtain a Job plot for the binding of Hoechst 33258 with CtDNA in phosphate buffer at pH 7.2 and the possible effect of osmolytes was observed (Fig. 4). This diagram shows an intersection point at 0.15 mol fraction of Hoechst 33258. This value corresponds to a 1 : 5 (Hoechst 33258 : CtDNA in bp) stoichiometry. Increasing concentration of sucrose 1.5 and 2.5 Osm shifted this intersection point to 0.25 and thus the corresponding binding stoichiometry is 1 : 3 CtDNA bp. TEG significantly reduced the binding affinity of Hoechst 33258 with CtDNA. The stoichiometry is also reduced to 1 : 0.66 CtDNA (bp) in 2.5 Osm TEG.

Absorption Spectroscopy of Hoechst 33258–CtDNA Complex To assess the effect of osmolyte on the environment at the binding sites, spectral characteristics of Hoechst 33258–CtDNA complex were studied by absorption spectroscopy. Figures 5A and B show the absorption spectra of Hoechst 33258–CtDNA complex at 0—3 Osm concentrations of sucrose and TEG respectively. The absorbance at 260 nm increased significantly with concentration of sucrose (Fig. 5A). The absorbance at 354 nm increased significantly with concentration of sucrose and no spectral shift was observed in absorbance maxima at 354 nm.

Similar absorption spectral measurements were carried out in the presence of TEG (Fig. 5B). Hyperchromicity at 260 nm was observed with increase in concentration of TEG. A decrease in absorbance from 0.3 to 0.24 was observed at 354 nm associated with red spectral shift from 354 to 368 nm (Fig. 5B).

Fluorescence Spectral Measurement of Hoechst 33258–CtDNA Complex Hoechst 33258 has a characteristic emission band at 500 nm in aqueous solution and its fluorescence intensity and spectral positions depends on interaction with DNA.[45] Thus, fluorescence spectral measurements are expected to provide additional information on the Hoechst 33258–CtDNA complex in different osmolytes. The fluorescence intensity of Hoechst 33258 increases with a blue spectral shift when it forms complex with DNA as compared to free Hoechst 33258.[46] The emission spectra of Hoechst 33258 (10 μM)–CtDNA complex in the presence of 0—3 Osm sucrose showed decrease in the fluorescence intensity (Fig. 6A) with red spectral shift from 470 (0 Osm) to 480 nm

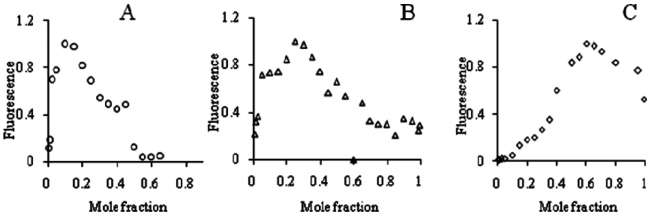

Fig. 4. Job Plot for Hoechst 33258 Binding to CtDNA in Presence of Osmolyte Sucrose and TEG

The sum of the concentrations of the two components was kept constant at [CtDNA] : [Hoechst 33258] ratio 2 μM. (A) no osmolyte, (B) 2.5 Osm sucrose, (C) 2.5 Osm TEG.

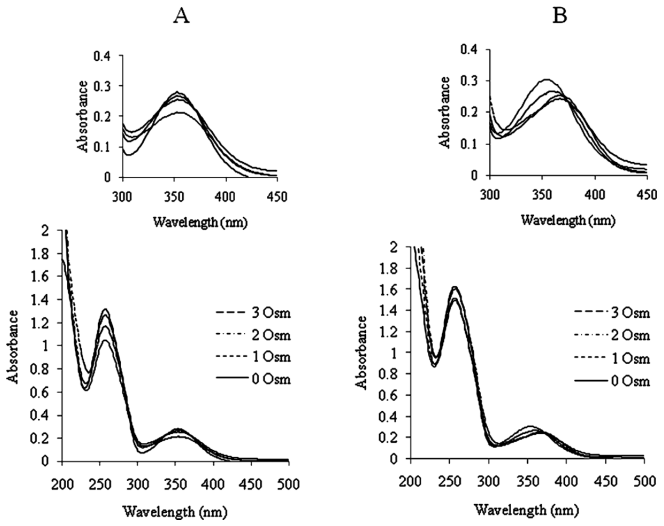

Fig. 5. (A) Changes in Absorption Spectra of Hoechst 33258 (10 μM)–CtDNA Complex at Different Concentration of Sucrose (———) 0 Osm, (--------) 1 Osm, (-·-·-·-·-) 2 Osm and (— —) 3 Osm and (B) Changes in Absorption Spectra of Hoechst 33258 (10 μM)–CtDNA Complex at Different Concentration of TEG (———) 0 Osm, (--------) 1 Osm, (-·-·-·-·-) 2 Osm and (— —) 3 Osm

Inset shows the magnified absorbance of the Hoechst 33258–CtDNA complex in presence of (A) sucrose and (B) TEG. All experiments were carried out in phosphate buffer containing 1 mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 20 mM NaCl. The concentration of CtDNA was 100 μM (bp).

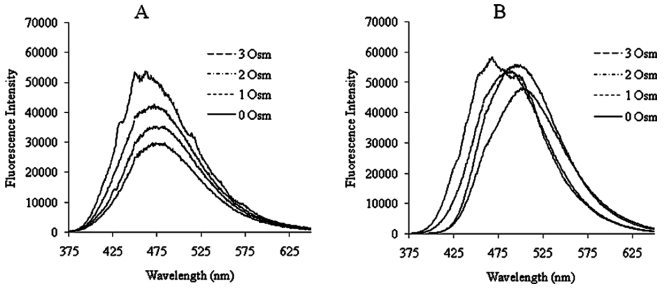

Fig. 6. (A) Changes in Emission Spectra of Hoechst 33258 (10 μM)–CtDNA Complex in Different Concentration of Sucrose (———) 0 Osm, (--------) 1 Osm, (-·-·-·-·-) 2 Osm and (— —) 3 Osm and (B) Changes in Emission Spectra of Hoechst 33258 (10 μM)-CtDNA Complex at Different Concentration of TEG (———) 0 Osm, (--------) 1 Osm, (-·-·-·-·-) 2 Osm and (— —) 3 Osm

All experiments were carried out in phosphate buffer containing 1 mM Na_2HPO_4/NaH_2PO_4 pH 7.2, 20 mM NaCl. The concentration of CtDNA was 100 μM (bp).

(3 Osm). Figure 6B shows the emission spectra of Hoechst 33258 (10 μM)–CtDNA complex in the presence of 0—3 Osm of TEG with a red spectral shift from 466 (0 Osm) to 502 nm (3 Osm) and fluorescence intensity decreased with increase in the concentration of TEG.

Fluorescence Anisotropy Measurement of Hoechst 33258–CtDNA Complex Fluorescence anisotropy measurements reveal changes in the orientation of fluorescent molecule when bound to macromolecules like DNA, proteins and membranes.[47] The fluorescence anisotropy of Hoechst 33258 bound to DNA is higher as compared to free Hoechst 33258.[48,49] The measured values of anisotropy are shown in Table 5 along with the fluorescence lifetime data. The anisotropy value 0.3 and 0.25 for the two concentrations of Hoechst 33258 in Hoechst 33258–CtDNA complex remained unaltered in presence of sucrose and TEG suggesting unaltered orientation.

Excited State Fluorescence Lifetime Measurement of Hoechst 33258–CtDNA Complex Fluorescence lifetime measurement enables us to understand the dynamics of fluorescent complex. Hoechst 33258 is known to bind with DNA in two different conformations *viz.*, planar and non planar and these can be identified with distinct fluorescence lifetimes and in the present context these measurements were carried out to elucidate the possible changes in the contribution of these two different conformers of Hoechst 33258 with CtDNA in presence of osmolyte. Fluorescence lifetime of free Hoechst 33258 in phosphate buffer has characteristic short (τ_1) and long (τ_2) decay constants.[43,47,50] Cosa *et al.* showed two modes of binding of Hoechst 33258 on the basis of double lifetime decay characteristics.[49] The excited state fluorescence lifetime of Hoechst 33258–CtDNA complex in phosphate buffer was measured with excitation at 354 nm. The fluorescence decay profiles after analysis revealed double exponentials having a short (τ_1) and long (τ_2) decay component. The numerical values of τ_1 and τ_2 and their relative distribution altered with the concentration of Hoechst 33258, for example, at lower concentration of Hoechst 33258 (1 μM), the values of τ_1 and τ_2 components were 2.5 ns and 4.4 ns with relative contribution of 43% and 57% respectively. At higher concentration of Hoechst 33258 (10 μM), the numerical values of these decay components decreased to 1.3 ns and 3.9 ns with similar relative contribution of 32% and 68% respectively. The fluorescence lifetime measurement of Hoechst 33258–CtDNA complex in presence of osmolyte revealed interesting observation. Sucrose (3 Osm) is able to

Table 5. Excited State Fluorescence Lifetime and Fluorescence Anisotropy Measurement of Hoechst 33258–CtDNA Complex in the Presence of Different Concentration of TEG and Sucrose

Hoechst (μM)	Osm	TEG						Sucrose					
		τ_1	A_1	t_2	A_2	χ^2	Ani	τ_1	A_1	τ_2	A_2	χ^2	Ani
1	0	2.5	43	4.4	57	0.98	0.34	2.5	43	4.4	57	0.98	0.3
	1	1.5	8	4.2	92	0.8	0.34	2.3	42	4.4	58	0.99	0.3
	2	1.6	5	4.0	95	0.9	0.37	2.0	36	4.3	64	1.04	0.3
	3	2.2	14	4.0	86	0.91	0.37	3.0	72	5.6	28	1.02	0.3
10	0	1.3	32	3.9	68	0.83	0.26	1.3	32	3.9	68	0.83	0.25
	1	1.7	12	4.2	88	0.90	0.25	1.2	29	3.8	71	0.90	0.25
	2	2.4	19	4.2	81	0.98	0.28	1.2	32	3.8	68	0.89	0.25
	3	2.6	26	4.1	74	1	0.26	1.3	39	3.9	61	0.99	0.26

χ^2 is the statistical fitting parameter. The numerical values of τ is in ns and A indicate the % contribution of the two components of lifetimes.

alter the excited state lifetime only at lower concentration of Hoechst 33258 *i.e.* τ_1 was 3.0 ns and τ_2 was 5.6 ns (Table 5) whereas TEG have shown the more effect on τ_1 of Hoechst 33258–CtDNA complex in both the ratios but τ_2 remains unaffected (Table 5). At higher concentration of Hoechst 33258, in presence of 1 Osm of TEG the τ_1 and τ_2 values were 1.7 ns and 4.2 ns respectively with the relative contribution of 12% and 88%. But at 3 Osm TEG the τ_1 and τ_2 values were 2.6 ns and 4.1 ns and the relative contribution to τ_1 increased to 26% and for τ_2 the relative contribution was 74%.

Discussion

Understanding the role of water in ligand–DNA interaction has gained importance in DNA targeted rational drug designing.[51,52] In this study, we have investigated the role of water on Hoechst 33258–CtDNA interactions. The results of UV thermal denaturation studies have clearly showed the distinct effects of two different osmolytes sucrose and TEG on CtDNA stability (Table 1). TEG destabilized CtDNA ($\Delta T_{m,TEG} = -16$ °C) by much higher extent as compared to sucrose ($\Delta T_{m,Suc} = -5$ °C). These solutes are known to lower the water activity outside the hydration shell of DNA and thereby facilitate water removal from the hydration shell.[53] The hydroxyl groups of polyhydric alcohols (TEG and sucrose) can compete with water for the formation of hydrogen bonds with the side groups of the DNA nitrogenous bases both in the minor and major grooves, thereby increasing the dehydration of DNA and thus destabilizes the DNA. The observed destabilizing ability of these osmolytes also seems to be related to the molecular size and number of hydroxyl groups present in the osmolyte. The reduced water activity in presence of osmolytes can in turn alter the forces responsible for stabilizing the DNA double helix, namely hydrogen bonds, vander Waals, hydrophobic and electrostatic interactions. As the water activity reduces the repulsion between the negative charges of the phosphate groups are expected to be enhanced and thus a gradual destabilization of the DNA double helix occurs.

Hoechst 33258 has been extensively studied for elucidating different aspects including physicochemical processes underlying its interaction with DNA by using a variety of experimental techniques.[4,5,7] Binding of Hoechst 33258 increases the stability of CtDNA in a concentration dependent manner.[43,44] In the presence of these osmolytes interesting observation were made in respect of lowering of T_m. For example, though Hoechst 33258 increased the T_m in concentration dependent manner in CtDNA, TEG caused higher ($\Delta T_m = -36.7$ to -37.3 °C) as compared to marginal decrease in sucrose ($\Delta T_m = -4.9$ to -6.2 °C) (Table 2). These results suggest that the presence of osmolytes can affect the stability of Hoechst 33258–CtDNA complex. These osmolytes acts by altering the property of water whereas Hoechst 33258 interacts directly with the structure of CtDNA. The significant differences in the ability to reduce the stability of the complex in presence of TEG can be possibly related to its higher ability to alter water activity. In order to investigate this aspect in CtDNA, we have undertaken the binding titration in the similar experimental conditions. The results from the plot of $\ln K_s/K_o$ *vs.* osmolyte concentration using Eq. 6 provided the Δn_w value for the complex formation and TEG facilitated higher extent of water uptake ($\Delta n_w = 74 \pm 2$) in the binding site of Hoechst 33258–CtDNA complex. This has caused such lowering of stability of the Hoechst 33258–CtDNA complex (Table 2). The binding affinity of Hoechst 33258 with CtDNA has also decreased from 4.75 to 0.16×10^7 M^{-1} in the presence TEG (Table 3). The osmotic stress method shows that the complexation of Hoechst 33258 with CtDNA is accompanied by uptake of 30 ± 1 and 74 ± 2 water molecules in presence of sucrose and TEG respectively. This result is in agreement to previous studies with oligonucleotides and for intercalators–CtDNA interaction.[27,37] Thus there is a correlation between osmolyte induced lowering CtDNA stability, binding affinity and water activity.

The complementary fit of Hoechst 33258 is known to displace most of the interfacial water as demonstrated by X-ray crystallography, thermodynamics and molecular dynamic simulation studies.[4,5,7,54] Volumetric and compressibility studies have also reported acquisition of 21—34 waters in the Hoechst 33258–DNA complex.[31] In addition, the volume changes measured using hydrostatic pressure indicated enhanced hydration of the Hoechst 33258 complex with poly(d*A*)–poly(d*T*).[55] This aspect is also demonstrated in femtosecond resolved fluorescence studies indicate the presence of weakly bound waters in the Hoechst 33258–DNA complex.[56,57] All these studies have indicated association of water in the Hoechst 33258–DNA complex.

The thermodynamics parameters obtained from van't Hoff plot (Table 4A) suggest a negative enthalpy and positive entropy for Hoechst 33258–CtDNA interaction. Favorable enthalpy is an indication of hydrogen bonding. This result is in close agreement with the thermodynamic data obtained from van't Hoff plot for the binding of Hoechst 33258 with poly [d(*A*–*T*)].[24] Thermodynamic study based on Hoechst 33258–oligonucleotides interactions indicates the positive enthalpy and positive entropy, indicating that binding is driven by hydrophobic interactions with a favorable change in binding free energy. Favorable entropy changes are primarily due to hydrophobic interactions, as a result an increase in solvent entropy from burial of hydrophobic groups and release of water upon binding. But the result from the osmotic stress measurement clearly reveals the uptake of water for Hoechst 33258–CtDNA interaction. This suggests that thermodynamic study and osmotic stress studies are sensitive to different kind of bound water. Thermodynamic study measures the total hydration changes for reactions involving macromolecules.

The thermodynamic data obtained in the presence of osmolyte (Table 4B) suggest that osmolyte effect the hydrogen bond formation between ligand and DNA. Our thermodynamic data reveals that in presence of sucrose the hydrogen bonding between the Hoechst 33258 and CtDNA remains same at two 0.5 and 1 Osm sucrose concentration while change in ΔS revealed the binding of Hoechst 33258 with CtDNA is less hydrophobic in presence of sucrose as compared to without osmolyte. TEG has remarkable effect on thermodynamics of Hoechst 33258–CtDNA interaction. In presence of TEG, ΔH becomes more and more negative with the concentration of TEG (Table 4B) and hydrophobic nature of interaction decreases. This suggests that in presence of osmolyte the interaction between Hoechst 33258 and CtDNA is predominantly due to hydrogen bonding. Changes in binding

free energy suggest that the interaction of Hoechst 33258 with CtDNA is more or less equally feasible in absence and presence of osmolyte.

The use of the continuous variation method (Fig. 4) confirms the well-defined 1 : 5 Hoechst 33258–CtDNA binding stoichiometry.[24] The stoichiometry of Hoechst 33258–CtDNA complex is altered in presence of osmolyte and this is in accordance with the osmolyte induced decreased binding affinity (Fig. 4). TEG being more effective in lowering the stoichiometry of Hoechst 33258–CtDNA complexation than sucrose and therefore the observed reduced binding affinity of Hoechst 33258 with CtDNA is related to lowered stoichiometry. This lowering of binding stoichiometry is in support of the results obtained from the thermal denaturation studies and binding titration.

Spectroscopic studies of Hoechst 33258–CtDNA were undertaken to investigate the affect of altered water activity around the complex. Latt and Wohlab[45] showed that the absorbance of Hoechst 33258–DNA decreases with red spectral shift of absorption maxima.[46] In the present study, the increased absorbance (hyperchromicity) at 260 nm of CtDNA (Figs. 5A, B) upon addition of osmolytes is thus associated with the lowering of thermal stability of CtDNA (Table 1). The absorption band at 354 nm arising from the bound Hoechst 33258, showed further red shift with TEG increasing concentration (Fig. 5B). This red spectral shift in absorption spectra indicated that the polarity of the binding site has increased in presence of osmolyte with respect to Hoechst 33258–CtDNA complex in aqueous solution.

The decrease in fluorescence intensity with red spectral shift in emission spectra is again associated with the decreased binding affinity of Hoechst 33258 with CtDNA in presence of osmolyte. This is also related with increase in hydration of the complex upon addition of osmolytes (Figs. 6A, B). The observed value of higher anisotropy *viz.*, 0.3 (Table 5) of Hoechst 33258 clearly indicate the bound state of this ligand[43] even though the binding affinity has lowered significantly (Fig. 3). The value of anisotropy was not changed by osmolytes and thereby indicates that the presence of osmolytes did not alter the orientation of the bound Hoechst 33258. These measured parameters thus infer the following effect of osmolyte on Hoechst 33258–CtDNA complex: Osmolyte lowers T_m accompanied by increase in net hydration of CtDNA depending on the nature of osmolytes and binding affinity was though reduced but the Hoechst 33258 remained bound in the same orientation.

Hoechst 33258 is known to exists in two conformers (planar and non planar) in solutions. These two conformers are with respect to the two benzimidazole moieties present in the Hoechst 33258 molecule. When both the benzimidazoles are in same plane then it is called as planar and otherwise nonplanar. Fluorescence lifetimes of these conformers are distinctly different.[49] For example, Hoechst 33258 in phosphate buffer has the two decay components 0.14 ns (τ_1) and 2.5 ns (τ_2).[49] The short and long decay components *i.e.*, τ_1 and τ_2 correspond to non planar and planar conformers of Hoechst 33258 respectively[50] suggesting the coexistence of both the conformers in solution. The numerical values and relative contribution of both the conformers of Hoechst 33258 change upon binding with CtDNA.[42] Further, these characteristics are strongly dependent on the nature of the binding sites and the ligand.[58] In order to examine the effects of osmolytes on the different modes of binding of Hoechst 33258 fluorescence lifetimes measurements on Hoechst 33258–CtDNA complex were undertaken. The detailed analysis of fluorescence lifetimes of Hoechst 33258–CtDNA in presence of these osmolytes clearly showed that TEG caused decrease in contribution from non-planar conformation in the bound Hoechst 33258 with CtDNA (Table 5). From these observations, the first and foremost conclusion is that Hoechst 33258 remained in the bound state with CtDNA in presence of high concentration of TEG in planar form. Thus it is an interesting situation, where osmolytes though affected the stability of the Hoechst 33258–CtDNA complex but Hoechst 33258 remained in the bound state. Thus in osmotic stress condition induced by TEG around CtDNA Hoechst 33258 still able to bind with CtDNA in planar conformation. The binding affinity of this conformer is however much low. This is a new insight on the effect of osmolytes induced changes in the mode of binding of Hoechst 33258 with CtDNA.

Conclusion

Most of the minor groove binding ligands have shape and structure similar to Hoechst 33258, therefore this study assumes importance in understanding how variation of water activity in and around DNA affects the interaction process of minor groove binding ligands with DNA. This is a first detailed study with genomic DNA where multiple binding sites for the ligand exist. In the present study, osmolyte lowered the stability of CtDNA even in the presence of Hoechst 33258. Hoechst 33258 remained in the bound state with decreased affinity with calf thymus and the lowering of stability is primarily governed by the reduced water activity. This study has provided an insight on how altered water activity is able to change the Hoechst 33258–CtDNA interaction. These finding are expected to contribute in understanding on the role of water on the interaction of minor groove binding ligands with genomic DNA. This study will also be helpful for the ordered DNA structures being recognized by proteins or small molecules in a cell.

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