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Binding Characteristics of Hoechst33342 in Nucleiof GammaIrradiated Human GliomaCells (BMG-1):A novel method for fluorescence spectroscopy ofnuclei

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Abstract

DNA minor groove binding ligand Hoechst33342 is one of the most extensively studied DNA ligands for nucleic acid targeted drug designing. Most of the understanding on Hoechst-DNA interaction is based on binding characteristics of Hoechst with DNA in solutions. In the present study, we have made an attempt to investigate the binding characteristics of gamma irradiated Hoechst33342-Nuclei complex (entrapped in agarose-gel) using fluorescence spectroscopy. We have also investigated the effect of Hoechst33342 on gamma radiation induced DNA damage and repairusing single cell gel electrophoresis (comet assay), and correlated further with binding characteristics of Hoechst33342-Nuclei complex. Results from fluorescence spectroscopy revealed that Hoechst33342 remained bound with DNA in nuclei even after irradiation. The percent tail DNA analysis ofcomets showed that Hoechst33342 enhanced the radiation induced initial DNA damage and also inhibited the DNA repair in concentrations and radiation dose dependent manner. These findings indicate that DNA bound Hoechst33342 not only altered the induction of DNA damage and but also interfered with DNA repair processes in BMG-1 cells. This agarose gel based methodfor fluorescence spectroscopy of nuclei can be a useful tool for elucidation of nature of binding of Hoechst33342 and its analoguewith DNAin various *in vitro* studies.

Key Words: Hoechst33342, gamma radiation, fluorescence spectroscopy, Hoechst-DNA binding characteristics

1.Introduction

Hoechst33258 and its derivatives bind with DNA in AT rich region of minor groove and the detailed nature of binding are well characterized in solution [1-7].This sequence specificity and high affinity of binding of Hoechst33258 has attracted attention of nucleicacid based drug designers for anticancer and other diseases[8]. Various Hoechst derivatives have been

synthesizedand their interactions with DNA were studied primarily in solutionsand attempts have been made to correlate with cellular responses. Among the various derivatives of Hoechst, most of the cellular studies have been carried with Hoechst33342 in different cell systems[9-15]. Hoechst33342 is structurally similar to Hoechst33258 and our understanding of interaction with nuclear DNA in cells is based on its binding characteristics in solution. Due to binding of Hoechst33342 with DNA in cells, various interference on cellular processes related to DNA transactions e.g., DNA replication, transcription, DNA repair, restriction enzymatic cleavage of DNA, inhibition of topoisomerase activity[16-18]are well established. Hoechst33342 also interfere with mitochondrial processes[19]and alter mitochondrial function. Athar et al have shown that Hoechst33342 binds with mitochondrial DNA[20,21]. The cellular consequences of interaction of Hoechst33342 with DNA e.g., interference in DNA repair, apoptosis, inhibition of topoisomerases are expected to contribute overall cytotoxicity and cellular dysfunctions and these effects are attributed to binding of Hoechst with nuclear DNA in cells.

Distinct sequence specificity at the binding sites, requiring 4-5AT sequences in the minor groove region of DNA with high binding affinity are important features of interaction of Hoechst 33258 with DNA in solutions[4,7]. X-ray crystallography and NMR spectroscopy have provided atomic level details of binding and interaction forces between Hoechst33258 and DNA [22,23]. Biophysical studies using spectroscopic methods viz., absorption, fluorescence, circular dichroism and thermodynamics provided binding affinities, energetics and physicochemical properties of the environment at the binding sites specific to this molecule [24-26]. Based on these understandings, a variety of such ligands were synthesized, characterized and cellular responses were studied by various research groups. The much desired structure function correlation could not be achieved. One main reason being the complex environment of DNA in nuclei and cells, no such spectroscopic studies have been attempted for elucidating binding characteristics of ligands with DNA in cellular nuclei and therefore no direct correlation with Hoechst33342 binding nature and cellular responses were possible.

In this study we have designed a simple method for undertaking fluorescence spectroscopic studies for characterization of Hoechst33342-DNA interactions in cell-nuclei. This method is based on entrapment of nucleiisolated from in agarose plug for detailed fluorescence spectroscopic measurements similar to solution. Using this method we have investigated the binding characteristics of Hoechst33342 in nuclei isolated from malignant glioma cells (BMG-1) treated with this ligand. Further, using this method, we have also investigated effect of gamma irradiation on binding characteristics of Hoechst33342 in BMG-1 cells. Hoechst33342 treatment was given prior to gamma irradiation. This class of molecule has ability to protect Chinese hamster cells V79 from deleterious effects of gamma irradiation [12]. This was an important finding for developing DNA minor groove binding ligand as safe radioprotector for radiation countermeasure. The present study has thus provided a new method to access binding characteristics of Hoechst33342 directly in cell nuclei using fluorescence spectroscopy. The particular information obtained from this study with respect to binding characteristics in irradiated cells suggested that binding of Hoechst33342 to DNA in minor groove can sensitize mammalian cells against radiation effects. This new method will find applications in various bio-molecular studies based on fluorescence spectroscopy in cells and insights into nature of binding and functioning of cells.

Materials and methods

2.1Cell culture

The human glioma cells line, BMG-1 was maintained as monolayer at 37ºC in plastic petri dishes PD 35 (Tarsons, India) in the Dulbecco's Modified Eagle's Medium (Sigma, USA) supplemented with 5% fetal bovine serum (Sigma, USA), 10mM HEPES buffer, pH 7.4 (Sigma, USA) and antibiotics (Penicillin-sodium salt 50,000 units, Streptomycin sulphate (4,777 units/mg) 50,000µg/L, Nystatin (2000µg/L from 500,000 USP units). All these chemicals were from Sigma Chemical Co., USA. Cells were passaged routinely in exponential growth phase using 0.05% trypsin solution (with 0.02% EDTA, 5.5mM glucose, and 0.002% phenol red) in phosphate-buffered saline (PBS).

2.2 Chemicals and bio-chemicals

Hoechst 33342, Dulbecco's Modified Eagle's Medium (Sigma, USA) and all antibiotics, citric acid and tween-20, the fetal bovine serum were procured from Sigma Chemical Co. USA. Calf thymus DNA (Biochemistry grade) was obtained from E. Merck (Germany). Phosphate buffered saline (PBS) and Hanks Balanced Salt Solution (HBSS) was obtained from Hi-Media, Mumbai, India. Low melting temperature agarose (low EEO) was from Sigma, USA).

2.3 Treatment of cells

Exponentially growing BMG-1 monolayer cultures were seeded at cell density of $0.1x10^6$cells/ PD35 (plastic petri dishes, Tarsons, India). After 16-18 hrs exponentially growing monolayer cells were treated with different concentrations of Hoechst33342 (in HBSS) for 45 min and washed twice with HBSS after the treatments.

2.4 Irradiation of cells

Exponentially growing BMG-1 cells were irradiated using ^{60}Co-teletherapy unit (Model, Theratron-780C, AECL, Canada). The dose-rate was in the range 1.2 - 0.9 Gy min^{-1}. Cells were pre-cooled and irradiated at ice temperature to ensure minimal processing of DNA damage. After irradiation, cells were kept at 4^0C studying initial DNA damage or at 37^0 C for DNA repair until further processing for various measurements.

2.5 Single cell gel electrophoresis (comet assay)

The comet assay, also known as single cell gel electrophoresis is widely being used for analysis of DNA strand breaks at single cell level[27,28]. The electrophoresis of cells embedded and lysed in agarose gel leads to migration of DNA (away from nuclear core in to the nuclear halo region towards opposite electrode under the influence of electric field), such that DNA fragments migrates relatively further. This fashion of migration of DNA fragments forms an appearance like 'comet'. The measure of migrated DNA fragments determines the extent of initial DNA damage (measured immediate after treatment) and repair of DNA damage (measured the residual DNA damage at different time intervals i.e. 0, 30 and 120 min., in randomly selected ~50 comets. Comet shaped electrophoregram formed by the DNA fragments of each individual cell and the amount of DNA present in the tail directly corresponds with the amount of DNA breaks induced by treatments. After the treatment, cells were trypsinized and re-suspended in HBSS (an aliquots of 30,000-40,000 cells) were mixed in 0.75% (~ 600 µL) warm low gelling agarose (BDH Electran, England and gelling temp, 17°C) and layered on microscopic slides (pre-coated with 0.1% agarose, kept at 45°C). Slides were transferred immediately on cooling plate

(maintained at 4°C) for 5 minutes to allow gelling of agarose along with the embedded cells. The slides were then immersed in SDS lysis buffer (2.5% sodium dodecyl sulphate (SDS), 1% sodium sarcosinate and 25mM ethylenediaminetetra acetic acid (EDTA) for 15 minutes at room temperature (25°C) and subsequently washed twice with double distilled water for 5 minutes at 4°C and then electrophoresed at 1.2V/cm for 5 minutes. After the electrophoresis slides were washed twice with double distilled water and then and air-dried at 45°C. Before analysis, the slides were rehydrated by immersing in distilled water at room temperature for 8-10 minutes, and then stained with 25µg/mL propidium iodide (PI) dye for 5 minutes. The cometswere randomly selected (n=50) andimages were acquiredat 200x under BX60 fluorescence microscope, green light and images of cells using the images analysis system comprising of a monochrome CCD camera (FA 87, Grundig, Germany). After acquisition, the comets were analyzed using Optimas Software and thepercent tail DNA was calculated from data generated by the software.

2.6 Isolation of nuclei from BMG-1 cells

The isolation of nuclei from BMG-1cells was carried out to perform fluorescence spectroscopic measurements of Hoechst33342in nuclei. Cells were trypsinized and re-suspended in ice cooled phosphate buffer saline (PBS). Cell suspension was centrifuged and pellet was re-suspended in lysis buffer (Tris-HCl 10mM pH 7.4, EDTA 10mM, $MgCl_2$ 2.5mM, NP40 0.2 %, PMSF 1µM) for 15 minutes and homogenized by gentle pipetting. The nuclei suspension was centrifuged at 660g for 5 minutes. The supernatant was discarded and nuclear pellet re-suspended in ice cooled nuclei storage buffer STM (Sodium chloride-Tris-Magnesium chloride buffer: Tris-HCl 10 mM, NaCl 10 mM, $MgCl_2$ 3 mM). Nuclei were counted, redistributed at density of 10^6 nuclei / ml of STM buffer and kept on ice temperature until further use.

2.7 Preparation of agarose plugs with entrapped nuclei

Nuclei entrapped in 1.2% warm low gelling agarose (Sigma, Low EEO agarose, gelling temperature 17°C) were used for making plugs for fluorescence spectroscopic measurements. These agarose plugs were then allowed for gelation at 4°C. The agarose plugs were then submerged in STM buffer at ice cool temperature for measurements using fluorescence spectroscopy.

2.8 Preparation of Hoechst 33342-DNA complex in phosphate buffer saline

The stock solution of Hoechst33342 (1mM) was prepared in M.Q. water. The stock solution of DNA was prepared in PBS by slowly stirring the solution 24 hrs to ensure complete solubilization of DNA. The actual concentrations of DNA and Hoechst33342 were assessed spectrophotometricallyby measuring O.D. at 260 nm and 340 nm respectively. The extinction coefficients used were $42000 M^{-1}cm^{-1}$ and $6420\ M^{-1}cm^{-1}$ for Hoechst33342 and DNA respectively. The initial concentration of DNA was kept at 200 µM and different concentrations of Hoechst33342 were added by mixing equal volume of both the solutions. The final concentration of DNA thus became 100µM and the concentration of Hoechst33342 was in the range 0.1 µM to 30 µM. Thus, the Hoechst33342 to DNA ratio, R varied from 0.001 to 0.3. The final concentration of DNA was 100 µM in each solution.

2.9Fluorescence spectroscopic (emission spectral and anisotropy) measurement

The fluorescence spectroscopic measurements (emission and anisotropy) in solutions containing Hoechst33342-DNA and Hoechst33342-nuclei (BMG-1) were performed using fluorescence

spectrofluorimeter model FS900CDT (Edinburgh Analytical Instruments, UK). The excitation wavelength was 360 nm and the fluorescence emission spectra were recorded in the range 375-675 nm. Emission anisotropy measurements depict the orientation of the Hoechst33342 bound to DNA. These measurements were performed by using a pair of Glan Thompson polarizers; one each was introduced in the excitation and emission paths. The polarizers allow intensity or spectral measurements in four different directions, viz., I_{VV}, I_{VH}, I_{HH} and I_{HH}. The subscripts V and H correspond to vertical and horizontal directions of measured light intensity respectively "Eq.1".

2.10 Fluorescence lifetime measurement

Fluorescence lifetime measurements of Hoechst33342-DNA in solutions and Hoechst33342 innuclei were performed in time resolved spectrofluorimeter model FL900CDT (Edinburgh Analytical Instruments, UK). Both, the models FS900CDT and FL900CDT were integrated in a single custom designed modular system by Edinburgh Analytical Instruments, UK. Fluorescence lifetime measurements of excited states allow direct estimation of excited state dynamics of a fluorescent molecule. The range of fluorescence lifetime of fluorescent molecule varies from few pico second to few hundreds of nanosecond and the structural dynamics of biomolecules (DNA, proteins) are in the similar range. Fluorescent lifetime is a very important parameter studying dynamics in structural biology. This is because fluorescence lifetime is very sensitive to alterations due to changes in physicochemical properties around fluorescent molecules. The excitation wavelength of Hoechst33342, was 360 nm and this was obtained from a hydrogen gas filled (0.4 bar) flash lamp operating at 40 KHz repetition frequency. The short-lived fluorescence signal (emitted photon) is collected after each excited pulse by using a technique called as time correlated single photon counting (TCSPC). This technique basically creates a histogram of time of arrivals of a statistically relevant ensemble of photons, for a given number of event, 5000 counts, and the histogram is analyzed by an exponentially decaying function. This is because the average lifetime (τ) is exponential in nature and often multi exponentials " Eq.2".

3. Results

In the present study,we have first demonstrated the utility of the new agarose based gel plug method for fluorescence measurements. Further the possible effect of Hoechst33342 on induction and repair of DNA damage in gamma irradiated human glioma cells (BMG-1) was assessed using comet assay and fluorescence spectroscopy. Detailed fluorescence spectroscopy was performed to investigate the binding characteristics of Hoechst33342-DNA incell nucleiand also made an attempt to study possible effects of irradiation on these binding characteristics.

3.1 Assessment of DNA damage and repair

In neutral comet assay, the percent tail DNA is a measure of DNA double strand breaks (dsb) which can be inferred from the distribution of percent tail DNA (mean percent tail DNA). The mean of percent tail DNA was calculated from data generated by Optimas Software using randomly selected50 comets in eachexperiment group(figure 1, Table 1). The percent tail DNA increasedwith dose of gamma radiation (figure 1A) and the trend was similar to earlier report from our laboratory[29]. Dose dependent increase in percent tail DNA, due to induction of initial DNA damage (dsbs), measured immediately after irradiation at 2 and 10 Gy. The percent tail DNA after treatment of cells with increasing concentration of Hoechst33342 observed at 1μM and 5μM suggested that Hoechst33342 possibly induced damages to DNA (figure 1B). In order to assess the effect of Hoechst33342 on induction as well as repair of DNA damage after gamma

irradiation, we have compared the percent tail DNA values of Hoechst33342 pretreated and untreated gamma irradiated BMG-1 cells.

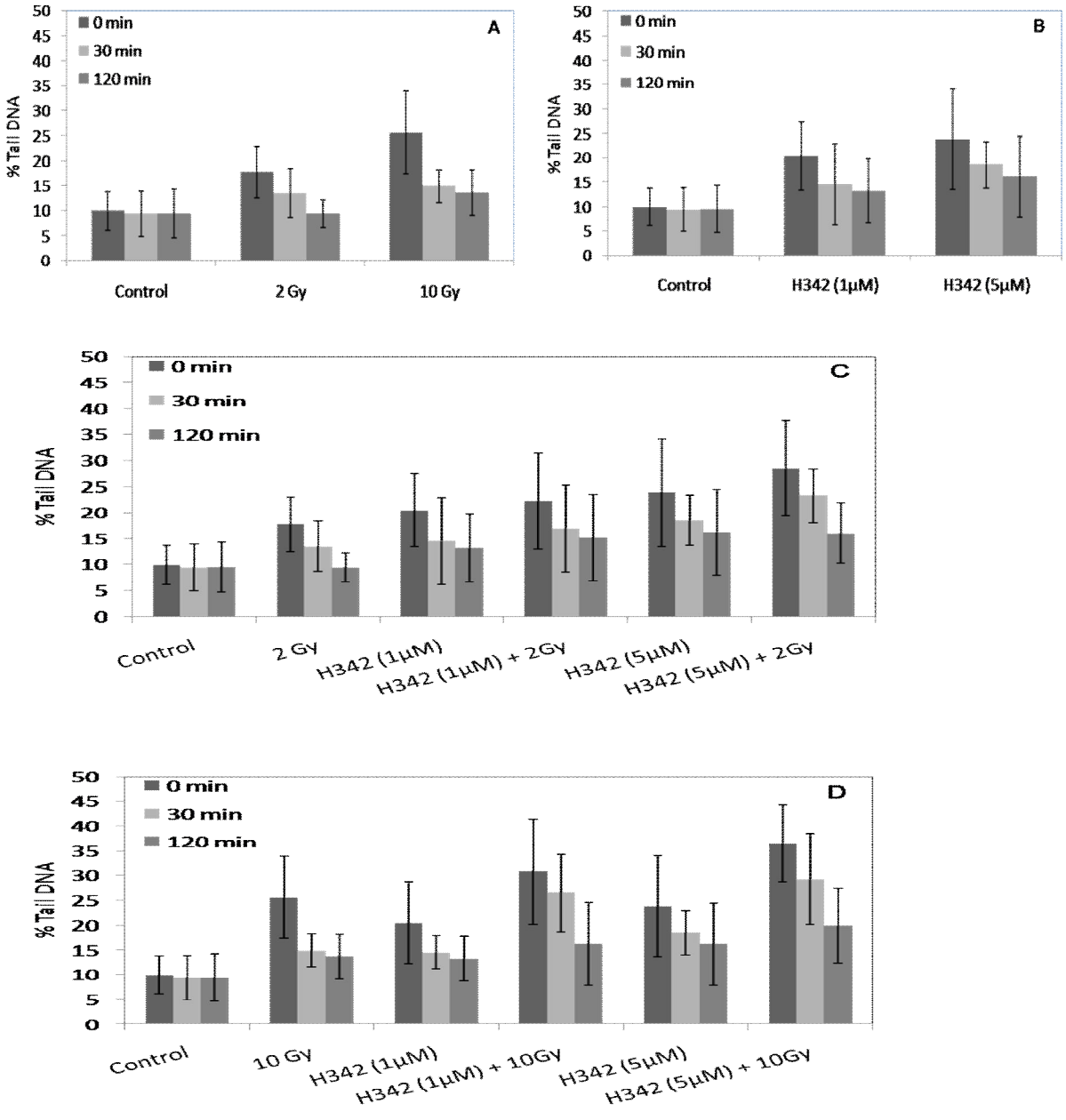

Figure 1. Percent tail DNA in BMG-1 comets depicting DNA damage following various treatments.Data shows mean±SD % tail DNA from 50 comets each. **(A)** Gamma-irradiation at 2Gy and 10Gy; **(B)** Hoechst 33342 (H342) treatment; **(C)** Hoechst33342 treatment (1µM and 5µM) followed by γ-irradiation at 2Gy; **(D)** Hoechst33342 treatment (1µM and 5µM) followed by γ-irradiation at 10Gy.

There was increase in percent tail DNA in a concentration and radiation dose dependent manner (figure 1C, 1D) in cells treated with Hoechst33342 followed by gamma irradiation, possibly due to the induction of DNA damage by Hoechst33342. Further observations on DNA repair kinetics (by measuring the residual DNA damage at different post irradiation time intervals viz., 30 min and 120 min) indicated possible interferences of Hoechst33342 in repair of DNA damage (figure 1C, 1D, Table 1). The frequency histogram of distribution of DNA damage within these population of cells showed heterogeneity in residual DNA damage.

Table 1

Mean percent tail DNA±SD (DNA damage) in 50 comets from human glioma cells (BMG-1) treated with different concentrations of Hoechst33342 and doses ofγ-radiation showing initial and residual DNA damage

Concentrations of Hoechst 33342	Gamma radiation Dose (Gy)	Mean % tail DNA±SD		
		Repair time (minutes)		
		0 min.	30 min.	120 min.
0 µM	0 Gy	9.9±3.8	9.4±4.5	9.5±4.8
	2 Gy	17.7±5.2	13.5±4.9	9.4±2.8
	10 Gy	25.6±8.3	14.9±3.3	13.6±4.5
1 µM	0 Gy	20.4±7.0	14.5±8.3	13.2±6.6
	2 Gy	22.2±9.2	16.9±8.4	15.1±8.3
	10Gy	30.8±10.6	26.5±7.8	16.2±8.36
5 µM	0 Gy	23.8±10.3	18.5±4.8	16.2±8.3
	2 Gy	28.5±9.2	23.2±5.2	16±5.8
	10 Gy	36.5±7.8	29.3±9.2	19.9±7.6

3.2 Fluorescence emission spectra and anisotropy of Hoechst 33342-DNA complex in solutions

In this study we have first treated BMG-1 cells with Hoechst33342and subsequentlynuclei were isolated and embedded in agarose plugs for fluorescence spectroscopy. Since, the fluorescence spectroscopic characteristics of Hoechst33342-DNA in solutions are not available and therefore first fluorescence spectroscopic measurements (emission spectra, anisotropy and fluorescence lifetimes) of Hoechst33342-DNA (calf thymus DNA) in phosphate buffer solutions were carried outto establish the characteristics of bound complex. These spectroscopic characteristics werefurther compared with the Hoechst33342-DNA in isolated nuclei both in un-irradiated and irradiated BMG-1 cells. Figure 2 shows fluorescence emission spectra of Hoechst33342 bound to calf thymus DNA. As the concentration of Hoechst33342 increased from 0.1µM (R=0.001) to 30 µM (R=0.3), the fluorescence emission maximum shifted from 452 nm to 475 nm. The intensity of emission gradually increased till R=0.1 and then decreased at higher ratio (R>0.1). Similar emission spectral characteristics were observed in our earlier study with Hoechst33258-DNA[30]. The anisotropy value of the Hoechst33342-DNA solutions remained unchanged(at0.3) at higher concentration of Hoechst33342 (Table 2). The overall fluorescence characteristics are similar to Hoechst33258-DNA in solutions.

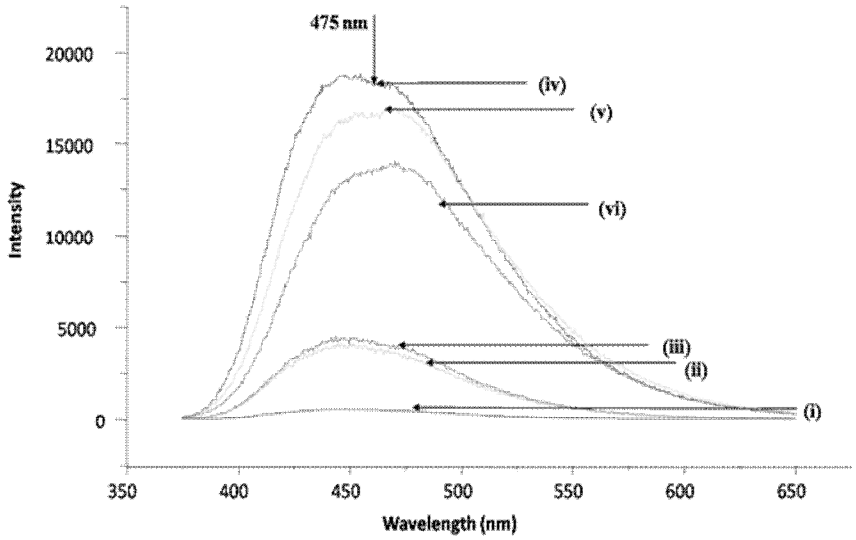

Figure 2. Fluorescence emission spectra of Hoechst33342-DNA (calf thymus DNA) in phosphate buffer at different ratios (R) of Hoechst33342 to fixed DNA (100 µM) i.e. (i) 0.001, (ii) 0.005, (iii) 0.1, (iv)0.2, (v) 0.3. The excitation wavelength was 360 nm.

Table 2

Fluorescence characteristics (emission max., anisotropy and lifetimes) of Hoechst33342

Hoechst33342	Emission max. (nm) / Intensity	Anisotropy	τ_1(ns), (%)	τ_2(ns), (%)
1 µM	462/3490	0.3	2.0 (81)	4.5, (19)
5 µM	462/9880	0.3	1. 5 (62)	3.0 (38)
10 µM	465/12730	0.3	1.5, (77)	3.3, (23)
20 µM	470/11020	0.3	1.6, (68)	3.9, (32)
40 µM	475/9130	0.3	1.6, (69)	3.9, (31)

Note: The fluorescence decay τ_1 and $\tau_2$of Hoechst 33342 in buffer alone are 0.5 ns and 5.2 ns.

3.3 Fluorescence lifetime measurement of Hoechst 33342-DNA complex in solution

The excited state fluorescence lifetime provides insights on dynamics of interaction a fluorescent molecule with macromolecule. Fluorescence lifetime of Hoechst33342 strongly depend on the physicochemical nature of binding interactions with DNA and its surrounding environment. Fluorescence lifetime characteristic of a molecule is very specific and sensitive in nature. The excited state fluorescence lifetime of Hoechst33342 showed characteristics of double decay constants 0.5 ns (τ_1)and5.2 ns (τ_2) due to existence of two conformers in water [31, 32] and when Hoechst33342 binds with DNA both the decay components τ_1 and τ_2 altered significantly (Table 2). For example, the values of τ_1 andτ_2 become 2.0ns and 4.5ns respectively. As the concentration of Hoechst33342 increased, both τ_1 andτ_2 decreased to 1.5ns and 3.0ns in solution. The typical decay profiles of Hoechst33342 and Hoechst33342-DNA are shown in figure 3.

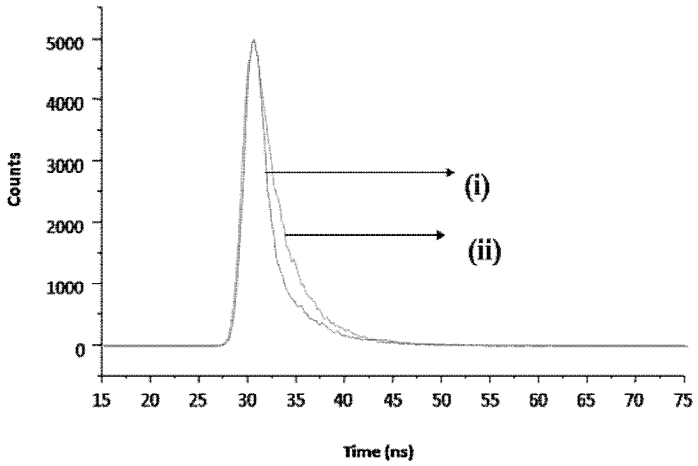

Figure 3. A typical fluorescence life time decay profile of (i) Hoechst33342 and (ii) Hoechst33342-DNA in calf thymus DNA.

3.4 Fluorescence emission spectra of Hoechst 33342 DNA in BMG-1 nuclei

The nuclei isolated from Hoechst33342 treated BMG1 cells both un-irradiated and irradiated by gamma radiation wereembedded in agarose plugs for fluorescence spectroscopy. In order to investigate the binding characteristics in nuclei, the fluorescence emission spectra, anisotropy and fluorescence lifetimes were measured. The emission spectra were recorded at excitation wavelength 360 nm. The emission intensity of spectral maximum observed at 455 nm increased initially with increasing concentration of Hoechst33342 up to 10μM (figure 4) and then decreased at further higher concentrations (40μM). The emission maximum at higher concentrations showed red shift at 475 nm at higher concentrations. The anisotropy value of Hoechst33342 remained unaltered at 0.3 in all the measurements. The overall spectral characteristics are similar to Hoechst 33342-CtDNA in solutions. These spectral characteristics did not change in irradiated nuclei(Figure 5).

3.5 Fluorescence lifetime measurement in Hoechst 33342-nuclei complex

The fluorescence lifetimes of Hoechst33342-nuclei complex was expected to be heterogeneous due to inherent complexity arising from binding sites of DNA in nuclei. Analysis of fluorescence decay profiles showed two exponentially decaying constants, 2.0 ns (τ_1) and 4.5 ns (τ_2) at 1μM Hoechst33342. The numerical values of both the decay components decreased at higher concentrations of Hoechst33342 (up to 40 μM). This was also associated with changes in relative contributions viz., the short decay component (τ_1) decreased at higher concentrations. The results of anisotropy and lifetime measurements along with emission maximum of spectral band and intensity are shown in Table 3.Fluorescence lifetime decay characteristics were examined as a function of repair time in irradiated cells at two time points 30 and 120 minutes. An interesting pattern was observed on these life time components, at first showed dependency on the concentrations of Hoechst33342 (1 and 5 μM) and doses of gamma radiation (2 and10 Gy).In nuclei pretreated with 1μM of Hoechst33342 in BMG1 cells followed by irradiation at 2 Gy of gamma radiation dose, the two decay components 2.0 ns (τ_1) and 4.5 ns (τ_2) decreased to 1.4 ns and 3.2 ns with respect to un-irradiated control respectively. When these cells were allowed for DNA repair for

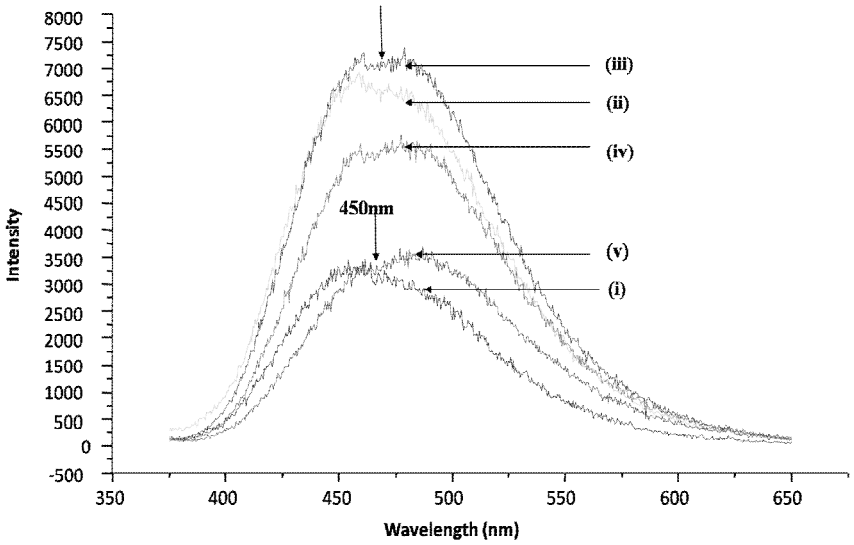

Figure 4. Fluorescence emission spectra of Hoechst33342-DNA at different concentrations of Hoechst 33342 in isolated nuclei entrapped in agarose gel plug. The concentrations were: (i) 2 µM, (ii) 5 µM, (iii) 10 µM, (iv) 20 µM, (v) 40 µM to DNA in fixed number of nuclei ($1x10^6$). The excitation wavelength was 360 nm. Emission spectra were recorded at different concentrations of Hoechst33342.

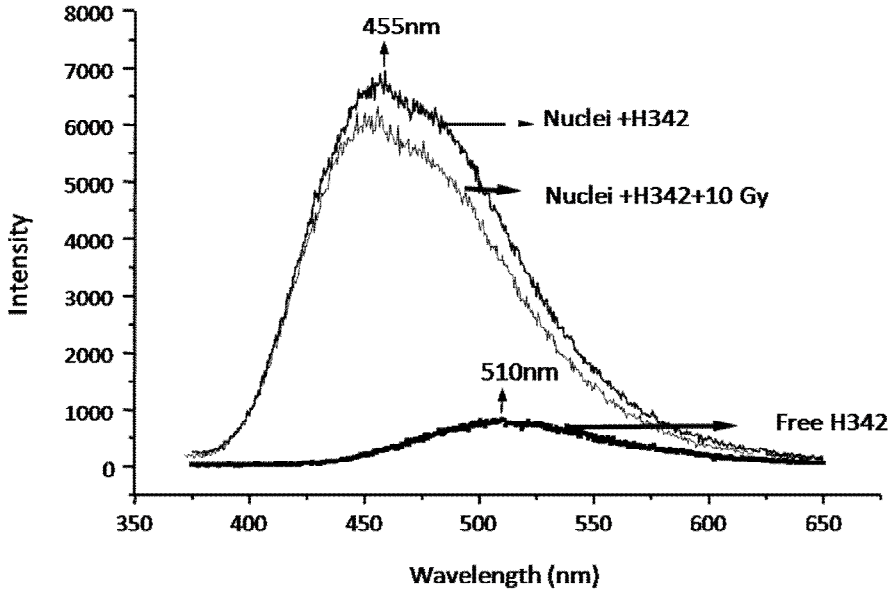

Figure5. Comparison of fluorescence emission spectra of Hoechst33342-DNA in isolated nuclei from Hoechst 33342 pre-treated irradiated and un-irradiated BMG1

30 and 120 minutes, the decay components gradually reverted to its original values 2.2ns and 4.6ns (at 120 minutes). Similar trend was observed at higher concentration (5µM) of Hoechst33342, the decay components 1.7 ns (τ_1) and 4.2 ns (τ_2) at 2 Gy reverted to 1.5ns and

3.4ns almost similar to the original values. At higher radiation dose 10 Gy, such trend in decay characteristics were not observed (Table 3).

Table 3

Fluorescence decay characteristics of Hoechst 33342–DNA complex in nuclei as a function of DNA repair time

Concentrations of Hoechst 33342	Gamma radiation Dose(Gy)	DNA Repair Time (min.)	Florescence decay characteristics	
			τ_1 (A_1)	τ_2 (A_2)
1 µM	0	0	2.0 (81)	4.5 (19)
	2	0	1.4 (41)	3.2 (59)
	2	30	1.9 (69)	4.6 (31)
	2	120	2.2 (70)	4.6 (30)
	10	0	1.8 (65)	3.8 (35)
	10	30	2.2 (74)	4.8 (26)
	10	120	2.1 (77)	5.3 (23)
5 µM	0	0	1.5 (62)	3.0 (38)
	2	0	1.7 (68)	4.2 (32)
	2	30	2.1 (74)	4.9 (26)
	2	120	1.5 (44)	3.4 (56)
	10	0	1.9 (71)	3.4 (29)
	10	30	1.8 (51)	3.8 (49)
	10	120	1.8 (73)	4.4 (27)

Note: τ and A are expressed as nsec (ns) and % respectively, the subscripts refer to decay component 1 and 2.

4.Discussion

DNA minor groove binder Hoechst33258 and its derivatives interact with DNA and may cause reversible inhibition of various DNA dependent functions e.g. DNA-DNA and DNA-Protein interactions. DNA in cells is organized in a complex higher ordered structure and thereforedirect as well as accurate information on binding characteristics at cellular level is required for better and clear understanding of DNA ligand structure-function activity relationship. But at present no method is available to perform studies in such complex environment.

Fluorescence spectroscopy is a versatile technique and can be performed in both homogeneous solutions as well as in heterogeneous biological samples. Studies on Hoechst-DNA complexes in solutions using fluorescence spectroscopy have provided important information on binding characteristics, nature of molecular interactions, and physicochemical properties of local environment of Hoechst33258-DNA [30-34]. These biophysical studies were carried out in solutions of oligonucleotides and Calfthymus DNA (ctDNA). In the present study, we have made an attempt to investigate the binding characteristics and physicochemical properties of Hoechst33342 in nuclei isolated from un-irradiated and irradiated BMG-1 cells to elucidate the effects of Hoechst33342 on radiation induced DNA damage and repair in these

cells. At first, the binding characteristics of Hoechst33342 in with ctDNA was first established and further compared with isolated Hoechst33342-nuclei complex. The aim was to elucidate the nature of binding characteristics and effects of irradiation on physicochemical properties of Hoechst33342-DNA complexes in BMG1 cell nuclei. At first, we have assessed the induction and repair of DNA damage in presence of Hoechst33342 using comet assay in BMG1 cells and correlated it with the binding characteristics.

4.1 Assessment of induction and repair of DNA damage

Therepair of gamma radiation induced DNA damage and its kinetics mainly depend on types of DNA damage (lethal, sub-lethal and potentially lethal damage), availability and accessibility of repair enzymes, activity of topoisomerases, the chromatin organization and most importantly the post irradiation environmental condition[35,36]. In view of this, to discriminate precisely, the level of induction of DNA damage and subsequent repair, we have chosen the comet assay because of its ability to distinguish the intercellular heterogeneity in distribution of DNA damages and repair (residual DNA damage) with in single population of cells [37]. The results from comet assay indicated that gamma radiation dose dependent increase in percent tail DNA suggestive of increased level of induction of DNA dsbs in BMG-1 cells at 2 and 10 Gy, similar to previous observations from our laboratory [29]. The level of initial DNA damage was further enhanced in Hoechst33342 treated gamma irradiated cells (Table1), suggesting that Hoechst33342 induced DNA damage and further enhancement of damage in irradiated cells. This is possible DNA binding molecules can inhibitfunctioning of topoisomerases, helicases and other repair enzymes involved in gamma radiation induced DNA repair[35,38]. Hoechst33342 induced considerable level of DNA damage (figure 1). These observations appeared to be in line with this hypothesis that is topoisomerase poisoning by stabilization of the cleavable complex in earlier studies[35, 39, 40].

We further analyzed the repair kinetics of DNA damage at 30 and 120 min. in BMG-1 cells treated with different concentrations Hoechst33342 and doses gamma radiation. The percent tail DNA values in comets from BMG-1 cells exposed to different doses of gamma radiation alone showed that cells could repair the DNA damage with time and radiation dose dependent manner (figure 1) and the frequency histograms of percent tail DNA showed homogeneity in DNA distribution of residual DNA damage with in this population. Whereas cells treated with Hoechst33342 alone showed non-homogenous distribution of residual DNA damage, indicating the interferences of DNA repair might arising from DNA bound Hoechst 33342. Interestingly, the less percent tail DNA and more heterogeneity in distribution of DNA damage was found among comets, in cells treated with gamma radiation alone compared with cells pre-treated with Hoechst33342 followed by gamma radiation (Table 1). These observations suggested that Hoechst33342 not only enhanced the induction of initial DNA damage but also interfered in processing of the DNA damage (figure 1A to 1D). Although, the percent tail DNA of repaired cells are numerically almost similar but the distribution of frequency histogram has been distinctly more non-homogeneous as comparedto un-treated control. These observations indicated that Hoechst33342 possibly remained irreversibly bound with DNA in nuclei and thus interfered with processing of DNA repair enzymes and proteins.

4.2 Binding characteristics of Hoechst 33342 with DNA in solution

Fluorescence spectroscopic studies have provided both complimentary and additional information of binding characteristics and local environment of bound Hoechst33258 in minor groove of DNA [41]. In order to systematically investigate the fluorescence spectroscopic

characteristics of Hoechst33342 bound to DNA complex in nuclei, we have first carried out detailed spectroscopic measurements on Hoechst33342-ctDNA (calf thymus DNA) in phosphate buffer and then undertaken these spectral measurements in Hoechst33342–nuclei complex. The fluorescence intensity increased with Hoechst33342 to DNA ratio at fixed concentration 100µM of DNA (in base pairs) (figure 2). As the concentration of Hoechst33342 increased, the fluorescence intensity initially increased till R=0.1 and then decreased at R>0.1. This was also associated with shift in spectral maximum from 452 nm to 475 nm. Similar spectral behaviour was observed in our previous study with Hoechst33258-DNA (calf thymus) in phosphate buffer solutions [30, 42]The fluorescence emission intensity enhanced by few fold upon binding to DNA due to decrease in polarity at the binding site of Hoechst33258[1]. This is because Hoechst 33258 displaces water molecules from the binding site in DNA and alters the order of hydration around DNA. This class of DNA ligand increases the stability of DNA through molecular interactions viz., Van Der Waal's forces and bifurcated H-bonding involving the two imidazole moieties of Hoechst molecule.Upon bindingHoechst attains an orientation of about 45^{O} with respect to DNA helix and this corresponds to the anisotropy value of about 0.3 [43,44]. Decrease in anisotropy corresponds to lowering of this angle and hence the overall change in conformation.

Fluorescence excited state lifetime characteristics provide the dynamics of molecular states and also heterogeneity of surrounding local environment [22]. This is evident from the analysis of fluorescence decay lifetimes obtained from free Hoechst33342 in solutions as well as when bound to DNA. Most importantly, the decay characteristics are double exponentials in nature suggesting inherent heterogeneity in solutions. The two decay components of Hoechst 33342 in solutions are 0.5 ns (τ_1)and5.2 ns (τ_2) and in bound state, change to 2.0 ns (τ_1) and 4.5 ns (τ_2) respectively. The relative distributions of these two decay components also differ in their free and bound states. These two decay components viz., τ_1 andτ_2 have been attributed to the two conformers of Hoechst33258 viz., planar and non planar coexisting in solutions [33]. Hoechst33258 contains three rotational sites due to the presence of two benzimidazoles and one piperazine moieties. The two benzimidazoles can be in same plane or at 90^{o} with respect to each other and thereby resulting into at least two different conformers of Hoechst 33258 [22]. The short component (τ_1)has been attributed to non planar and the planar conformer corresponds to long component (τ_2). Both the conformers co-exist in solutions, upon binding with DNA depending on the buffer characteristics, the relative distribution of these conformers and their environment changes. Since both Hoechst33258 and Hoechst33342 are structurally similar, but differences in the substituent at the phenyl ring in Hoechst33342, the magnitude and their relative distribution of τ_1 and τ_2 might change when bound to DNA. In the present study at higher ratio (R>0.1), only a single component at 2 nswas observed suggesting occurrence of only planar conformation. These fluorescence parameters are therefore represent these binding characteristics.

4.3 Binding characteristics of Hoechst 33342 with DNA in nuclei

The fluorescence spectroscopic characteristics of Hoechst33342-DNA complex in cells are expected to be complicated due to the presence of complex environment around DNA in nucleus. Moreover, the dynamic nature of cells is likely to further complicate the spectral characteristics of Hoechst33342 at different time of observations. We have made an attempt to investigate the physicochemical properties of Hoechst33342 bound to DNA in nuclei and compared it further with Hoechst33342-DNA complex in aqueous solutions. Hoechst33342 in nuclei showed emission maximum in the spectral range 462-475 nm, this spectral maximum as expected will strongly depend on the concentrations of Hoechst33342 and DNA. Therefore, in order to keep

the amount of DNA content similar in all experimental groups, the cell number was kept approximately about $1x10^6$ and concentration of Hoechst33342 was increased. The emission intensity of bound Hoechst33342 increased with concentration up to 10µM. The spectral shift i.e. 450 nm to 475 nm in Hoechst33342–nuclei complex correspond to increase in concentration of Hoechst33342 with respect to DNA in nuclei (figure 3), this trend was found almost similar to the observations of Hoechst33342-DNA in solutions (figure 2) and the spectral shift was also similar to increasing concentrations of Hoechst33342 in DNA at fixed concentration in solutions (Table 2). This spectral maximum position corresponds to hydrophobic environment of Hoechst 33342 in nuclei. Further, the measured anisotropy value 0.3 is exactly similar to that of Hoechst 33342-ctDNA in solutions and correspond to the angular orientation of bound Hoechst33342 (approximately 45^0 with the DNA helix)in nuclei and this was also similar to Hoechst33342-DNA solutions (Table 1). Most importantly, the anisotropy value remained same at different concentrations of Hoechst33342 in nuclei suggesting unaltered orientation of bound Hoechst 33342. The fluorescence decay characteristics viz. 2.3 ns (τ_1) and 5.5 ns (τ_2) are very important parameters as it correlate with excited state dynamics of bound Hoechst33342. The analysis showed decrease in their magnitudes with increasing concentrations up to 10-20 µM (Table 2). These two decay components as suggested in earlier studies due to co-existence of both planar (long component) and non planar (short component) conformers in the bound complex with different proportions in earlier studies [30]. In summary, the binding characteristics of Hoechst33342 in nuclei are largely similar to Hoechst33342 bound to DNA in solutions.

4.4 Effects of gamma irradiation on Hoechst 33342 -nuclei complex

The fluorescence spectroscopic characteristics when compared with irradiated Hoechst33342-nuclei complex, no significant changes were observed in emission spectral and anisotropy properties. In a similar study on Hoechst33258-DNA solutions, the spectral properties of Hoechst33258-DNA were also observed unaltered in irradiated solutions (120Gy) [30]. These observations clearly suggested that Hoechst33342 remained in the bound state in irradiated cells, but radiation induced DNA damage in a dose dependent manner as observed in comet histograms (figure 1) in presence of Hoechst33342. In addition, the Hoechst33342-DNA complex remained unaltered in orientation as evidenced from the same anisotropy value (Table 3). Fluorescence spectral shifts as well as intensity are also strongly influenced by the polarity surrounding Hoechst molecule [45]. Since binding of Hoechst33258 is known to reduce the polarity at the binding site due to its ability to remove the water molecules from vicinity of binding sites and therefore no change in spectral parameters in irradiated solutions also supports unaltered physicochemical properties surrounding Hoechst 33342-DNA in nuclei. These observations directly implies that Hoechst33342 remained bound to DNA in nuclei but damages still occurred as evident from DNA damage assays by Comet possibly at sites away from the bound Hoechst33342 (figures1-3). Therefore in-spite of the known radiochemical properties of Hoechst [46]which are responsible for radioprotection of DNA in solutions and inV79 cells, our observations suggest thatHoechst 33342 could not reduce DNA damage in BMG-1.

Most interestingly, when cells treated with Hoechst33342 (5µM) followed by gamma irradiation (2Gy) and allowed to repair for two different time intervals (30 and 120 min), the fluorescence lifetime though initially altered systematically but reverted back to almost original values after 120 min (Table 3) but no such dynamics could be observed at 10 Gy. In most of the mammalian cells, 10 Gy is largely considered to be a highly lethal dose. Neutral comet assay clearly demonstrated that treated cells could not undertake DNA dsbs repair processes at both the

time intervals depending upon the concentration of Hoechst33342 and radiation dose compared to control and radiation alone. The variation of mean percent tail DNA and its distribution (frequency histogram) indicated partial repair of damaged DNA depending upon the radiation dose. The observations of repair time dependent changes in fluorescence lifetimes viz., initial decrease in τ_1 and τ_2 and reverting to their original values after 120 minutes suggested two important features of bound Hoechst33342 viz., presence of Hoechst33342 (5 µM) interfered with repair processes. The excited state dynamics of the two conformers (fluorescence life time)are influenced temporarily during the repair processes and interestingly, Hoechst33342 remained bound to nuclei in irradiated BMG1 cells and did not allow repair.

4.5 Implications in elucidating the influence of Hoechst 33342 on irradiated cells

Hoechst33342 and other molecules of bis-benzimidazole family are known to inhibit DNA replication and repair processes[14,15]. It is therefore important to know micro-environment of Hoechst-DNA complex and further correlate it with cellular responses. In present study the fluorescence spectroscopic investigations together with DNA damage and repair assays using comet assay depicted for the first time the binding characteristics of Hoechst33342 in nuclei complex isolated from cells. Results indicated that Hoechst33342 remained bound to DNA in nuclei and the overall binding characteristics even after irradiation did not alter. Because of tight binding, Hoechst33342 appears to inhibit the DNA repair processes. This combinatorial approaches viz., binding characteristics(fluorescence spectroscopy) and assessment of cellular DNA damagemay be useful for a various kind of studies that involve DNA minor groove binding ligands like Hoechst.

5. Conclusion

The results from present study on binding characteristics of Hoechst33342-Nuclei complex indicated that Hoechst33342 remained bound to DNA in nuclei through-out the time and overall binding characteristics of Hoechst33342-DNA complex did not alter even after gamma irradiation. These observations also suggest that tight binding of Hoechst33342 in the minor groove of DNA may interferes in processing of radiation induced DNA lesions. Further, studies are needed to be carried out to develop better understanding in this direction.

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7. Equations

"1"Equation for calculating the anisotropy value.

$$R= G.I_{VV}-I_{VH}/(G.I_{VV}+2I_{VH})$$

Where, G is the correction factor involving the intensity response function of monochromators in the different directions and calculated automatically from these different intensity files using provisions provided in the operating software.

"2"Equation for calculating the fluorescence life time value.

$$I_t = I_0 \Sigma A_i(-t/\tau_i),$$

where, I_t = Intensity at any time t
I_0= Intensity at time t = 0
τ_i= Fluorescence lifetime of i_{th} decay component
A_i= Pre-exponential factors for each decay components (i=1,2)

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