Interaction of DNA Minor Groove Binder Hoechst 33258 with Bovine Serum Albumin

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Hoechst 33258 belongs to bisbenzimidazole class of molecules having anticancer properties for their ability to inhibit topoisomerase and many other cellular processes. The aim of the present study is to understand the nature of Hoechst 33258-bovine serum albumin (BSA) binding interactions by using absorption, fluorescence and circular dichrorism (CD) measurements under simulative physiological conditions. The absorption spectra of BSA indicated the binding of Hoechst 33258 with BSA. The analysis of fluorescence data indicated the presence of both dynamic and static quenching mechanism in the binding. The associative binding constant and number of binding sites were found to be $K=2.08=10^7\ \mathrm{M}^{-1}$ and $n=1.36$ respectively. Biexponential fluorescence lifetime distribution of Hoechst 33258 in the presence of BSA has altered *viz.* τ_1 was increased significantly from 0.3 ns (60%) to 1.2 ns (13%) whereas a marginal increase in τ_2 from 3.6 ns (40%) to 4.0 ns (87%). Fluorescence anisotropy value of Hoechst 33258 has increased from 0.14 to 0.34 upon the addition of BSA. Thermodynamic parameters were also calculated using Van't Hoff plot by conducting fluorescence titration at four different temperatures, $\Delta H=+102.785\ \mathrm{kJ\ mol^{-1}}$, $\Delta S=+490.18\ \mathrm{kJ\ mol^{-1}}$, $\Delta G=-491.708\ \mathrm{kJ\ mol^{-1}}$. The CD spectrum of BSA revealed that the binding of Hoechst 33258 to BSA causes loss in the secondary structure but increases the thermal stability of the protein. The results indicated that hydrophobic interactions were the predominant intermolecular forces in stabilizing BSA-Hoechst 33258 complex. The possible implications of these results will be on designing better therapeutic minor groove binding drug molecules.

Key words bovine serum albumin; Hoechst 33258; quenching; associative binding constant; thermodynamics; circular dichroism

Bisbenzimidazole family of molecules is potential anticancer drugs.[1,2] Because of their strong binding in the AT rich minor groove of DNA, they can affect transcription and translation,[3] blocking topoisomerase I[4] and helicases activity.[5,6] These properties are expected to contribute in anticancer activity. Many such DNA ligands have been synthesized and tested for their topoisomerase inhibitions through *in vitro* studies.[7] Considerable efforts are being made to design potent anticancer drugs.[8,9] Our group has also studied cellular responses of a few synthesized bisbenzimidazole derivatives in *in-vitro* systems.[10,11] Hoechst 33258 is a bisbenzimidazole molecule that binds strongly in the minor groove region of duplex DNA with predominant specificity for AT rich sequences.[12—14]

Bisbenzimidazole compounds have also been reported to increase radiation induced cytotoxicity in several cancerous cell lines[15] and thus have an ability to improve radiotherapy. Another important observation related to protection of cells pretreated with these minor groove binders from radiation exposure has been reported by Martin and coworkers.[16,17] Based on mechanistic studies, Martin *et al.* has designed proamine, a more potent derivative of Hoechst 33258, which has demonstrated much higher ability to reduce the deleterious effects of radiation both in *in-vitro* and *in-vivo* models.[17] Hence, Hoechst 33258 and its derivatives have another potentially useful application as radioprotector and therefore continue to be a molecule of central importance for drug development for various potential applications.[18]

DNA-Hoechst 33258 non covalent interactions have been studied extensively using various physicochemical tools and have provided detailed account on the nature of interactions.[19] NMR studies in solution along with X-ray crystallographic studies of complexes with several AT containing oligonucleotides and molecular dynamic simulations provided useful information of detailed nature on mode of binding of Hoechst 33258 with DNA. Molecular dynamic studies have also furnished information on thermodynamical aspect of the binding process.[20] In most of these DNA-ligand studies, the ultimate aim was to develop understanding about designing improved derivatives for better sequence recognition and site specific binding in DNA because of its potential applications in medicine.[21] Despite these studies, correlation of structures of a variety of such ligands and cellular responses is still a distant goal. However, due to potential drug candidate, studies with such minor groove binding ligand have spanned a wide range of investigations ranging from *in-silico* molecular dynamic simulation, thermodynamics, spectroscopy and physical chemistry, to cellular responses by different research groups.

Interaction of a drug or a small molecule with serum proteins (serum albumin protein) is known to influence the bioavailability and efficacy of the drug *in-vivo* system.[22] This study especially becomes important in the case of when a drug is administered through routes other than intravenously. Thus it becomes imperative to assess the concentration of the drug in systemic circulation for achieving better correlation of toxicity and efficacy with the dosage. In view of the importance of this class of molecule, as a potential candidate for drug action, the focus of this study is to elucidate the nature of interactions of the parent molecule

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Hoechst 33258 with bovine serum albumin (BSA) which is an analogue to human serum albumin. Absorption and fluorescence spectroscopic studies were undertaken to elucidate the interaction of Hoechst 33258 with BSA. Thermodynamic nature of this binding interaction was revealed through fluorescence titration measurements at different temperatures. Circular dichroism (CD) studies were undertaken to complement the findings of binding from absorption and fluorescence spectroscopic measurements. This study is likely to benefit nucleic acid targeted drug designers, particularly, bisbenzimidazole family, engaged in targeting topo inhibitors as well as modifying radiation effects.

Results and Discussion

UV–Vis Absorption Spectral Studies Absorption spectral measurements on BSA in the presence of small molecules provide useful information related to the nature of interaction between a ligand and BSA. In the present study BSA solution (10 μM) was titrated against Hoechst 33258 in 0.1 M phosphate buffer pH 7.4 (Fig. 1). The absorption maxima (λ_{max}) at 278 nm and 340 nm are due to the protein (BSA) and Hoechst 33258 respectively.[23] The absorbance at 340 nm increased with the concentration of Hoechst 33258. The absorbance of BSA at 278 nm also progressively increased when the concentration of Hoechst 33258 solution was increased from 0 to 20 μM. Besides this increase in the absorbance of BSA upon the addition of Hoechst 33258 a distinct blue shift of BSA-Hoechst 33258 complex in the 278 nm region was also observed. According to the studies in which small molecules *viz.* 1-benzoyl-4-*p*-chlorophenylthiosemicarbazide and nimesulide as reported by Cui *et al.*[24] and Shaikh *et al.*[25] respectively with and BSA, similar increase in absorbance of BSA at 278 nm associated with blue shift has been reported upon binding with the ligands. In view of similar observations in the Hoechst 33258 and BSA titrations, binding interaction of Hoechst 33258 with the serum protein (BSA) is suggested.

Fluorescence Spectral Measurements Intrinsic fluorescence of the protein provides considerable information about protein structure and dynamics and has been used extensively to study protein folding and association reactions.[26] Various measurable parameters of fluorescence *viz.* quenching, enhancement of intensity, spectral shift, anisotropy, and fluorescence lifetime are used for interpretation of related structure-dynamics in proteins.[26] The phenomenon of decrease in fluorescence intensity of a fluorophore is known as quenching. It is a common phenomenon that occurs due to various processes such as by excited-state reactions, molecular rearrangements, energy transfer, ground-state complex formation, and collisional quenching.[26,27]

In the present study, fluorescence spectroscopic measurements were undertaken to gain insight into the detailed nature of binding of Hoechst 33258 with BSA as indicated in UV–Vis absorption measurements. The fluorescence spectra of BSA-Hoechst 33258 are characterized by a strong emission band (λ_{em}) at 336 nm due to the tryptophan residue of BSA protein[26] and a relatively less intense band in the region 480—490 region due to the intrinsic fluorescence of Hoechst 33258[23] (Fig. 2). Addition of Hoechst 33258 quenched the emission intensity of BSA at 336 nm. This intensity decrease was accompanied with blue shift in the maxima from 336 to 321 nm. In several studies with small molecules *viz.* PAAB, vitamin B_{12} or cyanocobalamin and colchicines as reported by Zhang *et al.*,[28] Kamat and Seetharamappa,[29] and Hu *et al.*[30] in emission spectrum of BSA a blue shift of 8.5 nm, 5 nm, and 7 nm was observed, respectively. The blue spectral shift indicated decrease in the polarity around the tryptophan residues and increased hydrophobicity. In our study, similar blue shift of magnitude 15 nm was obtained and it is comparable to above mentioned reported blue shift values. Thus, it is suggested that the binding interaction between the Hoechst 33258 and BSA resulted in enhancement of hydrophobicity around the tryptophan residues of BSA. Further a well defined isobestic point is observed at 410 nm wavelength in the emission spectrum of BSA, which is also a direct evidence of Hoechst 33258 binding with BSA.[31]

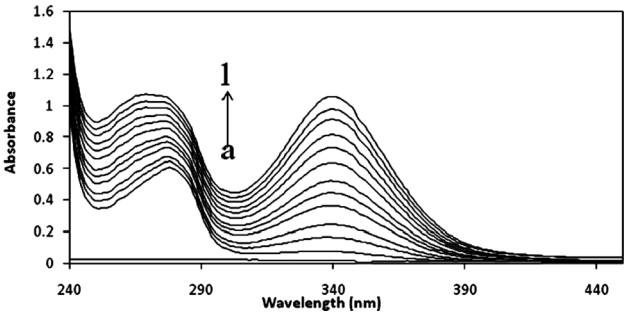

Fig. 1. Absorption Spectra of BSA-Hoechst 33258 in Phosphate Buffer Solution at pH 7.4

BSA concentration was kept fixed at 10 μM and Hoechst 33258 concentration was varied from (a) 0 to (l) 20 μM.

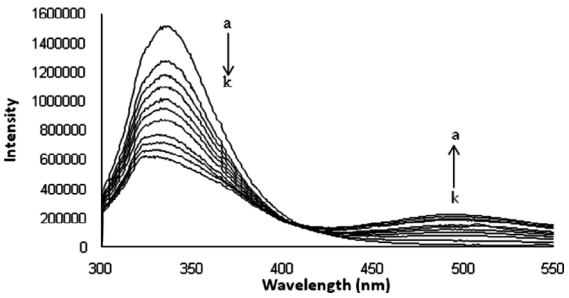

Fig. 2. Fluorescence Spectra of BSA in the Presence of Hoechst 33258 at pH 7.4 in Phosphate Buffer Containing 150 mM NaCl

BSA concentration was kept fixed at 10 μM and Hoechst 33258 concentration was varied from (a) 0 to (k) 20 μM. The measurement conditions were: excitation wavelength at 295 nm, slit width 4.0 nm, dwell time 0.5 s and wavelength increment 0.5 nm.

Fluorescence anisotropy of Hoechst 33258 is very sensitive to binding with biomolecules *viz.*, DNA and the anisotropy value has shown to increase upon binding.[32] In view of this, the emission anisotropy value of Hoechst 33258 was measured and compared with that of Hoechst 33258-BSA (Table 1).

Fluorescence excited state lifetimes are very sensitive to the structure and dynamics of a fluorescent molecule. Fluorescence decay profiles of Hoechst 33258 were obtained both for free and in the presence of BSA and when they were deconvulated for analysis with the instrument response function (IRF) revealed double exponentially decaying constants having a short (τ_1) and a long (τ_2) decay components as shown in Table 1. The double exponential decay components have been correlated with planar and non planar structures of

Table 1. Fluorescence Anisotropy and Life Time Data of 20 μM Hoechst 33258 Alone and with 10 μM BSA

Samples	Anisotropy	Lifetime (ns)		Amplitude (%)		χ^2
		τ_1	τ_2	A_1	A_2	
Hoechst 33258	0.14	0.3	3.6	60	40	0.923
Hoechst 33258-BSA	0.35	1.2	4.0	13	87	1.10

The excitation and emission wavelengths for Hoechst 33258 alone and Hoechst 33258-BSA were 340 nm, 503 and 340 nm, 475 nm, respectively.

Hoechst 33258.[33,34] This is because Hoechst 33258 is a heterocyclic compound containing two benzimidazole rings along with a phenol and piperazine moieties at the two ends and can have more than one conformer in solution because of rotational sites along the benzimidazole axis. For free Hoechst 33258, the values of τ_1 and τ_2 are 0.3 ns and 3.6 ns respectively. The relative contributions from these components were 60% and 40% respectively. The numerical values of τ_1 and τ_2 and their relative distribution have varied in the presence of BSA. The numerical values of τ_1 and τ_2 were increased to 1.2 ns and 4.0 ns respectively. However, the relative contributions of τ_1 decreased to 13% and the contribution from τ_2 has increased to 87% as depicted in Table 1. The analysis of fluorescence decay lifetime of Hoechst 33258 alone and Hoechst 33258-BSA clearly demonstrated that the decay components although depicted heterogeneity, the decrease in the relative contribution of τ_1 and increase in relative contribution of τ_2 upon binding with BSA indicates that Hoechst 33258 existed mostly in the non planer conformation in the complex form. The significant change in the values of both decay components and their relative contributions of Hoechst 33258 upon binding with BSA indicated the change in the microenvironment of Hoechst 33258 in the presence of BSA.

The fluorescence lifetime data suggested the significant transformation of planer conformation to the non planer conformation of Hoechst 33258 upon binding with BSA. Thus, binding of Hoechst 33258 is expected to increase the rigidity of Hoechst 33258 corroborating with an increase in the fluorescence anisotropy value of Hoechst 33258 (Table 1). The anisotropy value of Hoechst 33258-BSA at 475 nm emission maximum was 0.35 where as the corresponding value of the free Hoechst 33258 was 0.14 at 503 nm, the emission maximum of free Hoechst 33258. The higher anisotropy depicted that the bound Hoechst 33258 was rotationally restricted with respect to BSA. The altered emission spectral characteristics (emission maxima, intensity, anisotropy), strongly suggested environment around the Hoechst 33258 changed in the presence of BSA. The binding affinity between BSA and Hoechst 33258 resulting into significant alteration in the anisotropy values along with the associated emission wavelengths are as shown in Table 1.[32]

The fluorescence quenching data have been further analyzed by the Stern–Volmer equation

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

where, F_0 and F are the fluorescence intensities of BSA at 336 nm in the absence and presence of the quencher (Hoechst 33258) respectively. K_{sv} is the Stern–Volmer quenching constant and [Q] is the concentration of the quencher Hoechst 33258. The linearity or deviation from the

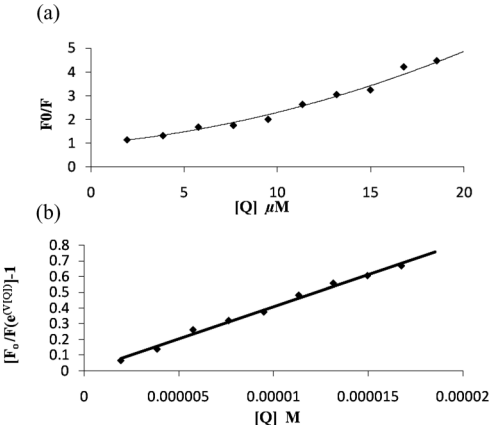

Fig. 3. Stern–Volmer Plot F_0/F *versus* [Q] for the Binding of BSA with Hoechst 33258 (a) and Modified Stern–Volmer Plot $[F_0/F(e^{V[Q]})-1$ *versus* [Q] for the Binding of BSA with Hoechst 33258 (b)

linearity of the Stern–Volmer plot F_0/F *versus* [Q] for BSA as shown in various studies revealed the type of quenching *viz.* static or dynamic.[35] The Stern–Volmer plot (Fig. 3a) in the present study has an upward curvature for the complete concentration range of Hoechst 33258,[36—38] it therefore indicates the presence of both static and dynamic quenching by the same fluorophore. The modified Stern–Volmer plot based on Eq. 2[39] facilitate better description of the quenching data when both dynamic and static quenching are present simultaneously.

$$F_0/F=(1+K_q[Q])e^{V[Q]} \quad (2)$$

where, K_q is the collisional quenching constant or Stern–Volmer quenching constant and V is the static quenching constant. The value of V was obtained from Eq. 2 by plotting $(F_0/Fe^{V[Q]})-1$ *versus* [Q] for varying V until a linear plot was obtained. The value of K_q was obtained from the slope of $(F_0/Fe^{V[Q]})-1$ *versus* [Q] (Fig. 3b). The values of V and K_q so obtained are found to be $9.0\times10^4\ M^{-1}$ and $18.8\times10^4\ M^{-1}$ respectively.

According to Eftink and Ghiron[40] the upward curvature in the Stern–Volmer plot indicates that both tryptophan residues of BSA are exposed to quencher and the quenching constant of each tryptophan residue is nearly identical and downward curvature indicates buried tryptophan residues. After addition of Hoechst 33258 to 20 μM BSA solutions around 70% of the fluorescence intensity was quenched. The maximum quenching was obtained by extrapolating a plot of $(F_0-F)/F_0$ *versus* 1/[Q] to 1/[Q]=0, corresponding to infinite concentration of Hoechst 33258. It was observed that at infinite concentration of Hoechst 33258, fluorescence quenching was approximately 90%. This again shows that both the tryptophan

residues of BSA are accessible to Hoechst 33258.

Fluorescence bimolecular quenching rate constant k_q measurement was evaluated using the equation,

$$k_q = K_{sv}/\tau_0 \quad (3)$$

where, τ_0 is the lifetime of BSA in the absence of quencher and k_q is the rate constant for the quenching. The τ_0 value for BSA is reported in many studies and a general τ_0 value was found to be in 10^{-9} s and hence the value of k_q was observed to be of the order of $10^{13}\,M^{-1}\,s^{-1}$. The value of k_q depends on the probability of a collision between fluorophore (in this case it is tryptophan) and quencher and this probability depends on their rate of diffusion (D), size and concentration.

$$K_q = 4\pi\sigma D N_a \times 10^{-3} \quad (4)$$

where, D is the sum of the diffusion coefficients of quencher and fluorophore σ is the sum of molecular radii and N_a is the Avogadro's number. Since the upper limit of k_q expected for diffusion controlled bimolecular process is $10^{10}\,M^{-1}\,s^{-1}$.[41] The high magnitude of K_q in the present study ($10^{13}\,M^{-1}\,s^{-1}$) can be attributed to a specific long-range interaction between drug molecules and tryptophan residues on protein and that the quenching is initiated predominantly by static collision due to complex formation.

Analysis of Binding Equilibria When small molecules bind independently to a set of equivalent sites on a macromolecule, the equilibrium between the free and bound molecules is established. The association constant and number of binding sites for Hoechst 33258-BSA interaction, K and n, was determined according to Feng *et al.*,[42] using the equation,

$$\log(F_0 - F)/F = \log K + n \log[Q] \quad (5)$$

where, F_0 and F denote the fluorescence intensities of protein in a solution without and with the ligand, respectively. Thus, the plot of $\log(F_0-F)/F$ *versus* log[Q] yielded the K and n values. In our study the K and n are found to be $2.08\times10^7\,M^{-1}$ and 1.36, respectively. The value of n is approximately equal to 1 indicating that there is one class of binding site to the Hoechst 33258 in BSA. From this n value it is proposed that Hoechst 33258 most likely binds to the hydrophobic pocket located in sub domain II A; that is to say near to tryptophan at site 214.[25,43]

Type of Interaction Force between Hoechst 33258 and BSA The interaction forces between a drug molecule and biomolecules may include hydrophobic force, electrostatic interactions, van der Waals interactions, hydrogen bonds, *etc.*[44] 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,

$$\ln K = -\frac{\Delta H}{RT} + \frac{\Delta S}{R} \quad (6)$$

where, K is the association binding constant at a particular temperature and R is the gas constant. The temperatures used in the study were 294 K, 298 K, 301 K and 310 K. The enthalpy change (ΔH) and the entropy change (ΔS) are calculated from the slope and intercept of the plot $\log K$ *versus* $1/T$, respectively (Fig. 4). The free energy change (ΔG) is determined from the following equation,

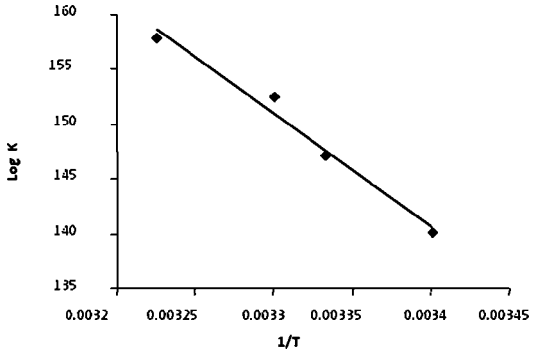

Fig. 4. Van't Hoff Plot for the Interaction of BSA and Hoechst 33258 in PBS Buffer at pH 7.4

$$\Delta G = \Delta H - T\Delta S \quad (7)$$

The thermodynamic parameters ΔH, ΔS and ΔG values so calculated are found to be $+102.785\,kJ\,mol^{-1}$, $+490.18\,J\,mol^{-1}\,K^{-1}$ and $-491.708\,kJ\,mol^{-1}$ respectively. The negative value of free energy (ΔG) as observed supports the assertion that the binding process is spontaneous. The positive values for enthalpy change (ΔH) and entropy change (ΔS) indicated that the interaction is only entropically driven.[41,45] Positive entropy change (ΔS) value also indicated the predominant hydrophobic character of binding because of the increased freedom of the water molecules, which were in the neighbourhood of the non-polar groups before association. Therefore, the binding force involved in the formation of Hoechst 33258-BSA complex is essentially hydrophobic in nature.

Circular Dichroism CD spectra of BSA in the absence and presence of different concentrations of Hoechst 33258 were determined in the far-UV region (Fig. 5a). Interestingly, the spectrum of the native BSA shows characteristic peaks at 222 and 208 nm, which is an indication of the presence of α-helical content in the protein (Fig. 5a). It is seen in this figure that on the addition of Hoechst 33258 above 10 μM the observed negative CD signals at these wavelengths decreased. Furthermore, CD spectrum of BSA in 10 μM does not differ significantly from that of the native protein. However, a significant change observed upon the addition of 20—30 μM of Hoechst 33258, clearly indicates a conformation change. The changes in the secondary structure in terms of α-helix content were measured by determining the $[\theta]_{222}$ as function of the ligand concentration with the help of an equation given by Chen *et al.*[46] The native protein is found to have 73% α-helix which is in excellent agreement with the previous work as reported by Shaikh *et al.*[20] Furthermore, a progressive loss in α-helical content occurs with a successive addition of Hoechst 33258 from 10 to 30 μM (Fig. 5a, inset).

Our fluorescence binding studies has revealed that Hoechst 33258 can strongly bind to BSA through hydrophobic interactions which in turn may stabilize the protein. In order to determine the extent of stability gained by BSA upon binding with Hoechst 33258, we have determined the effect of temperature on the native BSA and BSA in the presence of 30 μM Hoechst 33258. The heat-induced denaturation of the protein was measured by following changes $[\theta]_{222}$. It is seen

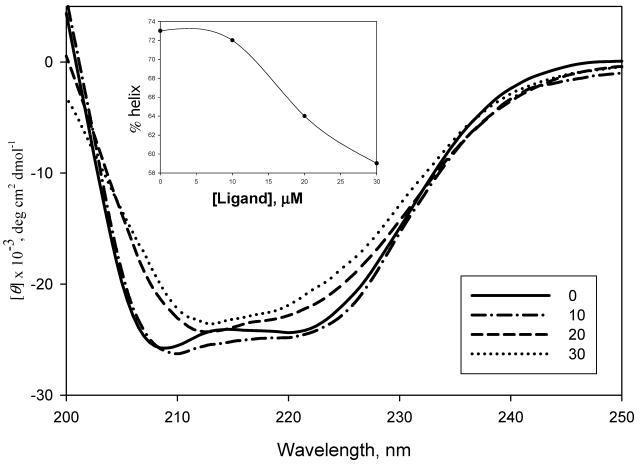

Fig. 5a. CD Spectra of BSA-Hoechst 33258 in the Absence and Presence of Different Concentration of Hoechst 33258 in 0.01 M Phosphate Buffer, pH 7.4 at 25±0.1°C

The inset shows a plot of % α-helix *versus* Hoechst 33258.

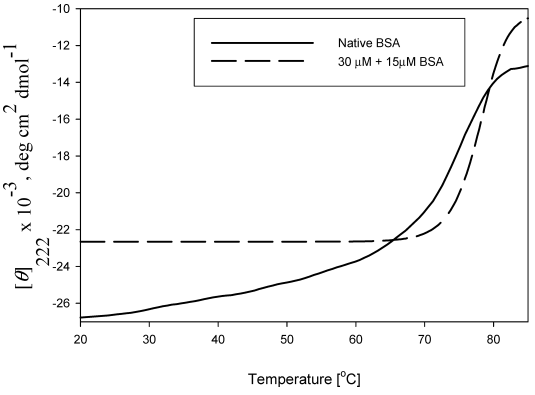

Fig. 5b. Heat-Induced Denaturation Curves of the Native BSA and BSA-Hoechst 33258 Complex in the Phosphate Buffer

The BSA concentration was kept fixed at 15 μM in all the experiments.

in Fig. 5b that there occurs an increase in melting temperature (T_m) from 70 °C of the native BSA to 75 °C for the protein in the presence of 30 μM Hoechst 33258. This increase in T_m reveals that the binding of Hoechst 33258 to BSA increases its stability considerably. Although, significant conformational changes have been observed on binding of Hoechst 33258 to BSA, its binding is considerably strong which most probably stabilizes the protein by forming a network of hydrophobic interactions. These findings are in agreement with our fluorescence binding studies.

Experimental

Materials Hoechst 33258 and Bovine Serum albumin (BSA, Fraction V, approximately 99%; protease free and essentially γ-GLOBUMIN free) were purchased from Sigma Chemical Company (U.S.A.). The water used for preparation of solution was of 18 ohm MQ grade derived from Milli Pore water system. For fluorescence and absorption measurements, the BSA solution of pH 7.4 was prepared in 0.1 M phosphate buffer (mixture of sodium dihydrogen phosphate dehydrate and anhydrous disodium hydrogen phosphate both were of analytical grade and from E. Merk) containing 0.15 M NaCl (analytical grade from Sigma). Hoechst 33258 stock solution was prepared in MQ water and then working solution was made by further dilution with 0.1 M phosphate buffer contain 0.15 M NaCl. The concentration of Hoechst 33258 and BSA solutions were determined on a Cary Varian double beam spectrophotometer (Cary Bio 100, Australia) by using extinction coefficient of $\varepsilon_{280}^{1\%}$=6.8 for BSA[47] and ε_{340}=42000 $M^{-1}\,cm^{-1}$ for Hoechst 33258[48] respectively. For CD measurement the working solutions BSA and Hoechst 33258 were prepared in 0.01 M phosphate buffer at pH 7.4.

Spectral Measurements The absorption spectral measurements were recorded on Cary Varian double beam spectrophotometer (Cary BIO 100, Australia). The sample cuvette used was a pair quartz cells of 1.00 cm path length. All scanning parameters were optimized to obtain the best spectra and in general the parameters were scan range 200—800 nm, wavelength step 0.5 and all measurements were carried out at room temperature (23±1 °C).

Fluorescence Measurements for Studying Quenching Mechanism Fluorescence measurements were performed using Spectrofluorimeter model FS920 of Edinburgh Instruments, U.K. equipped with xenon arc lamp. The temperature of the sample holder was regulated with a peltier cooled thermostat. Quartz cuvettes of 3 ml capacity, path length 1 cm were used for all measurements.

Fluorescence Excited State Lifetime Measurements The fluorescence lifetime measurements were performed using a customized integrated steady state spectrofluorimeter (model FS900CDT) and fluorescence lifetime instrument (model FL900CDT) from Edinburgh Analytical Instruments, U.K. The excitation source was hydrogen gas filled nanosecond flash lamp (model nF900) filled with low H_2 gas pressure of 0.4 bar operating at frequency 40 kHz. The silt width for both excitation and emission monochromators were kept fully open. The intensity decay curves were obtained at emission maximum and fitted as sum of exponentials as

$$I_t = I_0 \sum A_i \exp(-t/\tau_i)$$

where, τ_i and A_i are representing the fluorescence lifetime and pre-exponential factor for *i*th decay component. The detailed procedures of measurement and analysis of decay parameters are discussed elsewhere.[49] The excited state lifetime values were calculated by deconvoluting the intensity decay profiles. More precisely multiple-exponential fittings are applied along with the instrument response function of the excitation wavelength in the deconvolution process. The instrument response function was recorded with Ludox as scattering medium. The analysis of the fitted data was tested using statistical parameters provided in the analysis software.

Fluorescence Anisotropy Measurements Fluorescence anisotropy measurements were performed on spectrofluorimeter model FS900CDT by using a pair of Glan–Thompson prisms in parallel and perpendicular directions, *viz.* I_{VV}, I_{VH}, I_{HV} and I_{HH} and the anisotropy values *r* were calculated by using these intensity files in anisotropy calculation mode provided in the software by the following equation.

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

where, *G* is the correction factor obtained from the anisotropy software provided in the instrument.

CD Measurements The CD spectra of BSA in the absence and presence of Hoechst 33258 were measured in the far-UV (210—250 nm) region. The far-UV spectra were measured at a protein concentration of 15 μM in 10 mM sodium phosphate buffer (pH 7.5) containing 150 mM NaCl with a successive addition of Hoechst 33258 (10 to 30 μM) in a 1-cm quartz cuvette at 0.1 nm wavelength intervals in JASCO model 715 CD spectropolarimeter. The scan speed has been kept at 50 nm/min with an average response time of 1 s. For each sample a minimum of five consecutive scans were accumulated, and the average spectra were stored. All the CD measurements were carried at 25 °C with an accuracy ±0.1 °C. The blank for each concentration of Hoechst 33258 was prepared in the same buffer and its spectrum was subtracted from that obtained at corresponding concentration upon binding to BSA. Effect of temperature on the native and protein–ligand complex was also carried out in the same spectropolarimeter (model J-715) equipped with a Peltier-type temperature controller with a heating rate of 1 °C per minute, a scan rate providing an adequate time for equilibration. Changes in $[\theta]_{222}$ of each protein sample were measured in the temperature range of 20—85 °C.

BSA-Hoechst 33258 Absorption Titration In 3 ml capacity quartz cuvette, to 2 ml BSA solution of concentration 10 μM at pH 7.4 stepwise additions were made from Hoechst 33258 stock solution. The stock solution used during the measurements was concentrated one to avoid volume error. The accumulated volume was made less than 200 μl. The addition of ligand solution and through mixing of protein–ligand solution in the cuvette was done manually by using micropipettes. All the UV measurements were done against the blank taking using phosphate buffer in other quartz of the same capacity cuvette. The sample was scanned under aforesaid experimental conditions as mentioned above.

BSA-Hoechst 33258 Fluorescence Titration In 3 ml capacity cuvette, 2 ml of BSA solution of 10 μM concentration at pH 7.4 was titrated by the successive additions of Hoechst 33258 stock solution. Titrations were done manually by using micropipettes. Hoechst 33258 was added from concentrated stock solution so that volume increment was negligible. In each individual titration step, 5 μl of Hoechst 33258 from solution was added to 2 ml BSA solution in the cuvette by using the micropipette of 2 to 20 μl capacity followed by thorough mixing of the solution by using micropipette of 100 to 1000 μl capacity. The sample was then placed in the sample chamber of Spectrofluorimeter (model FS920 of Edinburgh Instruments, U.K.). Fluorescence spectra of BSA were recorded in the emission range 300—550 nm with excitation at 295 nm. During the course of titration the concentration of Hoechst 33258 was varied from 0 to 20 μM, the total accumulated volume of 60 μl of Hoechst 33258 remained very less than in the total volume 3060 μl. For obtaining Van't Hoff plot, similar titrations were performed at four different temperatures *viz.*, 294 K, 298 K, 301 K and 310 K. From these temperature dependent titrations, the thermodynamical parameters were obtained.

Fluorescence BSA-Hoechst 33258 Anisotropy Measurements Fluorescence emission anisotropy of Hoechst 33258 (20 μM) with BSA (10 μM) was obtained by recording emission scan in all the four directions as mentioned above and from the emission anisotropy scan the value of anisotropy was obtained at the emission spectral maximum position *viz.*, 475 nm. Similar measurements were performed for Hoechst 33258 alone and the anisotropy was calculated at its maximum at 503 nm. The measurement temperature was 294 K and the excitation wavelength was 340 nm.

Fluorescence BSA-Hoechst 33258 Lifetime Measurements Fluorescence lifetime measurements of Hoechst 33258 (20 μM) were measured in the presence of BSA (10 μM) using Time Correlated Single Photon Counting (TCSPC) technique. In order to compare the results of measured lifetime of Hoechst 33258 in the presence of BSA with Hoechst 33258 alone, similar lifetime measurement was performed for Hoechst 33258 in the phosphate buffer. The excitation wavelength was 340 nm. All the lifetime measurements were done at 294 K temperature.

Conclusion

The present study has suggested that the Hoechst 33258 binds to BSA possibly by predominant hydrophobic interactions. These non-covalent forces are long range, specific and the binding characteristics correspond to reversible complexation. The CD study revealed that the complete binding of Hoechst 33258 to BSA is found to induce conformational changes in protein, at the same time Hoechst 33258 increases the thermal stability of BSA. These findings are likely to provide useful insight for interaction of DNA minor groove binding agents with serum albumin protein and thereby lead to design new ligands with improved pharmacokinetics parameters.

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