Structural stabilization by Hoechst 33258 in γ-irradiated DNA: Evidenced by spectroscopic studies

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Several studies have demonstrated radioprotective effects of DNA minor groove binding ligand Hoechst 33258 (H258) in isolated DNA and cells as well as in whole body irradiated mice. The mechanism underlying Hoechst-mediated radioprotection is still under intensive investigation. In this study, we have investigated the possible changes in the local environment at binding sites and influence of Hoechst-induced structural stabilization in γ-irradiated DNA. H258 was added at two different concentrations, viz. 4 μM and 80 μM in irradiated DNA (800 μM) and the corresponding ligand to DNA ratios were $R = 0.005$ and 0.1 (DNA-H258) respectively. Helix to coil transition temperature (T_m), absorption, fluorescence emission and excited state fluorescence lifetime measurements were carried out and compared with un-irradiated DNA-H258 solutions. Spectroscopic measurements indicated that the majority of spectral characteristics (spectral positions and intensity) were similar in DNA-H258 and H258-DNA complexes. The excited state fluorescence decay was double exponential having a short (τ_1) and a long (τ_2) component. Their numerical values strongly depended on the ratios $R = 0.005$ and 0.1 and remained unaltered in irradiated solutions. This data strongly suggested that the local environment of H258 at the binding sites in DNA remained unaltered upon irradiation and binding of H258 appeared to contribute to structural stabilization in a concentration-dependent manner.

DEVELOPMENT of non-toxic radioprotectors is of considerable interest for management of radiation accidents and in protection of normal tissues during cancer radiotherapy. DNA-binding agents appear to be promising candidates for radioprotection as the binding sites can be prevented from attacks of radiolytic products of water. Since the first report of radioprotection of DNA by Hoechst 33342 (H342)[1], several studies have been reported in the literature on radioprotection by Hoechst derivatives, viz. Hoechst 33342 (H342) and Hoechst 33258 (H258)[2–8]. The aim of most of these studies was to elucidate the mechanism underlying radioprotection[3,4,7]. H atom donation from DNA-binding ligand, H258 to radiation-induced carbon-centered radicals in DNA and electron transfer from the electron donating substituent in H258 derivative to DNA were attributed as possible mechanisms. Based on these understandings, a new analogue of Hoechst, methylproamine was designed and synthesized[4] and the radioprotective potency was shown to be much higher in comparison to H342. In this newly synthesized molecule, the ethoxy group in H342 was substituted by an electron donating *N*,*N*-dimethyl amino group. It is interesting to note that in all these studies, the DNA-binding ligands Hoechst 33258 and its new analogues were added prior to irradiation in DNA in solutions as well as in cells.

Hoechst derivatives are known to bind in the AT-rich region of the minor groove of DNA[9–11]. The H258–DNA complex becomes strongly fluorescent, thereby making this dye a useful stain for DNA in cells[12]. Several investigations have led to elucidation of the nature of binding of this ligand with DNA[13–15]. The forces, viz. van der Waals and H-bonding are responsible for rendering increased stability in the DNA–ligand complexes[14,15]. The ligand-induced stability was considered as an important contributory factor in earlier radioprotection studies with aminothiols, e.g. WR1065 as well as polyamines[16,17]. This aspect, viz. Hoechst-induced structural stabilization was not investigated in studies reported by earlier groups. In addition, Hoechst–DNA complex, the spectroscopic properties of H258, viz. spectral position, absorbance and fluorescence intensity are known to alter dramatically upon binding with DNA[18,19]. Further, excited state fluorescence lifetimes of H258 are also different in the bound form[20–22]. The fluorescence properties of Hoechst–DNA are strongly influenced by the nature of local environment at the binding sites[19,23,24]. Since spectroscopic properties, both steady state and excited states, are sensitive to processes like H-donation and electron transfer associated with H258, it is expected that these properties will be altered if such excited state reactions occur, upon irradiation in Hoechst–DNA complex. We are investigating the absorption and fluorescence spectroscopic properties in γ-irradiated H258–DNA complexes[25]. Results from these studies suggest unaltered local environment at the binding sites of H258 in γ-irradiated H258-DNA and influence of the ligand on structural stabilization of irradiated H258–DNA complexes.

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In the present study, DNA melting (T_m), binding affinity absorption and fluorescence spectroscopy including excited state fluorescence lifetime measurements have been undertaken in solutions where different concentrations of H258 were added to γ-irradiated DNA (DNA–H258) and these results were compared with unirradiated H258–DNA complexes. These measurements were carried out to examine the possible changes in the local environment at the binding sites in irradiated DNA and also the influence of ligand-induced stability in DNA–H258.

Material and methods

Preparation of Hoechst 33258 and DNA solutions

Hoechst 33258 (H258) was obtained from Sigma Chemicals, USA and calf thymus DNA (DNA) biochemistry grade and sodium perchlorate ($NaClO_4$) were procured from E. Merck (Germany). The stock solution of DNA was prepared in 1 mM phosphate buffer at pH 7.0 containing 20 mM $NaClO_4$ by slowly stirring the required amount of DNA. In order to ensure complete solubilization of DNA, absorbance at 260 nm was monitored until the entire DNA went into solution. The concentration of DNA (in nucleotides) was calculated from the measured O.D. using the extinction coefficient value (6420 M^{-1} cm^{-1}) at 260 nm[26]. The stock solution of H258 at concentration 1 mM was prepared in distilled water obtained from the Millipore MQ water purification system. The actual concentration was determined from the extinction coefficient (42000 M^{-1} cm^{-1}) at 340 nm[27]. The phosphate buffer was also prepared in water obtained from the Millipore water filtration system. The initial working concentration of DNA was 800 μM. This DNA solution was divided into two parts and in one portion of the solution H258 was added at two different concentrations, viz. 4 μM and 80 μM. The H258–DNA complexes at these two ratios $R = 0.005$ and 0.1 containing the same amount of DNA (400 μM) were prepared by slowly mixing the equal volume of the required H258 solution into the continuously stirred solution of DNA. The final concentration of $NaClO_4$ was 10 mM. The final concentration of H258 at ratios $R = 0.005$ and 0.1 was 2 μM and 40 μM respectively.

Gamma irradiation of aqueous solutions of DNA

Gamma irradiation was carried out in a Borosil petri dish containing fixed volume of DNA solutions. The radiation source was ^{60}Co Teletherapy unit (Model Eldorado 75, Canadian Atomic Energy, Canada) and the dose rate was 2.04–1.7 Gy/min.

Hoechst 33258 (H258) at different concentrations were added to these irradiated DNA solutions to make H258 to DNA ratio, R as 0.005 and 0.1 and designated as DNA–H258. H258–DNA solutions refer to H258 added to unirradiated DNA.

DNA melting temperature measurements

The DNA melting temperature measurements in different H258–DNA and irradiated DNA–H258 solutions were performed in a UV–Vis spectrophotometer (Model GBC UV/VIS 916, GBC Scientific Instruments, Australia) having DNA-melt software. The temperature was controlled to an accuracy of ± 0.2°C with the help of a software controlled thermo-electrical device. The melting temperature was calculated at the mid-point of sigmoidal phase diagram obtained from different solutions.

Absorption and fluorescence measurements

The steady state absorption and fluorescence spectral measurements in these solutions were carried out in spectrophotometer model GBC UV/VIS 916 (GBC Scientific Equipment, Australia) and spectrofluorimeter model FS900 (Edinburgh Analytical Instruments, UK) respectively. All emission spectra have been corrected using the correction file provided along with the instrument.

Determination of binding constant

Binding constants of H258–DNA and DNA–H258 were determined by fluorescence titration method. A fixed concentration of H258 (0.1 μM) was titrated against increasing DNA (un-irradiated) concentration in phosphate buffer. From the titration data, a Scatchard plot of r/c versus r was obtained, where r is the ratio of bound H258 concentration to the total DNA concentrations in base pairs and c 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 H258 as

$$I_t = I_o(C_t - C_b) + I_bC_b, \quad (1)$$

where I_o and I_b are the fluorescence intensities of free and fully bound H258 and C_t and C_b are the corresponding total and bound concentrations. From the slope of the plot, the binding constant k is determined for both unirradiated and irradiated DNA.

Fluorescence anisotropy measurements

Fluorescence emission anisotropy measurements were carried out 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.

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

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

Measurements of fluorescence lifetime

Both the steady state and lifetime measurements were performed in the specially modular custom designed spectrofluorimeter in which both steady state (Model FS900) and time resolved (Model FL900) units have been integrated by Edinburgh Analytical Instruments, UK. The excitation wavelength was 360 nm for Hoechst–DNA complexes. The intensity decay curves were fitted as sum of exponentials as

$$I_t = I_o \Sigma A_i \exp(-t/\tau_i) \quad (3)$$

where τ_I and A_i are fluorescence lifetime and pre-exponential factor for ith decay component.

The detailed procedures of measurement and analysis of decay parameters are discussed elsewhere[28].

Results

The structure of DNA binding ligand Hoechst33258 (H258) is shown in Figure 1. Helix to coil transition temperature (T_m) of DNA irradiated up to a radiation dose of 120 Gy was measured and compared with unirradiated DNA. The T_m of γ-irradiated DNA lowered to 63.2°C from 69°C. Addition of different concentrations of H258 (4 μM for $R = 0.005$ and 80 μM for $R = 0.1$), increased T_m from 69°C ($R = 0.005$) to 80.3°C ($R = 0.1$) (Table 1). Upon irradiation in H258–DNA the T_m at $R = 0.005$ lowered to the almost same value (63.1°C) as in irradiated DNA alone, whereas at $R = 0.1$, the T_m though showed a decrease compared to un-irradiated H258–DNA, i.e. 80.3°C remained at 75.8°C, much higher than 63.1°C. Interestingly, the T_m showed similar value, 77°C even when H258 was added to irradiated DNA at higher ratio. These values are shown in brackets in Table 1.

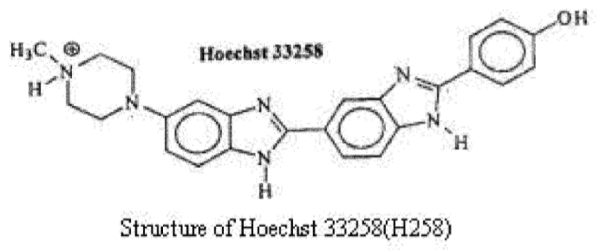

Figure 1. Structure of Hoechst 33258.

Absorption and emission spectral measurements

Absorption and fluorescence spectral measurements were carried out in γ-irradiated DNA solutions complexed with H258 (DNA–H258) and compared with unirradiated H258–DNA solutions at both the ratios.

Absorption spectra

The absorption spectra of H258–DNA showed spectral maximum at 356 nm at $R = 0.005$ and at higher ratio $R = 0.1$ slight blue shift (352 nm) has been observed. The spectra of irradiated DNA–H258 complexes showed no change in spectral position when compared to unirradiated H258–DNA solution. The absorbance however showed slight (insignificant) decrease only at $R = 0.005$ (Figure 2 *a*). In Figure 2 *a*, a second *y*-axis has been shown on the right to show absorbance at 356 nm.

Fluorescence spectra

The fluorescence emission spectral characteristics (spectral position of emission maximum and its intensity) are strongly dependent on ratios, R. The emission maximum due to H258–DNA at $R = 0.005$ was at 473 nm and showed a red shift at 483 nm at $R = 0.1$. The intensity of emission showed significant decrease when H258 was added in irradiated solution at $R = 0.005$ (Figure 3 *a*) and no spectral shift was observed. The emission spectra of DNA–H258 did not show any changes at $R = 0.1$ (Figure 3 *b*).

The anisotropy value of the DNA–H258 complex showed slight dependency on ratios, viz. 0.29 and 0.27 at $R = 0.005$ and $R = 0.1$ respectively. These values along with the absorption and fluorescence characteristics are shown in Table 2. The anisotropy of free H258 was 0.14.

Binding affinity measurement

Fluorescence titration was carried out at fixed concentration of H258 (0.1 μM) with increasing concentration of

Table 1. Helix to coil transition temperature (T_m) of Hoechst–DNA solutions

Ratio	Dose (Gy)	T_m (°C)
0	0	69.3
	120	63.2
0.005	0	69.0
	120	63.1 (61)
0.1	0	80.3
	120	75.8 (77)

Data corresponds to mean of two independent measurements.
Values of T_m shown in brackets are from irradiated DNA–H258 complex.

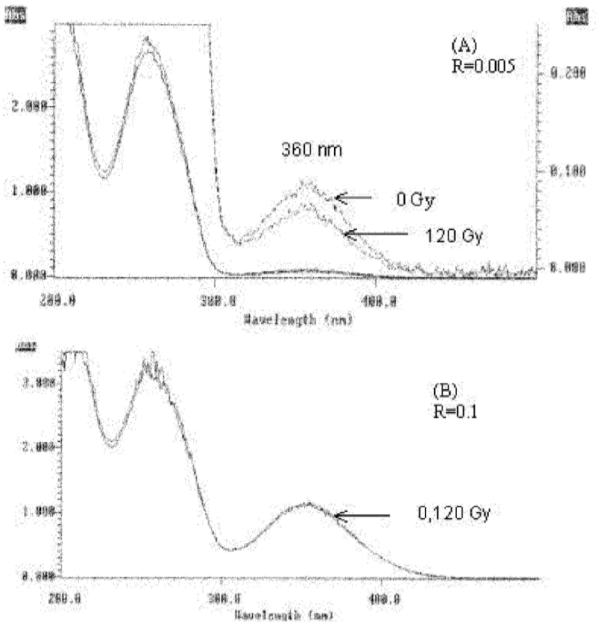

Figure 2. Absorption spectra of H258–DNA (unirradiated) and DNA-H258 (irradiated 120 Gy) solutions in phosphate buffer solutions at ratio $R = 0.005$ (*a*) and $R = 0.1$ (*b*). The second abscissa is for absorption band in the region 300–450 nm.

DNA. From the slope of the Scathard plot (r/c vs r), the binding constant was calculated for unirradiated and irradiated DNA. These values were $1.2 \times 10^8\ M^{-1}$ and $3.6 \times 10^8\ M^{-1}$ respectively. These values were similar to those reported in the literature[29].

Fluorescence lifetime measurements

The excited state fluorescence lifetime of Hoechst–DNA complexes in aqueous solutions was measured with excitation at 360 nm. The fluorescence decay profiles when deconvulated 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 varied strongly with the ratios, for example, at $R = 0.005$, the values of τ_1 and τ_2 component were 2.4 ns and 4.4 ns respectively. The relative contribution from these components was 35% and 65% respectively. At higher ratio, $R = 0.1$ the numerical values of these decay components decreased to 1.4 ns and 4.0 ns respectively. However, the relative contributions from these parameters remained almost changed as depicted in Table 3.

Interestingly, this decay parameter depicted only marginal variation at both the ratios in irradiated solutions (DNA–H258). For example, the values of τ_1 and τ_2 showed almost negligible decrease in irradiated solutions (DNA–H258) at 2.4 ns to 2.2 ns and 4.4 ns to 4.3 ns respectively at $R = 0.005$. The decay characteristics, i.e. numerical values and their relative contribution at higher ratio, $R = 0.1$ showed absolutely no change in irradiated solution.

Discussion

Hoechst derivatives, viz. Hoechst 33258 (H258) and Hoechst 33342 (H3342) have demonstrated radioprotection both *in vitro* and *in vivo*[31,32]. The possible mechanisms underlying Hoechst-mediated radioprotection were attributed to its scavenging and quenching properties for radiation-induced free radicals of water as well as DNA radicals[4,7,8]. The aim of this study was to investigate changes in binding environment of H258 in irradiated DNA. DNA melting temperature, absorption and fluorescence spectroscopy including binding affinity and excited fluorescence lifetime measurements were carried out on H258 bound to γ-irradiated DNA (DNA–H258) and compared with H258–DNA complexes. The possible role of structural stabilization by H258 in irradiated DNA was also examined.

The T_m shift depended on the amount of H258 added, viz. 69.0°C and 80.3°C at ratios $R = 0.005$ (2 μM) and 0.1 (40 μM) respectively. The DNA melting temperature T_m of γ-irradiated DNA–H258 complexes, i.e. when H258 was added in irradiated DNA solutions was almost similar to irradiated H258–DNA at both the ratios (Table 1). This T_m data, therefore suggested that H258 provided similar extent of structural stabilization in both H258–DNA and DNA–H258 complexes. Ionizing radiations are known to cause a variety of damages in DNA[30] and strand breaks, both single and double, are responsible for decrease in thermal stability. In general, it is expected that these strand breaks to be randomly distributed in DNA. The binding affinity of H258 in irradiated and unirradiated DNA was $1.2 \times 10^8\ M^{-1}$ and $3.6 \times 10^8\ M^{-1}$ respectively. Similar binding affinity of H258 was reported in calf thymus DNA[29]. The observed binding affinity in the present study was similar in both the DNA solutions, which suggests further that the forces responsible for binding remained unaltered. While performing the fluorescence titration measurements for determining the binding affinity, it was observed that the fluorescence intensity of bound H258 attained saturation at much lower concentration of irradiated DNA compared to un-irradiated DNA. H258 require 4–5 AT base pairs for binding and it appeared that these binding sites remained unaltered in both the DNA solutions. This data clearly suggested that H258 did not provide significantly different amount of structural stabilization when added in irradiated H258–DNA. Our present results, thus clearly showed that H258 provided similar concentration-dependent stability in both DNA–H258 and H258–DNA solutions.

In order to elucidate the possible changes at the binding sites in both the irradiated conditions in DNA, fluorescence spectral properties of H258 in DNA–H258 have been

Table 2. Absorption and fluorescence spectral characteristics of Hoechst–DNA complex

		Spectral parameters				
Ratio (R)	Dose (Gy)	$\lambda_{Abs.}$ (nm)	OD	λ_{em} (nm)	Intensity	Anisotropy
0.005	0	356	0.009	473	16500	0.29
	120	356	0.007	473	11000	0.29
0.1	0	352	1.05	483	13000	0.27
	120	352	1.05	483	13000	0.27

Emission intensity values correspond to the number of counts per second. The emission anisotropy of free H258 in phosphate buffer was 0.14.

Table 3. Fluorescence decay lifetime parameters of Hoechst–DNA solutions

		Decay parameters			
Ratio (R)	Dose (Gy)	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)
0.005	0	2.4 (± 0.08)	51.5 (± 6.5)	4.4 (± 0.02)	49.5 (± 6.5)
	120	2.2 (± 0.21)	48 (± 12)	4.3 (± 0.17)	52 (± 12)
0.1	0	1.4 (± 0.0)	35.5 (± 1.5)	4.0 (± 0.07)	64.5 (± 1.5)
	120	1.4 (± 0.08)	34.5 (± 0.5)	4.0 (± 0.11)	65.5 (± 0.5)

Data corresponds to mean of the two independent measurements. The fluorescence decay lifetimes of free H258 in water were 0.21 ns and 4.1 ns with 54% and 46% relative contribution[28].

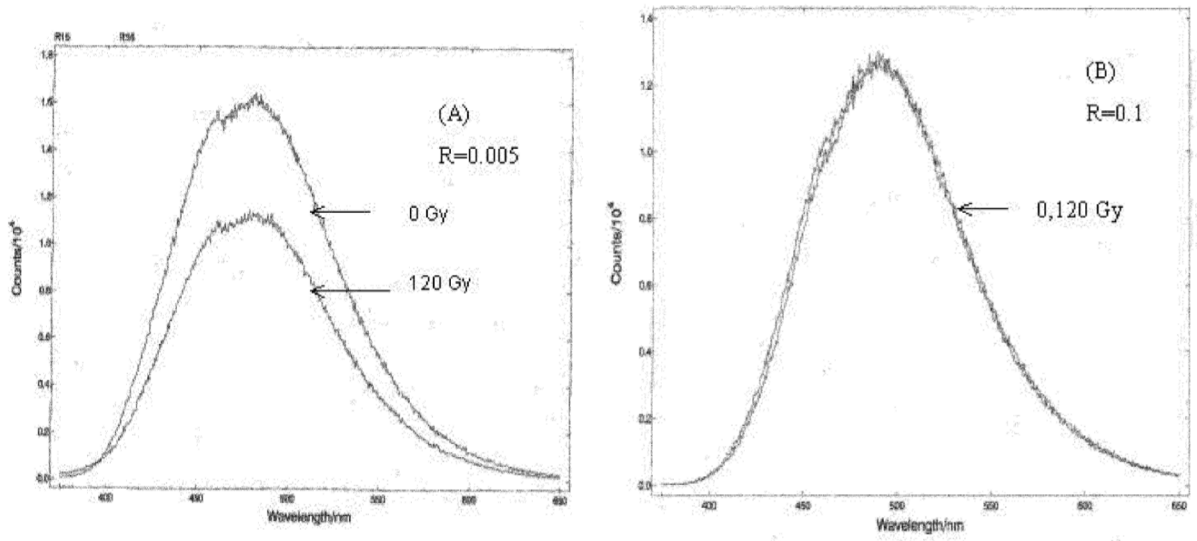

Figure 3. Fluorescence emission spectra of H258–DNA (unirradiated) and DNA–H258 (irradiated 120 Gy) at ratios $R = 0.005$ (***a***) and $R = 0.1$ (***b***). The measurement conditions were: excitation wavelength, 360 nm; slits, 0.5 mm; dwell time, 0.5 s; wavelength increment, 0.5 nm.

investigated. In several other studies, fluorescence spectral properties of H258 were used to elucidate the physicochemical properties like polarity, nature of base pairs, etc. at the binding sites in different DNA[18,24]. Some of the commonly observed spectral changes were, increase in fluorescence intensity of bound H258 with increase in AT contents in different DNA, blue spectral shift due to bound Hoechst which was interpreted as decrease in polarity at the binding sites compared to water surrounding DNA. Fluorescence intensity was also utilized for quantification of DNA in isolated cells as well as in solutions[33]. Various physical and chemical properties of solvent parameters of H258 are also known[24,28] to affect fluorescence properties of H258. Benzimidazole derivatives have been also used to probe microheterogenous structure within the homogeneous solutions of aqueous micelles[34,35] and depending upon the polarity and diffusion of water molecules in different regions in different micelles, processes like excited state reactions, charge transfer leading to altered fluorescence properties including excited state lifetimes were reported in the literature[34,35]. The results of absorption and fluorescence, both steady state and excited state lifetimes, are discussed in the following section.

In absorption and fluorescence spectra no spectral changes except for a very negligible decrease in absorbance (~ 3%) and considerably higher extent of decrease in intensity in

fluorescence (~ 20%) were observed in DNA–H258 solution (irradiated) at lower $R = 0.005$. Such decrease disappeared at $R = 0.1$ where the effective concentration of H258 was 40 μM. This ratio is very close to the reported value of $R = 0.2$, where all the binding sites were saturated[27]. The high affinity binding for H258 corresponds to minor groove binding at the AT-rich region[29]. There was no characteristic emission band at 510 nm due to unbound or free Hoechst 33258. The value of emission anisotropy ($r = 0.27$) also remained unaltered at this ratio. The corresponding anisotropy due to free/unbound H258 was 0.14. The higher anisotropy depicted that the bound H258 was rotationally restricted and oriented with respect to the DNA helix. It is interesting to observe the invariance in this anisotropy of bound H258 in irradiated DNA and in H258–DNA. The unaltered spectroscopic characteristics, viz. spectral intensity, position and anisotropy, strongly suggested that the environment at the binding sites in irradiated DNA did not undergo any change. This information along with the observed similar T_m values and binding affinity once again suggests that the increase in structural stabilization at higher ratio is associated with unchanged local environment at the binding site. This means that H258 primarily provided concentration-depended structural stability at the binding sites.

Fluorescence lifetime decay characteristics reveal excited state dynamics of different structures of a fluorescent molecule which otherwise has same steady emission properties and are very useful for validation of steady state hypothesis. In fluorescence decay lifetime analysis in Hoechst–DNA complexes, the intensity decay profiles were found to be usually double exponentials in nature. The double exponential decay components observed are still being investigated by different groups for correlating with possible structures[20,36] of H2582. This is because, Hoechst is a heterocyclic compound containing two benzimidazole rings along with a phenol and piperazine moieties at the two ends (Figure 1). Therefore, this molecule can, in principle, has more than one conformer in solution because of rotational sites along the benzimidazole axis. This is clearly evidenced by occurrence of double decay components, a short (τ_1) and a long (τ_2) one in water with characteristic values as 0.2 ns and 4.1 ns with relative contribution being 54% and 46% respectively[28]. Upon binding with DNA these decay components alter significantly[21,22]. Most importantly, the short component at 0.2 ns increases significantly however, the overall double exponential decay pattern still persists in H258-DNA complexes. The absence of such low short component around 0.2 ns rules out existence of free or release of bound H258 in our measurements. The exact reason for the observed double decay components in H258–DNA is still not solved, however, it appears that different types of conformers in H258 coexists in solutions and this might result into possible heterogeneity in binding of these conformers. Cosa *et al.*[20] have carried out a detailed study on characterization of these exponentials of Hoechst derivatives where two different conformers, viz. planar and nonplanar forms with respect to the two benzimidazole moieties were suggested[20]. The short (τ_1) and long (τ_2) components were attributed to these two forms respectively. Thus, the short and the long components observed in this study are possibly due to the planar and nonplanar conformers of H258 in the bound states. The analysis of fluorescence decay lifetime components of H258–DNA and irradiated DNA–H258 clearly demonstrated that the decay components although depicted heterogeneity remained unaltered in irradiated solutions. For example, 2.4 ns (τ_1) and 4.4 ns (τ_2) of H258–DNA remained almost the same in irradiated DNA–H258 at $R = 0.005$ (Table 3). The short component showed a lowered value, viz. 1.4 ns at higher ratio. It is to be mentioned that at this ratio the T_m was also very high and hence the increased stability. Although the variation in the decay characteristics with ratio is still not clearly understood, and if this component is associated with planar mode of binding, then the observed lowering only indicates that this planar mode of binding of H258 is altered at higher R. Therefore, the observed overall invariance in steady state and excited state characteristics in irradiated DNA–H258 and H258–DNA indicates that the binding environment did not undergo any significant changes, suggesting only structural stabilization at higher concentration as contributing factor in irradiated DNA solutions.

Conclusions

The results of these physicochemical studies suggested unaltered binding environment of H258 in irradiated DNA. This observation may have strong implication in understanding the mechanism underlying Hoechst-mediated radioprotection. However, Hoechst provided primarily, concentration-dependent structural stabilization in both DNA–H258 and H258–DNA solutions. These findings may have implication in design and synthesis of better radioprotectors as Hoechst 33258 has been reported to have mutagenic properties[37].

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