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Conformational changes to plasmid DNA induced by low energy carbon ions

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Abstract. The damage induced in supercoiled plasmid DNA molecules by low energy (<500 eV/u⁻¹) carbon ions has been investigated as a function of ion dose. The production of short linear fragments through multiple double strand breakage is suggested and exponential dose responses for each of the topoisomers are presented.

1. Introduction
The effect of ionising radiations upon biological material continues to evoke great scientific interest. The effects of high energy radiation have been linked to ionisation of the target material and production of reactive radical species. However recent studies [1] have shown that the low energy electrons (1-20 eV), produced by ionisation of DNA and surrounding material, are capable of damaging DNA and its components via a dissociative electron attachment process. Many other secondary species are produced by ionising radiation including low energy ions and excited neutral species. The production of these heavier secondary species is of particular importance when the primary radiation is composed of heavy particles, as in the case of hadrontherapy. Along the track of such primary radiation, secondary species with energies up to ~ 1 keV and with various charge states are produced [2]. Additionally low energy heavy species may be present from the primary beam ‘beyond the Bragg peak’ [3].

Reactive radicals are well known to cause DNA damage [4]. However the interactions with DNA of other low energy, heavy secondary species have attracted much less attention. Lacombe et al [5] have demonstrated that low energy (1 keV), singly charged, argon ions are capable of producing both single and double strand breaks in plasmid DNA. Huels et al [3] have carried out studies using the same ion species but at very low energies (less than 200 eV) demonstrating ion-stimulated desorption from films of DNA components. They showed that, even at these very low energies, the projectile ions were able to fragment the target molecules and it was suggested that these ions may produce complex DNA strand breakages.

In the present work we have employed carbon ions because of their relevance to heavy ion therapy [6,7]. Carbon is a major component of biological matter and will be one of the most commonly produced secondary ions when high energy heavy ions are employed therapeutically. The irradiation
target chosen for these studies is the plasmid DNA system (pBR322), a supercoiled circular DNA molecule comprising 4361 base pairs [8].

2. Experimental approach

The experimental setup consisted of two parts, an electron cyclotron resonance (ECR) [9] ion source coupled to a low energy ‘floating beamline’ ion accelerator and a dedicated sample chamber. Carbon monoxide gas was fed into the source as a source of ions. The ion beam was extracted from the source and focused onto the entrance slit of a 90° double focusing magnet. Following analysis, the beam was focused by a second set of electrostatic lenses and directed into the experimental system with a switching magnet. The beam then exited the ‘floating’ section, was collimated by two 4 mm diameter apertures and entered the sample chamber. A custom designed sample holder was mounted on a UHV XYZ rotatable manipulator and positioned 24 mm from the second aperture. This gold coated stainless steel sample holder consisted of two concentric rings of sample wells (4 mm diameter; 0.8 mm depth), equally spaced with 8 positions in each ring. The samples to be irradiated were spaced by ~ 8 mm whilst the outer (control) samples were spaced by ~ 13 mm.

Plasmid pBR322 (4361 base pairs) was purchased from Sigma Aldrich. This was replicated in Escherichia coli and subsequently extracted and purified using a PhoenIX™ Gigaprep kit (Qbiogene). 6 μl of dilute solution (20 ng μl⁻¹) was deposited in each of the wells occupying their full volume. The sample plate was then placed in a freeze dryer where samples were first frozen (in a nitrogen atmosphere) and subsequently vacuum dried (yielding a DNA film thickness approximately double the ion penetration depth) prior to transfer into the sample chamber which was operated at a pressure of ~1 × 10⁻⁶ mbar.

Samples were recovered in 10 μl of 10 mM Tris/1 mM EDTA (TE) buffer (pH 8.0) which had added to it 2 μl of gel loading buffer (0.25% (w/v) bromophenol blue, 40% (w/v) sucrose). The 14 samples were then loaded in a 16 well, 1% (w/v) agarose gel made up in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.3) pre-stained with 0.002% (w/v) ethidium bromide (EthBr). The remaining wells were used for external controls – a solution control of 6 μl of the plasmid solution which was not freeze dried and 5 μl of pBR322 digested with the restriction enzyme EcoRI (Sigma) in order to act as a marker for full length linear plasmid. The gel was then imaged using a ChemiDoc XRS (BioRad [10]) gel imager and fluorescence intensity plots obtained using the Quantity One (BioRad) software.

The intensities of each DNA conformation was obtained from the area of the corresponding peak. A correction factor [11,12] as applied to the supercoiled form intensity to allow for the decreased binding of the EthBr. The intensities were summed and averaged over all the controls and the result was used as a measure of the initial quantity of plasmid, $I_c^T$:  

$$I_c^T = I_c^S + I_c^N + I_c^L$$  

Where $I_c^S/I_c^N/I_c^L$ are the areas of the supercoiled, nicked and linear bands in the control samples. Hence each form was calculated from the corresponding band area as a percentage of $I_c^T$. Preliminary experiments demonstrated that the total irradiated intensity varied as a function of the irradiation dose. This was assigned to be due to the formation of short fragmented linear forms (indicative of multiple double strand breakage) and the percentage conversion of control material, $P^{Fr}$, was calculated as:

$$P^{Fr} = 100 \left[ 1 - \frac{(I_i^S + I_i^N + I_i^L)}{I_c^T} \right]$$  

Where $I_i^S/I_i^N/I_i^L$ are the intensities for supercoiled, nicked circle and full length linear in the irradiated samples.
3. Results and discussion

Figure 1. The effect of C⁺ ion dose upon the change in each of the forms. ● - supercoiled form, ○ - short linear fragments. The lines plotted are exponential growth/decay to saturations fitting the data sets, solid line - supercoiled, dashed line - short linear fragments.

Figure 1 shows the percentage change in each of the forms of the plasmid as the ion fluence is increased. Also shown are model fits to the data (shown as lines). Each data point results from the average of seven samples and the error bars are calculated as one standard deviation. A change is then defined by the subtraction of the control percentage from the irradiated percentage. The change in supercoiled form \( \Delta P^s \) was calculated by:

\[
\Delta P^s = 100 \left[ \frac{I_i^s - I_c^s}{I_c^s} \right]
\]

It is interesting to note that the data series reach ‘saturation’ at high ion fluences. The short linear fragments plot is of particular importance. The procedure used a quantity of DNA which would fill the well to approximately double the estimated ion penetration depth (to avoid secondary electron production from the metallic surface). At very high doses, it is expected that all the plasmid molecules subject to ion irradiation will be broken into small fragments. The saturation level at approximately half (56%) of the starting material (i.e. 44% of the plasmid DNA is ‘shielded’) is in good agreement with this interpretation. The fragment saturation level therefore corresponds to the quantity of the deposited DNA which is actually exposed to ions.

Over the entire fluence range, the supercoiling can be seen to decrease following irradiation. As the fluence increases, more supercoiling is lost, as would be expected. The change in this percentage follows an exponential decay to the minimum value illustrated on figure 1 so that the dependence on ion dose is given by:

\[
\Delta P^s = P_c^s (1 - e^{-b_i f})
\]

Where \( P_c^s \) is the percentage of the control material, exposed to the ion beam, in a supercoiled conformation, \( b_i \) is the cross section for conversion of a supercoiled form to nicked, linear or
fragmented form and \( f \) is the ion fluence. \( P_r^S \) is calculated to be \( 47 \pm 4 \% \) and the cross-section is calculated to be \( (2.2 \pm 0.5) \times 10^{-14} \text{ cm}^2 \) where the errors are estimated from the curve fitting process.

The general trend of a decay to a saturation level was also observed by Boudaïffa \textit{et al} [13] with low energy electrons where they calculated cross-sections for each of the studied energies. The total destruction cross section for loss of supercoiling was calculated to be \( \sim 4 \times 10^{-15} \text{ cm}^2 \) (at an electron energy of \( \sim 50 \text{ eV} \)). This value is five times smaller than that calculated for 2 keV C\(^+\) in the present work.

The induction of short linear fragments of the plasmid DNA as a function of ion fluence is also shown in figure 1 where they are assigned to be zero in the control. A strong dose dependence with 56% of the control material being converted to short linear fragments at higher doses is suggested. A good fit to the experimental data was obtained using an equation similar to equation 4.

Meaningful cross-sections cannot be extracted from this model however the saturation yield for fragmented forms with C\(^+\) is much higher than with low energy electrons [13] (viz. 10% compared to \( \sim 50\% \) with ions at saturation).

4. Conclusions
We have studied damage induced in plasmid DNA by low energy (<500 eVu\(^{-1}\)) carbon ions as a function of ion dose. A strong exponential dose response is observed and suggests fragmentation dominates at higher doses. This study with low keV carbon ions produces many more multiple double stand breakages (resulting in short linear fragments) than those reported for low energy electrons (with energies \( \sim 50 \text{ eV} \)).

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