A Basic Investigation on Low-energy Ion Irradiation Effect on Lives — Low-energy ion irradiation of naked DNA

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Abstract. Low-energy ions are everywhere, from arrivals of natural cosmic particles at the earth to biological and medical applications of manmade accelerator and plasma generated ions. When the low-energy ions irradiate biological cells, the basic effect is induction of mutation or killing of lives. Because of this effect, low-energy ion irradiation has widely been applied for mutation breeding and sterilization. However, some fundamentals involved in the applications are not yet well understood. Furthermore, biological effects from slow-down space particles and radiotherapy ions on genetic mutations are also not yet very clear. Some critical puzzles include whether the low-energy ion irradiation induced biological effect is a direct or indirect interaction consequence, what changes occur in DNA irradiated by low-energy ions, and what the lowest ion energy limit is to cause mutation. To look for answers, this investigation applies both experimental and computer simulation means, in which ions at energy from keV down to eV are used to bombard naked plasmid DNA, followed by checking DNA structure changes. In the experiment, nitrogen and argon ions at keV energy generated from low-energy ion accelerators or plasma bombarded naked plasmid DNA in vacuum to low fluences in orders of 10^{11}-10^{13} ions/cm^2 and the samples were analyzed using electrophoresis and sequencing. Results show that the low-energy ion irradiation of naked DNA can indeed cause DNA damage in the forms of single strand breakage, double strand breakage and multiple double strand breakage, which are the bases of mutation of biological organisms. Lighter nitrogen ions are found more effective in induction of mutation than heavier argon ions. Molecular dynamics simulation of ion bombardment of naked DNA reveals that ion interaction with DNA is not random but preferential. This presentation reports related details.

1. Introduction

Low-energy ion beam bombardment of biological organisms has recently been successfully applied to mutation breeding of both agricultural and horticultural plants [1]. Mutation can be introduced to DNA as a result of enzymatic processing of DNA lesions of post-bombarding replication. However, the mechanisms of low-energy ion bombardment-induced mutations are not well clarified at the molecular level such as X-ray induced mutation [2]. Many experiments using tens of keV ion beams have achieved mutations. Two types of mechanisms have been suggested for the mutation, namely direct and indirect effects of ion beam bombardment [3]. The direct effect refers to implanted ions directly interacting with DNA to cause changes in the DNA structures. The indirect effects involve ion implantation induced productions of x-ray, secondary electrons, heating and free radicals. This research was focused on the direct effect. The penetration depth of such low-energy ions in plant cell materials has been predicted to be several-fold that calculated for compressed solid of the cell materials [4]. Thus, it is statistically possible for a very small

portion of ions able to penetrate through the cell materials including the cell envelope and cell inner substance that cover the cell nucleus to reach DNA. In this case, both energy and fluence of these ions must be very low. A question raised is whether the few very low energy ions can still induce mutation for DNA. A good way to study this issue is to use low-energy and low-fluence ions to directly bombard naked DNA in vitro to simulate the final-step interaction between the ions and DNA. There have been some investigations done on various low-energy radiations including low-energy ion bombardment of DNA [5-10]. The results showed that low-energy ions could produce plasmid DNA strand breaks. However, our investigation is the first attempt to use very low energy nitrogen ions, which are the ion species most popularly applied for ion beam mutation practice and also the abundant element in DNA, and compare effects from N ions with inert and heavy Ar ions.

2. Methods and Materials

2.1 Low-energy Low-fluence Ion Irradiation of Naked DNA

An initial sample of plasmid pGFP (plasmid containing green fluorescence protein, 3344 base pairs), purchased from Clontech, was replicated followed by transformation into *Escherichia coli* (*E. coli*) and subsequently extracted and purified using a QIAGEN® Plasmid Purification kit according to the manufacturer's protocol. Aliquots of 1-µl plasmid DNA solution was deposited on sample pots of sample holders. In ion beam bombardment, the holder (Fig. 1a) was a set of glass tubes. On the tubes, pots, each with 5 mm in diameter and 5 mm in depth, were glass-worked in separation to prevent DNA molecules from jumping out due to beam sputtering effect. In plasma immersion ion bombardment, the holder (Fig. 1b) was made from stainless steel with uniformly arranged six pots of 5 mm in diameter and 5 mm in depth. Singly charged nitrogen ions at 2.5 keV and argon ions at 5 keV (both having almost the same ranges in most materials) to fluencies of 3, 6, 9 x 10^{13} ions/cm^2 bombarded the naked plasmid DNA at room temperature using the bioengineering-specialized low-energy ion beam line [11] (Fig. 1c). Nitrogen ions from plasma RF-formed in a plasma immersion ion implantation (PIII) facility [12] (Fig. 1d) bombarded the naked DNA samples with 2.5 kV bias or without bias to fluences of 10^{10} – 10^{13} ions/cm^2. After bombardment, the samples with vacuum control were then individually recovered. For analysis, samples added with gel and also the natural or solution control and digested control were loaded onto a gel electrophoresis apparatus [6]. This gel was run at a constant voltage (100V cm^{-1}) for about one hour. Images of the gels were captured using UV-transilluminator (for viewing DNA in agarose gels stained with ethidium bromide) and digital camera (for capturing). Fluorescence intensity plots were obtained using the Scion Image for Windows. There were totally four types of samples analyzed in electrophoresis: (1) ion bombarded, (2) vacuum or internal control, (3) natural or solution control, and (4) digested control. The digested control consisted of a sample of pGFP digested with restriction enzyme EcoRI (Sigma) in order to act as a marker for full length linear plasmid. The intensity corresponding to each form in the electrophoresis was quantified by integrating the area under the corresponding peak [6,13,14]. Some ion-bombarded DNA samples were transferred into *E. coli* JM109 competent cells. And the transformation mixture, containing 200 µg/ml IPTG (isopropyl *β*-D-thiogalactoside), was plated on plates. After overnight incubation at 37 ºC, white plaques were picked out and plated on plates again to check for their purity under ultra violet (UV) light.

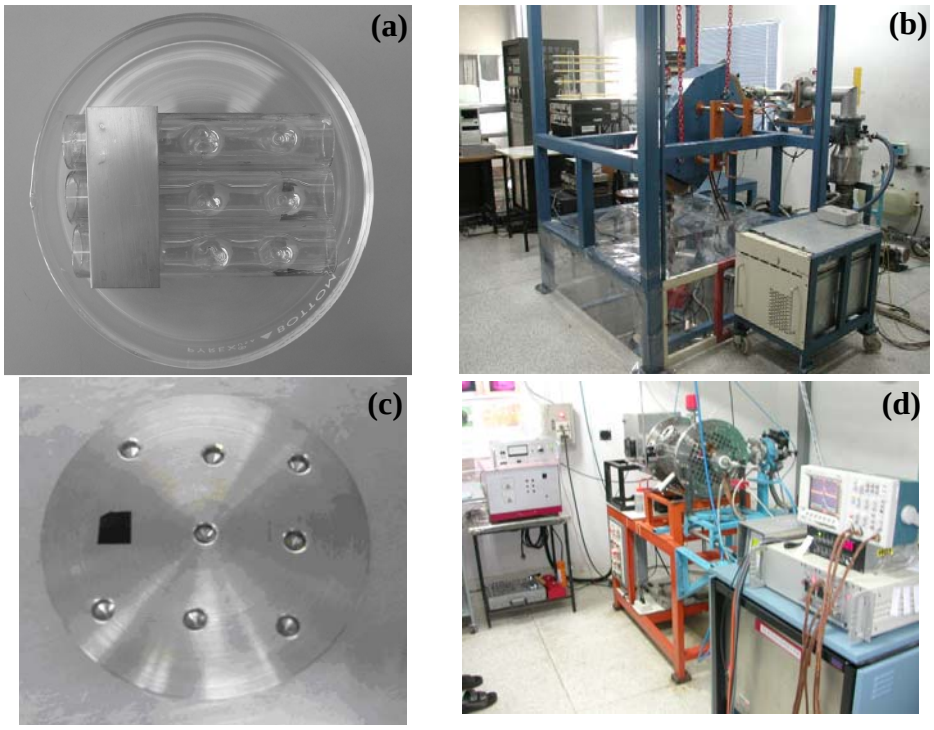

FIG. 1. Experimental facilities used in the low-energy ion irradiation of naked DNA. (a) The glass sample holder for low-energy ion beam irradiation of naked DNA. (b) The bioengineering ion implanter with a vertical beam line. (c) The stainless steel sample holder for plasma low-energy ion irradiation of naked DNA. (d) The plasma immersion ion irradiation facility.

2.2 Molecular Dynamics Simulation (MDS) of Low-energy Ion Irradiation of DNA

To simulate ion bombardment of DNA in vacuum, DNA in A form that is the DNA form in low humidity or low pressure environment was constructed. For investigating effect on the nitrogenous bases, 20 base pairs of alternating poly-AT and poly-GC double strands were constructed using HyperChem 7.0. The energy minimizations and MDS were performed, AMBER 9 software package [15], *in vacuo* to imitate the dried and evacuated condition in experimental bombarding chambers. For investigating effect on various bonds, a 30-base-pair-long DNA duplex was constructed in A-form with Discovery Studio 1.7 software package [16]. The selected part was the residues number 760 – 789 of the green fluorescent protein plasmid (pGFP) in the GenBank, sequenced by Chalfie et al. [17]. The CHARMm27 force field [18] was applied on this molecule. To obtain the DNA structure in the equilibrium state in vacuum, the energy minimization, heating, equilibration and production of MDS were performed using Standard Dynamic Cascade protocol. Two sets of ion parameters were used: carbon ion with energy of 2, 20, and 200 eV and nitrogen ion with 0.1, 1, 10 and 100 eV, the former for bombarding the bases and the latter for bombarding the various bonds of DNA. The simulation was performed using combined quantum mechanics and molecular mechanics (QM/MM) coupled potentials. The energy and geometry of the region were calculated by the PM3 semi-empirical Hamiltonians. The long range QM-QM and QM-MM electrostatic interactions were calculated by Ewald sum.

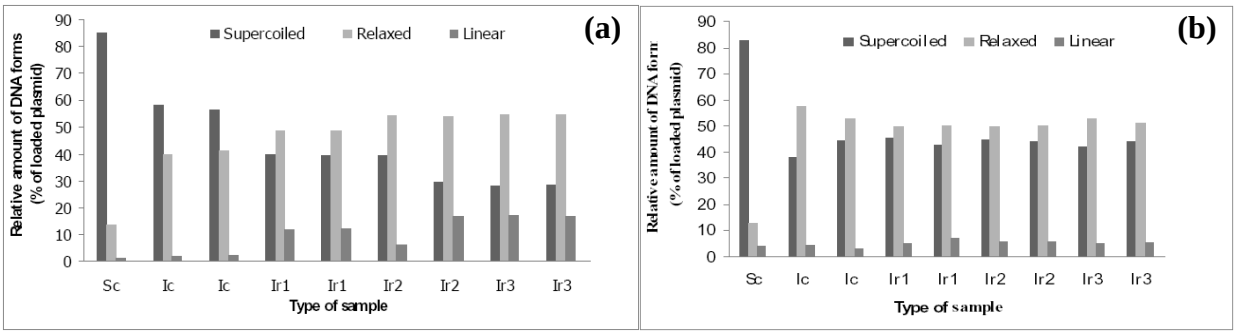

FIG. 2. Examples of electrophoresis results. Sc: solution control, Ic: internal control, Ir: irradiated with fluences of 3 ×10^{13} (Ir1), 6 × 10^{13} (Ir2), and 9 × 10^{13} (Ir3) ions/cm^2. (b) Quantification of various forms of plasmid DNA after N-ion bombardment. (d) Quantification of various forms of plasmid DNA after Ar-ion bombardment. In each electrophoresis analysis, two samples were used for each ion beam condition.

3. Results and Discussion

3.1 Low-energy and Low-fluence Ion Beam Irradiation of Naked DNA

The results from the electrophoresis analysis are shown in Fig. 2, where Figs. 2(b) and (d) are the normalized relative amounts of the DNA forms obtained from the fluorescence intensities of the electrophoresis bands. It is known that when a single-strand break (SSB) is induced, DNA converts into a relaxed form, and when a double strand break (DSB) or multiple DSBs are produced, DNA converts to a linear full-length form or fragments. From the figures, it is clearly seen that upon the very low-energy low-fluence ion bombardment both relaxed and linear forms are produced and hence SSB and DSB indeed occur. It is noticed that in the vacuum controls, the relaxed form is dominantly produced, indicating vacuum effect on DNA SSB. The changes in the amounts of the DNA forms as increasing the ion fluence are found related to ion species. In Figs. 2(b) and 2(d), the attention should be paid to the relative changes in the DNA forms for each ion species. As the experienced history of each set of samples from initial preparation, ion bombardment to electrophoresis might cause varied amount of the DNA forms, and hence, the amounts of the DNA forms of the vacuum control are considered only as a reference. As increasing the ion fluence, the amount of the original DNA supercoiled form decreases for the N-ion bombardment case but does not much change for the Ar-ion bombardment case; the amount of the relaxed form slightly increases for the N-ion case but does not change noticeably for the Ar-ion bombardment; the amount of the linear form increases for the N-ion case more than for the Ar-ion case. This comparison indicates that nitrogen ions, even with lower energy than that of argon ions, are more effective in producing double strand breaks and thus more capable to induce GFP gene mutation than argon ions. This result seems to be conflict with common knowledge that predicts higher-energy and heavier ions able to produce more damage than lower-energy and lighter ions. Whether more physics and biology are involved is being further investigated. One hypothesis is that because DNA contains much nitrogen at the nitrogenous bases, externally introduced nitrogen will have intimate interaction with the original nitrogen so that more effects can be produced. This implies that the direct interaction of the ions with the DNA is more complex than the indirect process.

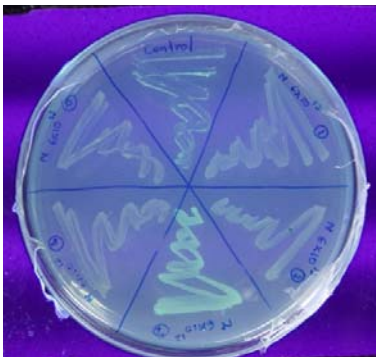

FIG. 3. UV observation of plasmid containing pGFP transferred into E. coli. The white colony indicates pGFP damaged (not functioning) and thus mutated, while the green colonies is non-mutant.

Following the model of the dependence of the change in the DNA forms on the ion fluence proposed in the study on carbon ion bombardment of naked DNA [6], we calculated the cross section of the loss of the supercoiled form of DNA for our N-ion bombardment case to be $(2.35 \pm 0.96) \times 10^{-14}$ cm^2. This result is very close to but roughly greater than that for the C-ion bombardment case, which gave $(2.2 \pm 0.5) \times 10^{-14}$ cm^2. For the case of Ar-ion bombardment, obviously the cross section is extremely small as almost no meaningful change is seen. Nitrogen and carbon are neighbors in the periodic table and both abundant in DNA, and thus expected to have similar effect on the molecular structure when they are used as ions to bombard DNA molecules. But, in the chemical structure in living matter, carbon has unique properties compared with all other elements [19]. Carbon is capable to make as many as four highly stable covalent bonds, while nitrogen has five valence electrons to make it less stably bonding or more active. It is then speculated that nitrogen ions may more actively interact with atoms in DNA, especially nitrogen atoms, than carbon ions.

The result of DNA transfer in *E. coli* showed that green (non-mutant) and white (mutant) colonies were produced. The white colonies were picked out and plated on plates again to check for their purity as shown in Fig. 3. The appearance of white colonies that are the evidence of the GFP gene damaged and thus not functioning confirms that low-energy ion beam bombardment indeed induced DNA mutation. Our gene sequencing showed that the sequences of the GFP gene in the mutants induced by both Ar-ion and N-ion bombardments were similar to that of the GFP in the control. This means that the GFP gene is not mutated. Therefore, the mutation can only be attributed to the Lac promoter, because GFP is expressed from the Lac promoter as a fusion with several additional amino acids, including the first five amino acids of the lacZ protein.

3.2 Low-energy Plasma Immersion Ion Irradiation of Naked DNA

Vacuum effect on damage in DNA and subsequently induced mutation of DNA-transferred bacteria *E. coli* was first checked. No mutation was found from the *E. coli* transferred with plasmid DNA pGFP which was exposed to vacuum at a pressure of 10^{-5} Torr up to one hour. In fact, under all of the conditions applied (varied low pressures and exposure time lengths), the DNA-transferred *E. coli* all showed green. This result demonstrates that certain long-time exposure of DNA to vacuum basically has no effect on mutation.

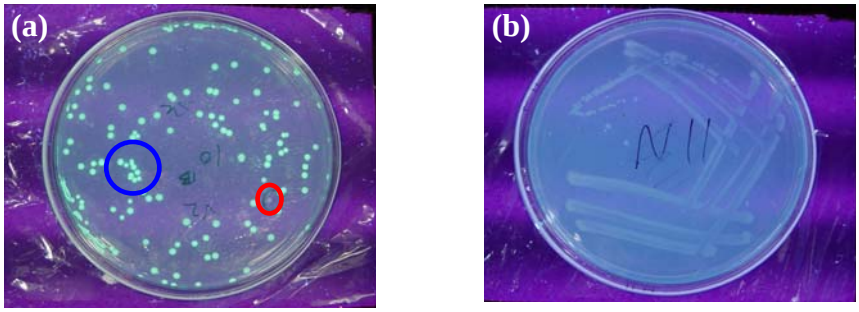

FIG. 4. Demonstration of effect from plasma immersion low-energy-ion bombardment on DNA mutation in DNA transferred E. coli. DNA pGFP was exposed to N-plasma in the conditions of a bias of 2.5 kV and an ion fluence of 10^{13} ions/cm^2. (a) Mutation selection from the DNA-transferred E. coli. White colonies as indicated by the red circle are the mutant, compared with the un-mutated green colonies as indicated by the blue circle. (b) Purified E. coli mutant from the red-circled mutant in (a) to show all of the bacterial cells white.

Effect from only plasma (without using bias) on DNA mutation was checked by placing the DNA samples in argon or nitrogen plasma generated from RF (radio-frequency) power input but without bias. The sample holder was or not grounded. In the former case, the ions with only the thermal energy were implanted in the DNA, while in the latter case, the ions only "blew" the DNA with the thermal energy. In both conditions, no mutation was found. This means that only with eV ion energy, DNA mutation cannot be induced within the treatment time periods.

At bias of a few kV, DNA mutation in transferred *E. coli* indicated by the white bacterial colonies was indeed observed, as shown in Fig. 4. Purification of the white colonies with picking up the colonies to grow in culture media LB exhibited all cells in green, demonstrating the white colonies not contaminated but really mutated. However, it was found that the mutation rate was very low as from only one condition of the PIII, i.e. the bias of 2.5 kV (which resulted in the ion energy of 1.25 keV for the majority of N-ions) and the fluence of 10^{13} ions/cm^2, among a number of conditions, including various fluences, pressures and gases, the mutation was observed. In biased plasma, there are normally not only ions but probably also electrons, X-ray and free radicals, which may also interact with DNA to induce DNA change in structure. But, from the result of the low mutation rate, we may speculate that the mutation source is the bias-accelerated ions which are implanted into DNA but not others, as if it was the latter, there might also be mutation in other conditions where the factors other than ions are also present. Our DNA sequencing analysis (data not shown here) revealed that the GFP fragment of the DNA was not broken but the promoter fragment had suspected breaks. The expression of green color of the plasmid DNA pGFP under UV is controlled by the promoter. If the promoter is damaged, the expression of green fluorescence from the DNA cannot be realized.

3.3 Molecular Dynamics Simulation of Low-energy Ion Irradiation of DNA

In MDS of C-ion irradiation of DNA, the root mean square displacements (RMSD) of the backbone atoms of poly-AT were found remaining in small fluctuation after 1.0 ns of the equilibration, while those of poly-GC were stable after 1.5 ns of the equilibration. The tendency of DNA strand splitting was inspected by measuring the distance between the backbone termini of

two strands, corresponding to A1-T40 and T20-A21 distances for poly-AT and G1-C40 and C20-G21 distances for poly-GC. The results are shown in Table I. It is seen that the poly-AT's T20-A21 backbone termini is the most sensitive to the ion irradiation as it exhibits the largest distance increase subjected to C-ion bombardment. The RMSDs of the base rings were measured to track the flexibility of bases. The behaviors of RMSD of poly-AT and poly-GC are different (Fig. 5). The base rings of poly-GC are quickly stabilized after about only 5 ps of ion bombardment, whereas those of poly-AT take the time more than ten times as poly-GC takes to stabilize. The average RMSD of poly-AT is about one angstrom more than that of poly-CG. All of these results indicate that poly-AT is more unstable and more tend to be broken than poly-GC when subjected to ion attack.

TABLE I: Distance (angstrom, Å) between the backbone termini of two DNA strand after 150 ps MDS. In the case of 200-eV C-ion bombarding poly-GC, the ion passed through DNA after 150 ps simulation. The average distance change is the ratio of the difference between the mean distance of all non-zero energies and the distance of the zero energy over the latter.

Distance between	Ion energy (eV)				Average distance change (%)
	0	2	20	200	
Poly-AT A1-T40	11.7	12.6	13.0	11.1	4.56
Poly-AT T20-A21	12.0	17.4	17.6	16.3	42.5
Poly-GC G1-C40	16.0	19.1	18.1	-	16.3
Poly-GC C20-G21	15.2	15.2	16.4	-	3.95

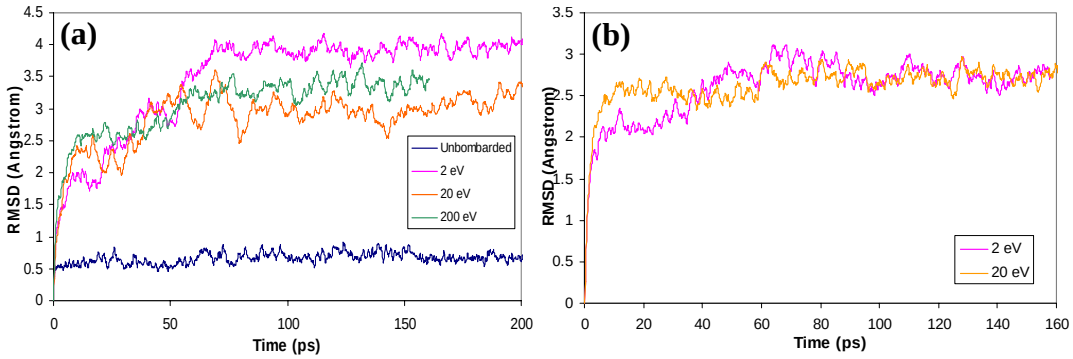

FIG. 5. The RMSD of the non-hydrogen atoms in the base rings of (a) poly-AT and (b) poly-GC.

TABLE II: Summary of the mean values of the distance of maximum radial distribution functions, r_{max}, and the integral of radial distribution functions from 0.0 to 4.0 Å, $I_{4Å}$, of each atom type.

Atom type	r_{max} (Å)	$I_{4Å}$ (Å)
N	3.85	1.83
O	4.75	2.8
O'	4.45	2.6
OP	3.3	3.25
C	3.9	1.5
C'	4.0	1.43

TABLE III: The bond lengths measured after certain time of N-ion irradiation.

Bond type	Average equilibrium length (Å)	Modal bond length after ion irradiation (Å)			
		0.1 eV, after 10 ps	1 eV, after 10 ps	10 eV, after 6 ps	Mean increase (%)
O-P	1.582	1.618	1.702	1.698	5.73
O-P (ar)	1.486	1.498	1.498	1.481	0.43
C-C	1.518	1.560	1.570	1.544	2.64
C-N	1.490	1.489	1.543	1.515	1.72
C-O	1.433	1.445	1.432	1.459	0.86
C-C (ar)	1.387	1.426	1.408	1.399	1.73
C-N (ar)	1.351	1.388	1.381	1.336	1.28
C=O	1.230	1.220	1.221	1.218	-0.84

In MDS of N-ion irradiation of DNA, radial distribution functions (RDF), the distances of maximum RDF, r_{max}, and the RDF integrals were studied, as shown in Table II. The results shown in the table are the mean values of two different doses. The higher RDF integral indicates the higher absorption preference of the implanted ion. It is seen that the preference of N-ion interaction with the DNA atoms is in an order of OP, O, O', N, C and C'. The shortest r_{max} of OP also indicates the strongest interaction with the incident ion as the distance represents the distance between the atom and the ion, obviously, the shorter the stronger the interaction force. The ranges and medians of bond lengths of eight types were studied. The studied types included oxygen-phosphorus single bonds (O-P), oxygen-phosphorus aromatic bonds (O-P (ar)), carbon-carbon single bonds (C-C), carbon-nitrogen single bond (C-N), carbon-oxygen single bonds (C-O), carbon-carbon aromatic bonds (C-C (ar)), carbon-nitrogen aromatic bonds (C-N (ar)) and carbon-oxygen double bonds (C=O). The maximum, minimum and modal bond lengths in each bombardment were measured. Table III summarizes the main results. It is clearly seen that the O-P bond is the weakest as it has the largest increase in the bond length after ion attack, and following the O-P bond are the C-C, C-C (aromatic) and C-N bonds, whereas the C=O bond is the strongest.

4. Conclusion

Irradiation of naked DNA with low-fluence ions of energy lower than a few keV can induce damage in DNA structure, such as individual single and double strand breaks and multiple double strand breaks, to result in mutation. This is a direct effect of ion interaction with DNA. The breaks of the DNA strand are not random but preferential. Strand poly-AT in the nitrogenous base pairs is more vulnerable than poly-GC and the O-P bond in the phosphate group is the weakest.

Acknowledgements

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