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Induction of Strand Breaks in DNA films by Low Energy Electrons and Soft X-ray under Nitrous Oxide Atmosphere

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Abstract

Five-monolayer (5ML) plasmid DNA films deposited on glass and tantalum substrates were exposed to Al K_α X-rays of 1.5 keV under gaseous nitrous oxide (N₂O) at atmospheric pressure and temperature. Whereas the damage yields for DNA deposited on glass are due to soft X-rays, those arising from DNA on tantalum are due to both the interaction of low energy photoelectrons from the metal and X-rays. Then, the differences in the yields of damage on glass and tantalum substrates, essentially arises from interaction of essentially low-energy electrons (LEEs) with DNA molecules and the surrounding atmosphere. The *G*-values (i.e., the number of moles of product per Joule of energy absorbed) for DNA strand breaks induced by LEEs (*G*_{LEE}) and the lower limit of *G*-values for soft X-ray photons (*G*_{XL}) were calculated and the results compared to those from previous studies under atmospheric conditions and other ambient gases, such as N₂ and O₂. Under N₂O, the *G*-values for loss of supercoiled DNA are 103±15 nmol/J for X-rays, and 737±110 nmol/J for LEEs. Compared to corresponding values in an O₂ atmosphere, the effectiveness of X-rays to damage DNA in N₂O is less, but the *G* value for LEEs in N₂O is more than twice the corresponding value for an oxygenated environment. This result indicates a higher effectiveness for LEEs relative to N₂ and O₂ environments in causing SSB and DSB in an N₂O environment. Thus, the previously observed radiosensitization of cells by N₂O may not be only due to OH• radicals but also to the reaction of LEE with N₂O molecules near DNA. The previous experiments with N₂ and O₂ and the present one demonstrate the possibility to investigate damage induced by LEEs to biomolecules under various type of surrounding atmospheres.

Keywords

Nitrous oxide; DNA Strand breaks; *G*-value; Radiosensitization

1. Introduction

Nitrous oxide (N₂O) is a molecule which has been widely studied for many reasons. It is a minor component of the atmosphere, known as the ‘laughing gas’, having many beneficial actions, but it is also a strong ‘greenhouse gas’ and its emission into the atmosphere should be strictly controlled (Leont’ev et al., 2001). It is about 200–300 times more powerful in

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global warming potential than of carbon dioxide (CO₂) and contributes to ozone loss (Bange, 2000). Nitrous oxide has been used in many radiation chemistry studies to scavenge solvated electrons formed by the radiolysis of water. This property allows increasing the production of OH• to show its importance in the lethality of the cells. When irradiated, dissolved N₂O reacts rapidly to eliminate e_{aq}⁻ and increases the yield of OH•, via

$$e_{aq}^{-} + N_2O \xrightarrow{H_2O} N_2 + OH^{\bullet} + OH^{-}$$
 (Watanabe et al., 1981; Ewing et al., 1991). Since OH• radicals are major contributors to lethality, it is therefore logical to expect N₂O to be a radiosensitizer. Powers et al. (1972) first observed the sensitizing action of N₂O on spores of *Bacillus megaterium* to X-rays. Samuni and Czapski (1978) then irradiated *Escherichia coli* B saturated with oxygen, argon, and nitrous oxide gases and reported a higher radiosensitivity for nitrous oxide-saturated bacterial cells relative to others. Their result showed that in cells saturated with either argon or nitrogen, the reactive free radical species are OH•, e_{aq}⁻ and H•, while in the nitrous oxide-saturated cells, the reactive species are only H• and OH•, with the concentration of the latter species twice that of the argon- or nitrogen-saturated cells. Therefore, a sensitizing action of N₂O was interpreted by a complete conversion of e_{aq}⁻ to OH•. Brustad and Wood (1976) found a small enhancement in the radiosensitivity of N₂O-saturated bacterial cells with respect to N₂-saturated cells. In a series of experiments, then Watanabe et al. (1981, 1982) reported a significant sensitizing effect of N₂O toward a radioresistant bacterium of *Pseudomonas radiora* O-1 in a dilute aqueous suspension. The sensitizing effect of N₂O anoxia with respect to N₂ anoxia was much higher in this bacterial system than the extent of sensitization found for mammalian cells in vitro in work of Roots et al. (1982).

However, attempts to generalize N₂O sensitization to several types of bacteria, has met with limited success. Radiosensitization by N₂O has not been found in vegetative cells (Watanabe et al., 1982) except for *E. coli* B and results appeared to conflict with those from other laboratories. For instance, Tilby et al. (1982) observed no more than a slight radiosensitizing effect by N₂O on anoxic or oxic survival at high radiation doses. Michaels et al. (1983) and Ewing et al. (1986, 1991) did not find any direct evidence of nitrous oxide sensitization of Chinese hamster ovarian cells. These results could not discern why several vegetative cells are not sensitized in the presence of N₂O, and differences in the effect of N₂O among several bacteria suggest that sensitization by N₂O was governed by some parameters such as, initial cell concentration of suspension, different test system, dose rate, irradiation temperature, and growth phase. Thus, despite numerous studies on radiation response of cells systems to nitrous oxide, its radiosensitization effect has yet to be resolved (Conere et al., 1991). It must be noted that in all studies, the role of N₂O was considered only with regard to its reaction with solvated electrons and the subsequent increase of OH• radicals in aqueous suspension. Obviously, the presence of N₂O within the densely packed structure of the chromosomes in the nucleus can engender other reactions.

Reactions near the DNA of cells are first initiated by highly reactive intermediate species produced along radiation tracks. These species include radicals, ions and secondary electrons (Sonntag, 2006; O'Neill and Fieldman, 1993). The latter are the most abundant of the secondary species and most of them have low energies (i.e., below 30 eV) (Sanche, 2009; Pimblott and LaVerne, 2007). Numerous studies on phenomena involving low energy

electrons (LEEs) have shown that these particles play a central role in determining the effects of ionizing radiation. Thus, in recent years much research interest has been focused on the fragmentation and modification of DNA by LEEs (Sanche, 2002, 2005). The initial experiments on LEE-DNA were performed on DNA molecules under dry high vacuum conditions (Sanche, 2009; Boudaïffa et al., 2000; Cai et al., 2005). However, the damaging effects of LEEs on the DNA molecule are known to be dependent on its environment (Sanche, 2002; Lehnert, 2008; Ptasińska and Sanche, 2007; Barilla and Lokajílek, 2000). Thus, to better understand the damaging effects of LEE on DNA, attempts have been made to perform LEE-irradiation experiments under conditions more closely approximating those found in the cell. For this reason, the effect of water and oxygen molecules on radiation-induced damage in biological systems has been extensively studied (Yokoya et al., 2002; Quintiliani, 1986; Yokoya et al. 2009; Alizadeh et al. 2011). Also in our laboratory some experiments have been completed under well-controlled environmental conditions at standard atmospheric temperature and pressure (STP) to show the induction of DNA damage by LEEs in presence of gases such as, air, pure N₂ and O₂. In preliminary experiments, Cai et al. (2005, 2006) studied the damage to DNA in vacuum resulting from the emission of secondary LEEs from a metal surface exposed to X-rays. Then, by improving the technique of Cai et al. (2006), Brun et al. (2009) and Alizadeh et al. (2011) were able to investigate dry plasmid DNA films irradiated at STP by LEEs (0–30eV) and soft X-rays (1.5 keV) under air, N₂ and O₂. Due to these advances in the technique it is now possible to study the influence of various gaseous atmospheres at STP on LEEs induced DNA damage.

In the present work, we apply this radically different experimental approach, to study other reactions than those of solvated electrons that may be associated with the radiosensitization induced by N₂O on irradiated DNA. The experiments are performed with dry DNA films deposited onto two different substrates, irradiated by 1.5 keV X-rays, under nitrous oxide-saturated atmosphere at STP. Total damage and single- and double- strand breaks are measured as a function of dose. The damage to DNA deposited on glass is due to the X-rays. From comparison of results obtained with DNA films deposited on glass and tantalum, the damage created by LEEs emitted from tantalum is deduced. More damage is induced in plasmid DNA under an N₂O atmosphere by LEEs than under N₂ and O₂ atmospheres, demonstrating strong radiosensitization by N₂O of plasmid DNA extracted from *E. coli* B, without the presence of H₂O and hence solvated electrons.

2. Materials and Methods

2.1. Plasmid DNA Preparation

pGEM-3Zf(–) bacterial plasmid DNA (3197 base pairs, ca. 1.97×10^6 amu, Promega) was obtained from *Escherichia coli* JM109 host, and followed by purification using the QIAfilter Plasmid Giga Kit (Qiagen, www.qiagen.com). TE buffer (Tris-EDTA: 10 mM/1 mM) was extracted from the plasmid with a home-made microcolumn of Sephadex G-50 resin on a bed of glass beads. Sephadex G-50 is highly efficient for the removal of the small molecules and thus purified DNA solution was considered containing a minimal amount of Tris-EDTA (Dumont et al., 2010). The DNA concentration was measured spectrophotometrically by measuring its absorbance at 260 nm, assuming a molar absorption coefficient of 5.3×10^7

$\text{L.mol}^{-1}.\text{cm}^{-1}$ at pH 7.0 for DNA (Manchester, 1996). DNA solution was diluted in ddH_2O until a final concentration of $50 \text{ ng.}\mu\text{L}^{-1}$ was reached. In the present study, about 96% of the extracted plasmid was in the supercoiled form and the rest was in the relaxed circular (C, > 3%) and concatemeric (CM, < 1%) configurations.

Owing to the very short range of LEEs, DNA films were prepared as thin and uniform as possible. The method of preparation is the same as that used by Mirsaleh-Kohan et al. (2011). These authors showed that 5-monolayer-thick films of lyophilized DNA bombarded by LEEs gave the same products as the well-ordered self-assembled monolayer films of DNA bombarded by the same 2–20 eV electrons. To obtain a fairly uniform thickness of approximately 5 monolayers (5ML) $10\mu\text{L}$ of solution containing 500 ng of purified DNA in nanopure water without any salts, was deposited on the glass and tantalum substrates. These substrates were cleaned by rinsing in the ethanol and then ddH_2O and after dried under N_2 flow. Samples were frozen at -70°C and then freeze-dried by pumping under the pressure of 1–3 mTorr for two hours. The film thickness has been estimated as previously described (Alizadeh et al. 2011; Goodhead, 1990) by taking $6.0 \pm 0.2 \text{ nm}$ as the diameter of the DNA film, a thickness of 2 nm for each ML of plasmid and the known density of 1.71 g/cm^3 of the plasmid extracted from *E. coli* (Adams et al., 1986). This procedure resulted in a dry film of 10 nm average thickness on the substrates, assuming minimal clustering of the plasmids.

2.2. Experimental Setup and Irradiation Conditions

All the experimental data were recorded with the apparatus recently developed by Alizadeh et al. (2011) which has already been described in detail. Only a short survey is given here. The apparatus, shown schematically in Figure 1, comprises a chamber evacuated to pressure < 5 mTorr, connected to a pressure gauge (A) and an adjustable leak valve (B) connected to a nitrogen gas source. A negative potential of 3.4 kV is applied to a concave aluminum cathode (C) through a high-voltage electrical feedthrough (D) fixed in a glass-ceramic (Macor) support (E) and placed as a cap on a long quartz tube (F). A nitrogen plasma discharge with 5.5 mA current is formed between the cathode and an aluminum foil target (G) to produce characteristic K_α X-rays (1.5 keV). A portion of the X-rays, emitted towards the He-filled volume (H), traverses the helium gas and reaches a thin foil of mylar (I); X-rays traversing (I) enter a chamber filled by N_2O at STP introduced by valves (J). The DNA samples are placed in this chamber on six aluminum plates (K) fixed on a brass rotating disc (L). The distance between (I) and the surface (K) is only $1.7 \pm 0.05 \text{ mm}$.

In a typical experimental run, 36 samples were exposed to X-rays, in the presence of pure N_2O without humidity. Plasmid DNA was deposited on two different substrates, tantalum and glass. The tantalum was a thin film of $450 \pm 50 \text{ nm}$ thickness evaporated on a 0.4 mm thickness silicon wafer. For each substrate, three samples were used as controls, i.e., were lyophilized, kept under the same atmospheric experimental conditions as the irradiated samples, and recovered, but were not irradiated by X-rays. The other samples were exposed in groups of three, to X-rays for varying periods of exposure (i.e., 7, 15, 30, 45 and 60 min). The number of incident photons on the samples or photon fluences, has been determined to be $1.14 \times 10^{10} \text{ photons/s.cm}^2$ using GAFCHROMIC[®] HD-810 dosimetry films (Cai et al., 2003). Calibration of the sensitivity of Gafchromic film was obtained by irradiating film

samples with X-rays produced by a PHI Model 04–548 Dual Anode X-ray photoelectron spectrometer (XPS, Perkin-Elmer) with an aluminum target and a factor 0.0393 min^{-1} , was used to convert the irradiation time to photon fluence ($\text{photon}/\text{cm}^2$) (Alizadeh et al., 2011).

2.3. Quantification of the Yields by Agarose Gel Electrophoresis

After irradiation, samples were immediately retrieved from the chamber and dissolved in TE buffer from the tantalum and glass surfaces with 95–98% efficiency. The undamaged plasmid exists in a supercoiled topological form (SC). Induction of a single strand break (SSB) converts the plasmid into a relaxed form or ‘nicked circular’ (C) form, while induction of a double strand break (DSB) within both the SC and C forms changes the plasmid into its linear (L) form. Other forms of plasmid DNA in the gel include concatemeric, cross-link form 1 (C-C) and cross-link form 2 (C-SC) of DNA. The relative proportion of each configuration induced in DNA was then determined as a percentage of the total amount of DNA by a 1% agarose gel electrophoresis run in TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) at $10 \text{ V}\cdot\text{cm}^{-1}$ for 7 min and $7.5 \text{ V}\cdot\text{cm}^{-1}$ for 68 min. The amount of SC DNA retrieved from the substrate is of course lower than its initial amount because some of it is converted to the other forms.

One μL from each recovered solution containing about 50 ng of DNA were prestained by 3 μL of $100\times$ SYBR[®] Green I (Molecular Probes[™]) for loading in each well. The samples were incubated with SYBR Green I for at least 15 minutes prior to electrophoresis. The gel itself was stained by 8 μL of $10,000\times$ concentration SYBR Green I. After electrophoresis, gels were scanned with the Typhoon-Trio laser scanner (GE Healthcare) using blue fluorescence mode at an excitation wavelength of 488 nm and filter type (520 BP 40). Various DNA forms were quantified using ImageQuant software (Molecular Dynamics). These values were corrected for the weaker binding of SYBR Green I to the supercoiled form of DNA compared to nicked circular and linear configurations. To obtain this correction factor, equal amounts of supercoiled DNA and digested plasmid with nicking enzyme, *EcoRI*, were loaded in the wells of agarose gel and stained as usual. Using *EcoRI* enzyme in appropriate buffer at 37°C during one hour, results in entire linearization of plasmid DNA (Alizadeh et al. 2011). After determining the intensity of each band, the area under the peak of linear DNA was divided by the area under the peak of supercoiled DNA minus any other forms of DNA. A correction factor of 1.4 was obtained for plasmid DNA used in this work.

2.4. X-ray Attenuation and SE Emission

As seen in Figure 1, X-rays emitted from the aluminum foil (G), pass through three different environments before reaching the DNA surface: the He-filled volume of 50 mm thickness, the Mylar foil of thickness $2.5 \mu\text{m}$, and finally the 1.25 mm N_2O atmosphere. Using the mass attenuation coefficient (μ/ρ) and density of these media (ρ) (NIST, <http://physics.nist.gov>), and applying the exponential attenuation law $I_t = I_0 e^{(-\mu/\rho \cdot \rho \cdot x)}$, the intensities of photons at the DNA surface (I_t) was found to be 54% of the initial intensity (I_0). From $\mu/\rho = 1252.3 \text{ cm}^2/\text{g}$ and $\rho = 1.83 \times 10^{-3} \text{ g}/\text{cm}^3$ for N_2O at STP, we find that before reaching the surface of the DNA films, 25% of soft X-rays were absorbed by N_2O molecules. With no salt and 2.5 bound water molecules per nucleotide under ultra-high

vacuum, our pGEM-3Zf(−) plasmid had a molecular weight of 2.25×10^6 Da. Thus, for 1.5 keV X-rays, DNA film absorbance was calculated to be $\mu/\rho = 1056 \text{ cm}^2/\text{g}$ based on the DNA's composition and μ/ρ of individual atoms. Therefore, for 5 ML DNA films (10 nm thickness), 0.2% of the soft X-ray photons arriving at the film's surface interact with DNA, while 99.8% pass through the film to reach the substrate. Additional photon absorption by N_2O molecules diffusing within the DNA film does not change subsequently this percentage (Alizadeh et al. 2011).

X-ray photons interacting with metal atoms produce energetic photoelectrons and Auger electrons inside the metal (mainly via the photoelectric effect). These electrons lose energy, producing at the surface a distribution consisting essentially of LEE. The secondary electron energy distribution curve from a tantalum substrate has been given in our previous work (Alizadeh et al. 2011). The electron energy distribution peaks at 1.4 eV and the average energy is 5.85 eV. The average energy for SEs with energies less than 10 eV is 3.1 eV. From comparison of the area under the curve in two energy regions, we find that more than 95% of electrons emitted from the tantalum have energies below 30 eV. A total electron yield of 0.049 electrons per photon is obtained from this distribution. Using the previously measured value for the current of SE emitted from a tantalum surface (Alizadeh et al. 2011), the electron flux and the quantum yield of electron emission were calculated to be $(0.54 \pm 0.2) \times 10^9 \text{ electrons.s}^{-1}.\text{cm}^{-2}$ and 0.047 ± 0.005 electron per photon, respectively. The calculated value is in good agreement with the yield obtained from the SE distribution curve, i.e., 0.0049 electrons per photon. This latter value used for G-value calculations.

3. Results

Figure 2 shows the variation in the proportions of SC and C forms of DNA, with photon fluence (in incident photons per cm^2), for irradiated DNA samples deposited on glass substrates. Figure 3 presents similar data for samples deposited on tantalum. Each point in these figures corresponds to the mean value of the yields from three samples, prepared under identical conditions; the error bars denote the standard deviation of these means. As shown in both figures, when the X-ray fluence increases, the amount of supercoiled DNA decreases over the entire range of fluence studied. In contrast, the formation of nicked circular damages increases linearly with X-ray fluence. Additionally, the loss of SC DNA from non-irradiated samples deposited onto tantalum is higher than the percentage of those deposited onto glass substrates. The reasons for the higher DNA damage of deposited plasmid on the tantalum than on the glass substrate with no irradiation, was discussed in our previous publication (Alizadeh et al., 2011). Figure 4 shows the yield of L form DNA created by DSBs, for plasmid deposited on either glass or tantalum, in the presence of N_2O . The number of DSB depends linearly on the fluence.

The linear-least-squares fitting has been applied to model the data in Figures 2–4. The slopes of the fitted lines, representing the yields of DNA strand-break induction per photon fluence, are listed in Table 1, together with their standard errors, as well as the enhancement factors (EF) that are calculated by dividing the yield obtained with the DNA on the tantalum substrate by that measured with DNA films on the glass. The uncertainties on the EFs were estimated from the uncertainties indicated on the percentage yields, shown in Table 1. The

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yields and EF values obtained previously with the same apparatus under O₂ and N₂ at STP are also shown for comparison (Alizadeh et al. 2011). As already mentioned, damages of DNA deposited on the glass substrate results from the interaction of absorbed X-rays, but damage on DNA deposited on the tantalum is induced by both X-ray photons and LEEs emitted from the metal substrate. From comparison of results obtained with DNA films deposited on two substrates, the damage produced by LEEs is deduced. Our data shows that LEE emission from the metal substrate under N₂O enhances the DNA damage (loss of SC) by a factor of 1.9 relative to X-rays. Enhancement in damaged DNA can be quantified as an increase in the G-values (i.e., in the number of moles of product per Joule of radiation energy deposited by each primary particle). Since the thermalization distance of LEEs is about the same as the film thickness, the energy they deposit in the film can be estimated and hence their G-values. However, as previously explained (Alizadeh et al. 2011), since X-rays produce fast electrons that do not deposit all their energy into the film, we only can provide lower limits. Table 2 lists the G values for LEEs (G_{LEE}) and the lower limit of G-values for 1.5 keV X-rays (G_{XL}), in nmol.J⁻¹, derived from the yields in Table 1. Details of the G-value calculations from these films have been described elsewhere (Alizadeh et al. 2011). Briefly, G-values for LEEs were obtained by subtracting the damage on the glass from the damage on tantalum substrate and dividing it by the energy deposited by the LEE in the film. G-values obtained previously with the same apparatus under O₂ and N₂ at STP are also shown in Table 2.

Under N₂O, the G-values for loss of supercoiled DNA are 103±15 and 737±110 nmol/J for X-rays and LEEs, respectively. Compared to our previous experimental results (Alizadeh et al. 2011) and considering the standard errors we can find that the effectiveness of X-rays on DNA in N₂O is comparable to that of the O₂ environment; although, the G value for LEEs in N₂O is almost twice the G-value found for an oxygenated environment. Furthermore, it would appear that from the G-values in Table 2, G_{LEE}/G_{XL} for the loss of supercoiled in an N₂O atmosphere is 7.2. This factor was almost 3.5 under N₂ and O₂ atmosphere (Alizadeh et al. 2011) and indicates a higher effectiveness of LEEs in N₂O than that in N₂ and O₂ atmosphere.

Finally, from the slopes of the exposure-response curves for circular and linear damages (Figures 2–4), the G-values for the formation of SSB and DSB were calculated. As shown in the Table 2, the G-values for LEEs are 5.9 and 6.3 times those for soft X-rays inducing SSB and DSB damage in plasmid DNA, respectively. One notable point here is that, considering the errors, value of G_{XL} for loss of SC is very close to the summation of G_{XL} for the formation of SSB and DSB, i.e., 99 nmol/J, while for G_{LEE} , the difference between these numbers is much larger. This indicates that in comparison to X-rays, LEEs not only cause a larger enhancement in loss of SC and formation of SSB and DSB, but also induce the other forms of damages, particularly, cross linked forms which produce bands in the gel. Additionally, electrons above about 15 eV which exist in the SE distribution emitted from tantalum substrate, possibly can contribute in the formation of multiple double strand breaks (MDSBs) via the direct interactions of a single incident electron with multiple sites in a single DNA molecule. The yield of MDSB can be determined from the integral of the entire broad feature representing the smear in the gel (Huels et al. 2003).

4. Discussion

The damage directly induced by X-rays and LEEs to DNA has been intensively studied in the past and therefore not discussed in this section. Rather, we give a description of the species created around DNA by the presence of irradiated N_2O that may further damage the molecule. In previous work (Alizadeh et al. 2011) damage to DNA was found to be similar in vacuum and N_2 atmosphere under LEE bombardment. We therefore can take the N_2 atmosphere as the minimum damage reference. As seen from Table 1, the N_2O atmosphere surrounding DNA increases total damage induced by X-rays and LEEs when the N_2 atmosphere is taken as a reference. Species resulting from N_2O radiolysis are present in two regions: the space between the Mylar foil and the surface of the DNA film and the empty space within the film. According to our calculations, 25% of the soft X-rays are absorbed by N_2O molecules before reaching to the plasmid surface. This is more than the X-ray absorption expected for an N_2 atmosphere (14.7%) and even more than that calculated for an O_2 atmosphere (22.9%) of similar pressure ($\sim 10^5$ Pa) (Alizadeh et al. 2011). Since more energy is absorbed in N_2O molecules, more reactive species may be produced close to the DNA surface, allowing a higher probability for these species to react with the DNA. X-rays dissociate N_2O molecules mainly via $N_2O + h\nu \rightarrow N_2 + O(^1D)$ (Gallagher et al., 1988) or photoionization $N_2O + h\nu \rightarrow N_2O^+ + e^-$. The kinetic energy of the emitted electron ranges from $h\nu - E_k$ and $h\nu - TE$, where E_k is the energy of the K -edge of oxygen and TE is the threshold energy for the ionization of N_2O (~ 13.75 eV). Additionally, some ionization reactions lead to generation of a huge amount of positive dissociative fragments and molecular ions, such as the formation of NO^+ by dissociative ionisation of N_2O^+ via $N_2O^+ \rightarrow NO^+ + N$, with two dissociation thresholds at 14.2 eV and 16.6 eV (Illenberger and Momigny, 1992). Primary ionization of N_2O molecules by electrons occurs via $e^- + N_2O \rightarrow N_2O^+ + 2e^-$ at ~ 12.6 eV. Considerable detailed discussions of the N_2O ionization processes have been given in literature (Galuska et al., 2005; Dutton et al., 1975; Curran and Fox, 1961). The point to be noted here is that, all of these species are formed due to the interaction of X-rays and the secondary electrons they generate with surrounding N_2O molecules. Thus the species mentioned in this paragraph only affect the induced damage to DNA by soft X-rays. Damage induced by LEEs from the metal substrate is confined within the film since the thermalization distance of LEEs is about 10 nm (Nikjoo and Lindborg, 2010).

N_2O molecules as well as the reactive species mentioned in previous paragraph can also diffuse within the DNA layers. These species created by X-rays outside the film could produce damage inside. However, inside the film LEEs interact strongly with DNA and N_2O molecule, especially those which adsorbed within the DNA structure. These interactions are responsible for the high value of G_{LEE} . Electron attachment to gas-phase N_2O has been studied extensively (Galuska et al., 2005; Hayakawa et al., 1986; Schulz, 1961; Brüning et al., 1998; Rapp and Briglia, 1965). Such studies show that dissociative electron attachment (DEA) to nitrous oxide leads to the formation of O^- via $e^- + N_2O \rightarrow N_2 + O^- (^3P)$ with a large electron capture cross section relative to that of O_2 . The dissociation energy required for this reaction was calculated to be 0.21 eV from N_2O molecules in the ground vibrational state (Schulz, 1961).⁴⁴ However, Chantry (1969) found that electrons with near zero eV

energy could attach to N_2O and the yield of oxygen negative ions production has a broad peak extending from ~ 0.0 eV to ~ 1.2 eV. Another more intense peak for this reaction occurs at 2.2 eV (Chaney and Christophorou, 1969). Absolute cross sections for the production O^- by DEA to O_2 and N_2O were recently measured by May et al. (2008). O_2 yielded the O^- ion at electron energy 6.5 eV with a cross section of $126 \times 10^{-20} \text{ cm}^2$ while, the O^- production from N_2O occurs at 2.2 eV with a cross section of $830 \times 10^{-20} \text{ cm}^2$. More importantly, for N_2O molecules condensed within the DNA films, studies on DEA to condensed N_2O indicates the presence of a peak in the yield of O^- production near 1 eV. Its cross section at 1 eV is $350 \times 10^{-18} \text{ cm}^2$ that represents an enhancement in DEA of more than 40 over the gas-phase value (Bass et al., 1997), while condensed-phase cross section for DEA to O_2 is $27 \times 10^{-18} \text{ cm}^2$ at 6 eV (Bass and Sanche, 1998). Thus, in the DNA films, the formation of O^- from condensed N_2O is possible with much lower energy than the formation of O^- from O_2 . It is much more probable owing to its ~ 13 -fold greater cross section than that from O_2 and the electrons distribution from tantalum substrate which peaks at 1.4 eV. This could be the main reason we observe a larger effectiveness of LEEs to damage DNA films in an N_2O atmosphere than that in O_2 .

Other anions are also arising from LEE- N_2O interactions. Some studies have shown that there is no evidence to the existence of N_2O^- at low electron energies (<1 eV) (Chantry, 1969), although, its formation has been reported in an ion-molecule reaction. In pure nitrous oxide and its mixtures with oxygen the ions O^- with kinetic energies between 4 to 12 eV, undergo fast negative ion-molecule reactions via $O^- + N_2O \rightarrow NO_2^- + N$ and $O^- + N_2O \rightarrow NO^- + NO$. NO^- may be converted via ion-molecule reaction to NO_2^- ion

$NO^- + N_2O \rightarrow NO_2^- + N_2$. Other anions formed by ion-molecule reactions are $N_2O_2^-$ and $N_3O_2^-$ resulting from O^- and NO^- interacting with N_2O molecules. It is therefore concluded that the formation of many reactive species from molecular N_2O involves the formation of O^- and any subsequent reactions with N_2O are dependent on the availability of oxygen anions in the environment. In other words, O^- could be a source of formation of some additional reactive radicals in the presence of N_2O . Particularly, when resulting O^- reacts with molecular N_2O via $O^- + N_2O \rightarrow N_2 + O_2^-$ (Burt and Kircher, 1958). $O_2^{\bullet-}$ is the same product formed from presence of O_2 under irradiation, and it is a highly reactive radical known to cause oxidative DNA damage through its combination with guanine neutral radicals (Misiaszek et al., 2004). In addition, some N_2O molecules can react with electronically excited oxygen atoms via $N_2O + O(^1D) \rightarrow 2NO$ and $N_2O + O(^1D) \rightarrow N_2 + O_2$ that the latter is a source of formation of molecular oxygen which can induce significant damage of DNA due to oxygen fixation hypothesis (Burt and Kircher, 1958). According to this hypothesis, oxygen sensitizes the DNA because the short-lived free radicals in DNA arising from the direct interaction of soft X-rays and LEEs, react with available oxygen molecules to change the chemical composition of DNA and generate 'non-restorable' lesions in the DNA molecule, i.e., peroxy radical ($DNA-O_2^{\bullet}$) (Bertout et al., 2008).

Another possibility to explain the observed increase in damage in the presence of N_2O is the formation of a DNA- N_2O complex that arises from an interaction between N_2O molecules and DNA. As already mentioned, freeze-dried DNA films contain almost 2.5 bound water per nucleotide, which are structural water molecules and are not known to participate in

radiolysis reactions. It is known that the addition of water to dry DNA results first in the binding of H₂O to the phosphate group. In other words, the ionic phosphate group is responsible for the majority of the first layer of hydration (Falk et al., 1963). Ptasi ska and Sanche (2007) showed that the formation of a DNA-H₂O complex permits the formation of a transient anion located on the phosphate group via LEE impact, and consequently, new dissociative transient anion states, which can cause an enhancement in the DNA damage. In the present work, the DNA is prepared without the addition of salt, so that the negative charge on the oxygen of the phosphate group is mainly counterbalanced by a proton. N₂O has a covalent valence-bond structure, ${}^{-}N=\overset{+}{N}=O \leftrightarrow N \equiv \overset{+}{N}-O^{-}$. A proton can thus bind to the N₂O molecule from either the oxygen side to form a structure NNOH⁺, or the nitrogen end, to form ⁺HNNO. Studies have shown that the O-protonated structure lies energetically below that of the N-protonated case. It could therefore be argued that the preferred site for protonation of N₂O is on the oxygen atom to form N₂OH⁺ (Bogey et al., 1986). N₂O has a very low dipole moment, 0.167 D (Mason and McDaniel, 1988), but the dipole moments of its protonated forms are expected to be 2.4 D for the O-protonated form and 3.6 D for the less stable N-protonated form (Mason and McDaniel, 1988). However, the dipole moment of charged species is origin dependant and we must therefore consider the above arguments as highly speculative without more details on the interaction of N₂O with DNA. Further investigations are therefore needed to determine the site of interaction of N₂O as well as its binding energy and the fragments produced from this site. Presently, we can only speculate that, if the same type of interaction with DNA seen for water molecules occurs for N₂O molecules, some new type of transient anions may form via LEE capture by DNA-N₂O complexes. The dissociative decay of such complex anions could be another reason to observe a higher damage of DNA under an N₂O atmosphere. This hypothesis should be tested in other experiments and with other techniques, such as mass spectrometric studies of desorbed fragments from N₂O condensed on DNA to study the interaction between the two molecules. Further investigations are also needed to determine the reactions involved between DNA and the species formed in the N₂O atmosphere; particularly, those which considerably increase the yields of single and double strand breaks.

5. Conclusions

The yields of strand breaks induced by soft X-ray (1.5 keV) and LEEs (0–30 eV) were measured in supercoiled plasmid DNA under a N₂O atmosphere at STP. Our results show that LEE emission from a tantalum substrate under this condition enhances DNA damage by a factor of 1.9 relative to X-rays alone; this is almost 1.5 times larger than the enhancement factor seen under an oxygen atmosphere (i.e., EF=1.3) (Alizadeh et al, 2011). Similarly, the higher G_{LEE}/G_{XL} ratio in N₂O compared to those found in O₂ and N₂ atmospheres, indicates that LEEs have the highest efficiency in N₂O to induce DNA damage. This high ratio is reflected in the large increase in the yield of both single and double strand breaks induced by LEEs.

Owing to their short range, LEEs are expected to thermalize in the DNA film. Hence, the larger enhancement in G-values for DNA deposited on tantalum in N₂O compared to N₂ and O₂ must be attributed to N₂O lying inside the DNA film, i.e., to the interaction of LEEs with

N₂O molecules diffused within DNA. Such an interaction leads to high cross section for the formation of reactive radicals, particularly those involving the production of O⁻. These latter, which appear as the most numerous and reactive, are formed with a 13-fold higher cross section in N₂O than in O₂. We therefore suggest that a major contribution in the increase of G_{LEE}/G_{XL} in N₂O compared to O₂, arises from the reactions involving initial production of O⁻. Formation of O₂^{•-} and O₂ in the N₂O environment within the film could also leads to fixation of radiation damage.

Radiosensitization by N₂O has been a subject of many debates. As discussed in the introduction, it may be a consequence of different test systems and irradiation conditions. In our different experimental approach, we find that even without the presence of water molecules, as the source of indirect damage to DNA, N₂O by itself can considerably increase the sensitivity of DNA to LEEs. Since pGEM-3Zf(-) bacterial plasmid is a very primary biological system, further research is needed in more complex systems and living cells to test the hypotheses advanced in this article.

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Figure 1. Schematic view of the apparatus used to irradiate DNA samples with 1.5 keV Al K α X-ray photons under N₂O gas at STP: (A) pressure gauge, (B) adjustable leak valve, (C) concave aluminum cathode, (D) high voltage feedthrough, (E) glass-ceramic (Macor) support, (F) quartz tube, (G) aluminum foil target, (H) He-filled enclosed volume, (I) thin foil of Mylar, (J) gas circulation valves, (K) aluminum sample holder and rotating disk (L).

Figure 2.

Exposure-response curves for the loss of supercoiled and formation of circular DNA by 1.5 keV X-rays under N₂O at STP for samples deposited on glass. The points represent the means of three yield measurements and the error bars represent the standard deviation of the means.

Figure 3.

Exposure-response curves for the loss of supercoiled and formation of circular DNA by 1.5 keV X-rays and LEEs under N₂O at STP for samples deposited on tantalum. The points represent the means of three yield measurements and the error bars represent the standard deviation of the means.

Figure 4.

Exposure-response curves for the formation of linear DNA (DSBs) for films irradiated by 1.5 keV X-rays under N₂O at STP, deposited on glass and tantalum substrates. The points represent the means of three yield measurements and the error bars represent the standard deviation of the means.

Table 1

Percentage yield per 10^{12} photons.cm⁻² for the loss of supercoiled DNA in 5ML films of the plasmid deposited on tantalum and glass substrates under N₂O, O₂ and N₂ gases at atmospheric pressure. The yields of SSB and DSB under a N₂O atmosphere are also shown. The data for O₂ and N₂ were taken from Alizadeh et al. (2011). The enhancement factor (EF) is calculated by dividing the percentage yields obtained with DNA on tantalum by that obtained with DNA deposited on glass.

Environment	DNA Form	Glass	Tantalum	EF
N ₂ O	SC	5.6 ± 0.4	10.5 ± 1.2	1.9 ± 0.2
	SSB	5.0 ± 0.5	8.0 ± 1.3	1.6 ± 0.3
	DSB	0.4 ± 0.1	0.6 ± 0.1	1.5 ± 0.4
O ₂	SC	6.8 ± 0.9	9.0 ± 0.8	1.3 ± 0.1
N ₂	SC	3.6 ± 0.6	4.9 ± 0.7	1.4 ± 0.2

Table 2

The lower limit of G -values for soft X-rays (G_{XL}) and G -values for LEEs (G_{LEE}) under N_2O , O_2 and N_2 atmospheres. The data for O_2 and N_2 were taken from Alizadeh et al. (2011).

Environment	DNA Form	G_{XL} (nmol/J)	G_{LEE} (nmol/J)	G_{LEE}/G_{XL}
N_2O	SC	103 ± 15	737 ± 110	7.2
	SSB	92 ± 13	540 ± 80	5.9
	DSB	7 ± 1	46 ± 6	6.3
O_2	SC	124 ± 9	415 ± 15	3.4
N_2	SC	65 ± 6	227 ± 15	3.5